REGULATORY MECHANISMS OF THE HEAT SHOCK RESPONSE

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Tianxin (Cynthia) Chen

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To my family

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ABSTRACT

Although Drosophila heat shock transcription factor (dHSF) is abundant in the early embryos, it does not enter the nucleus in response to heat shock. Using the nuclear localization signal (NLS) of dHSF as bait in a yeast two-hybrid system, we identified and cloned a nuclear transport protein, Drosophila Karyopherin α3 (dKap α3). dKap α3 binds specifically to the dHSF's NLS, but not to mutant NLSs that abolish transport in vivo. The early embryo is deficient in dKap α3 protein through cycle 12, resulting in dHSF nuclear exclusion. From cycle 13 onward the transport factor is present and the dHSF is localized within the nucleus, allowing the embryo to respond to heat shock. The functional domain organization of dKap α3 was mapped in detail using yeast two-hybrid analysis and immuno-fluorescence staining.

The function and specificity of the multiple Karyopherin αs found in higher eukaryotes is not understood. RNA interference was used to knock out each Kap α protein individually in Drosophila S2 cells. We found that RNA polymerase II, TATA binding protein and heat shock transcription factor were transported into the nucleus by different karyopherins, indicating that unique and non-overlapping pathways exist.

In *Saccharomyces cerevisiae*, the transcriptional activity of HSF is repressed at non-stress temperatures. Certain mutations of Arginine 274 in the DNA-binding domain (DBD) increase both basal and induced activities of HSF. We demonstrate that the mutations reduce the association between the DBD/oligomerization domain and the transcription activation domains. Our studies suggest that the DBD of HSF can interact with activation domains directly, and this interaction is important for the repression of HSF activity.

RNA polymerase II is bound to Drosophila Hsp70 promoters in the absence of heat shock, and paused after initiating a short transcript. Heat shock induces RNA polymerase II hyperphosphorylation and stimulates RNA polymerase II into productive elongation. We demonstrate that knocking out the expression of positive transcription elongation factor b (P-TEFb) significantly reduces Hsp70 mRNA transcription. P-TEFb is bound to HSF upon heat shock, and the complex can phosphorylate polymerase II C-terminal domain (CTD) in vitro. CTD kinase activity is inhibited by either RNAi targeting CDK9, or CDK9 inhibitor DRB.

TABLE OF CONTENTS

page

Acknowledgements..... iii

Abstract..... iv

Table of Contents..... vi

Chapter One: Introduction..... I-1

Transcriptional Regulations of Eukaryotic Genes..... I-2

Promoters and Other DNA Elements..... I-3

RNA Polymerase II and Initiation Cofactors..... I-5

RNA Polymerase II and Elongation Cofactors..... I-9

Transcription Factors and Inducible Gene Expression..... I-11

Heat Shock Response..... I-16

RNA Interference..... I-23

References..... I-26

Figures and Figure Legends..... I-34

Chapter Two: Developmental Regulation of the Heat Shock Response by Nuclear Transport Factor Karyopherin α3..... II-1

Abstract..... II-2

Introduction..... II-3

Materials and Methods..... II-5

Results II-11

Discussion..... II-19

References II-24

Figures and Figure Legends II-30

Chapter Three: Determination of the Functional Domain Organization of Nuclear Transport Factor Karyopherin α3..... III-1

Abstract..... III-2

Introduction III-2

Materials and Methods..... III-4

Results III-5

Conclusion..... III-9

References III-9

Figures and Figure Legends III-12

Chapter Four: Transcription Factors Enter the Nucleus by Unique and Specific Pathways Involving the Karyopherin α Family of Nuclear Transport Factors in Drosophila..... IV-1

Abstract..... IV-2

Introduction IV-3

Materials and Methods..... IV-5

Results IV-8

Discussion..... IV-12

References IV-15

Figures and Figure Legends IV-18

Chapter Five: Dynamic Association of Transcriptional Activation Domains and Regulatory Regions in *Saccharomyces cerevisiae* Heat Shock Factor..... V-1

Abstract..... V-2

Introduction V-3

Materials and Methods..... V-5

Results V-9

Discussion..... V-17

References V-21

Figures and Figure Legends V-26

Chapter Six: Drosophila Heat Shock Transcription Factor Interacts with the Positive Elongation Factor P-TEFb to Stimulate the Transcription Of Heat Shock Genes..... VI-1

Abstract..... VI-2

Introduction VI-3

Materials and Methods..... VI-6

Results VI-10

Discussion..... VI-16

References VI-19

Figures and Figure Legends VI-25

CHAPTER ONE

INTRODUCTION

TRANSCRIPTIONAL REGULATIONS OF EUKARYOTIC GENES

One of the fundamental principles of molecular biology is that DNA produces RNA, which in turn produces protein. The purification of the nuclear RNA polymerases in the 1960s provided the foundation for efforts to understand the regulation of eukaryotic gene expression. Three distinct DNA-dependent nuclear RNA polymerases were purified based on their ability to synthesize a polyribonucleotide copy of a calf thymus DNA template (Lee and Young, 2000). These purified RNA polymerases were incapable of initiating transcription selectively at promoters in vitro. Basal or general transcription factors (GTFs) that reconstituted efficient selective initiation by purified RNA polymerase II were identified by fractionation of cellular extracts (Conaway and Conaway, 1997). Purified RNA polymerase II and GTFs alone, however, did not fully reconstitute the response to transcriptional regulators observed in vivo, suggesting that an additional apparatus is necessary for gene regulation in living cells.

Knowledge that the DNA template is packaged into chromatin in vivo prompted genetic, biochemical, and structural studies to understand the roles of nucleosomes and nucleosome-modifying machinery in gene regulation (Latchman, 1998). Promoters for protein-coding genes contain sites bound by transcriptional activators, and activators recruit both chromatin-modifying factors and the transcriptional machinery (Figure 1). The importance of chromatin modification in gene expression was emphasized by the discovery that chromatin-modifying enzymes are components of multiple complexes involved in transcription initiation and elongation. These factors act by chemically modifying nucleosomes, as is the case with the Gcn5 acetyltransferase of the SAGA

complex, or through noncovalent modifications, as in the case with the Swi/Snf complex (Brown et al., 2000; Cairns, 1998).

The RNA polymerase II transcription apparatus recruited to promoters and the form of polymerase engaged in elongation are both considerably more complex than previously imagined. Transcriptional activators recruit RNA polymerase II to promoters in a holoenzyme form consisting of GTFs and a multiprotein complex called the Srb/Mediator (Bjorklund et al., 1999; Bjorklund and Kim, 1996). Actively elongating RNA polymerase II molecules are associated with elongation factors (Conaway et al., 2000; Conaway and Conaway, 1999). Protein complexes involved in RNA capping, polyadenylation and perhaps splicing can also associate with RNA polymerase II (Bentley, 1999).

PROMOTER AND OTHER DNA ELEMENTS

Basic Promoter Structure

There are at least three features common to most promoters for protein-coding genes: the transcription start site, the TATA box, and sequences bound by transcriptional regulators (Latchman, 1998). The core promoter element, which consists of the start site and the TATA box, is sufficient for directing transcription initiation by the basal transcription machinery. The sequences bound by transcriptional regulators include Upstream Activating Sequences (UASs), enhancers, Upstream Repressing Sequences (URSs), and silencers.

Core Promoter Element: TATA and Transcription Initiation Site

The average core promoter element encompasses approximately 100 bp and contains the transcription initiation site. An AT-rich site called the TATA box is located upstream of the start site; its location is 25 to 30 bp upstream in higher eukaryotes and 40 to 120 bp upstream in yeast. The TATA box is the binding site for the TATA-binding protein (TBP). Although a canonical sequence can be derived for TATA boxes, TBP can bind and function at a broad range of sequences, making it difficult to identify genuine TBP-binding sites from sequence alone (Cormack and Struhl, 1992).

In some genes, the transcription initiation site includes an initiator (Inr) element, defined here as an element encompassing the transcription start site that binds regulatory factors. Various factors can bind to Inr elements, and these may facilitate recruitment of the transcription apparatus (Aso et al., 1994; Carcamo et al., 1991).

Core promoters can contain TATA and Inr elements (composite), either element alone (TATA- or Inr-directed), or neither element (null). Composite promoters are found primarily in viral genes; most cellular class II genes contain TATA-directed promoters and a more limited number contain Inr-directed promoters. The null promoters often have multiple transcription start sites, suggestive of imprecise initiation.

Although core promoter elements are fundamental for binding of the general transcription apparatus, both the composition and context of the sequence can influence transcriptional regulation. Experiments that exchange TATA boxes and Inr elements reveal that the composition of core promoters can mediate lineage-specific, temporal, and spatial regulation of gene expression.

Upstream Activating Sequences and Enhancers

Transcriptional activators bind to sequences that have been termed UASs or enhancers (Blackwood and Kadonaga, 1998). The term UAS is typically used to describe elements bound by activators that influence transcription from nearby start sites. Enhancers are clusters of DNA-binding sites for transcriptional regulators that influence transcription independent of their orientation and at distances as great as 85 kb from the start site.

Upstream Repressing Sequences

DNA elements bound by sequence-specific gene repressors are called URSs. URS-bound factors inhibit transcription through various mechanisms, including interfering with activator binding, preventing recruitment of the transcription apparatus by the activator, and modifying chromatin structure (HannaRose and Hansen, 1996).

RNA POLYMERASE II AND INITIATION COFACTORS

Transcriptional activators recruit the RNA polymerase II–containing transcription initiation apparatus to promoters of protein-coding genes. The assembled apparatus contains the 12-subunit RNA polymerase II core enzyme, the general transcription factors, and one or more multisubunit complexes called coactivators or mediators. RNA polymerase II holoenzymes that contain most of these components of the initiation apparatus in a single complex have been purified from yeast and mammalian cells, suggesting that much of this apparatus can be recruited to promoters in one step.

The best-defined RNA polymerase II holoenzyme is from yeast and contains RNA polymerase II, a subset of the general transcription factors and the Srb/Mediator complex. The Srb/Mediator complex appears to integrate signals from transcriptional regulators at promoters, and its composition can be remodeled as cells encounter new environments to allow coordinate control of specific sets of genes. Recent studies have revealed that various mammalian coactivators, purified independently by multiple investigators for their ability to reconstitute activated transcription of different genes, are homologues of the yeast Srb/Mediator complex.

Core RNA Polymerase II

Purified eukaryotic core RNA polymerase II typically has 10 to 12 subunits. Core RNA polymerase II is capable of DNA-dependent RNA synthesis in vitro, but is incapable of specific promoter recognition in the absence of additional factors. Yeast and human RNA polymerase II consist of 12 subunits, Rpb1 to Rpb12. The genes encoding the 12 yeast subunits are all required for normal cell growth. Genes for each of the 12 human RNA polymerase II subunits have been isolated and exhibit remarkable structural and functional conservation, as most human subunit genes can functionally substitute for their counterparts in yeast (Lee and Young, 2000).

Eukaryotic RNA polymerase II molecules share several important features with their bacterial counterparts that provide clues to subunit functions. The bacterial RNA polymerases are composed of a specificity factor, σ, and a three-component core enzyme, structured as α β, β', $\alpha 2$ tetramer. The two largest subunits, Rpb1 and Rpb2, are homologous to the β' and β bacterial core subunits, respectively. Rpb3 and Rpb11 share

a weaker homology with the α bacterial core subunit and can form a Rpb3-Rpb11 heterodimer in vitro. The eukaryotic subunit orthologues of these bacterial core enzyme subunits are largely responsible for RNA catalysis.

The structure of yeast RNA polymerase II has been solved at 3 angstroms resolution and has revealed several interesting features (Cramer et al., 2000). Comparison of previously solved structures of prokaryotic RNA polymerase with the structure of eukaryotic RNA polymerase II reveals that the structures of the core subunits of these enzymes are very similar (Cramer et al., 2000). The two largest subunits, Rpb1 and Rpb2, form a cleft that contains the active site, identified in the structure by the location of a catalytic magnesium ion. The position of the active site relative to the projected pathway of DNA in the cleft suggests that DNA does not follow a straight path through the enzyme, and two candidate grooves have been identified as exits for nascent RNA. The floor of the Rpb1-Rpb2 cleft contains two pores formed by extensions of Rpb1 and Rpb2 that cross the gap between the two subunits. The pores are at the apex of a funnel-like space in the enzyme. Together, the pores and funnel may function as channels for nucleotides or as channels for RNA and factors that affect 3' to 5' cleavage of nascent RNA during proofreading or bypass of blocks to transcription.

Basal/General Transcription Factors (Core Promoter Factors)

The set of basal or General Transcription Factors (GTFs) required for specific promoter binding by RNA polymerase II in vitro includes TFIIA, TFIIB, TFIID, TFIIE, TFIIF, and TFIIH (Lee and Young, 2000). Despite the name, it is not yet clear that each of the GTFs functions at all genes in vivo, and it is likely that other components of the

transcription apparatus are as generally employed as the GTFs. Hence, these proteins and complexes are best described as core promoter factors, as the general feature of GTFs is that they have roles at core promoters.

A preinitiation complex containing the GTFs and RNA polymerase II can be assembled in a stepwise fashion on promoters in vitro (Buratowski, 1994). Order of addition experiments indicated that promoter elements were bound by TFIID or TBP, followed by TFIIA, TFIIB, a subcomplex of RNA polymerase II and TFIIF, TFIIE, and finally TFIIH (figure 2a). However, it is unlikely that the individual factors assemble in this fashion at promoters in vivo. Several of the GTFs and other factors required for transcriptional initiation were found associated with polymerase II as a holoenzyme, thus PIC assembly may actually require only a limited number of recruitment events (Figure 2b).

The assembled transcription apparatus proceeds through several steps on the way to forming productively elongating complexes. The complex melts promoter DNA to form the open complex in which 12–15 bp of promoter DNA are in the form of a single-stranded bubble (open complex formation). Initiation continues with the formation of the first few phosphodiester bonds. Typically, polymerases repeatedly initiate transcription and release the resulting small RNAs (abortive initiation). Eventually, the polymerase transitions from abortive initiation as it generates a longer RNA (promoter clearance). Polymerase tends to pause 25–30 bp from the start site at many promoters. These early elongation complexes need to make a critical transition to a fully competent elongating form of the apparatus that is able to escape the promoter (promoter escape) (Conaway et al., 1998).

RNA POLYMERASE II AND ELONGATION FACTORS

A Switch from Initiation to Elongation

To produce an RNA transcript, the formation of a stable transcription initiation complex must be followed by promoter clearance and processive elongation. Several lines of evidence indicate that the switch from initiation to elongation involves phosphorylation of the RNA polymerase II Carboxyl-terminal domain (CTD) and an exchange of cofactors associated with the polymerase. RNA polymerase II molecules found in initiation complexes lack phosphate on their CTDs (pol IIa form), while elongating polymerase molecules contain heavily phosphorylated CTDs (pol IIo form) (Dahmus, 1996). The Srb/Mediator complex is tightly associated with RNA polymerase II molecules that lack phosphate on their CTDs in the holoenzyme. In contrast, the elongator complex and various RNA processing factors become associated with RNA polymerase II molecules with hyperphosphorylated CTDs. CTD phosphorylation must occur during the transition from transcription initiation to elongation, because the phosphorylated CTD has a role in recruiting the mRNA capping enzyme to the nascent transcript, and mRNA capping occurs soon after promoter clearance.

Phosphorylation of RNA Polymerase II CTD

The RNA polymerase II CTD shows a remarkable feature: it consists of tandem repeats of a consensus heptapeptide sequence (Tyr-Ser-Pro-Thr-Ser-Pro-Ser) (Riedl and Egly, 2000). The CTD consensus sequence is highly conserved in eukaryotes, although the number of heptapeptide repeats varies from 26 or 27 in *S. cerevisiae* to 52 in humans. The CTD is essential for viability in yeast and metazoan cells (Riedl and Egly, 2000).

Five out of the seven amino acids in the heptapeptide repeats have the potential to be phosphorylated. Multiple CTD kinases have been identified so far and were shown to phosphorylate distinct positions within the repeats (Bregman et al., 2000). The first CTD kinase characterized was the cyclin-dependent kinase (CDK) $p34^{cdc2}$, however, its physiological significance remains unclear (Sterner et al., 1995). Three other CDKs, CDK7, CDK8, and CDK9, emerged as major candidates involved in CTD phosphorylation in vivo (Riedl and Egly, 2000). CDK7 (yeast KIN28), associated with cyclin H (yeast CCL1), is a subunit of the general transcription factor TFIIH. It is suggested that the CTD kinase activity of CDK7/cyclin H is responsible for converting pol IIa to pol IIo at the promoter clearance stage (Svejstrup et al., 1996). CDK8 (yeast SRB10) and its partner cyclin C (yeast SRB11) were initially isolated as suppressors of truncation mutation in the CTD, and later characterized as a CTD kinase within holoenzymes (Liao et al., 1995; Thompson et al., 1993). CTD phosphorylation by SRB10/SRB11 kinase prevents the assembly of the pre-initiation complex and thereby represses the transcription of specific genes (Hengartner et al., 1998). Positive Elongation Factor b (P-TEFb) is composed of CDK9 and one of several cyclins including T1, T2 and K (Fu et al., 1999; Peng et al., 1998). The kinase activity of P-TEFb is necessary to overcome the effect of negative elongation factor DSIF, a heterodimer composed of Spt4 and Spt5 (Figure 3) (Yamaguchi et al., 2001), and numerous studies have shown that P-TEFb is required to prevent the arrest of elongation pol II (Conaway et al., 2000). Interestingly, CDK9 preferentially phosphorylates CTD that has been already pre-phosphorylated by other kinases such as TFIIH (Marshall et al., 1996), suggesting

that while TFIIH probably act only at the initiation step, P-TEFb remains associated with the elongation polymerase (Ping and Rana, 1999).

Fcp1 is a CTD phosphatase with a general role in regulating transcription of protein-coding genes. Both yeast and human Fcp1 bind the largest subunit of the general transcription factor TFIIF and both phosphatases are stimulated by the addition of partially purified TFIIF. This stimulation can be blocked by addition of TFIIB, suggesting that Fcp1, TFIIF, and TFIIB may all regulate polymerase recycling. Genome-wide expression analysis with yeast Fcp1 mutants demonstrates that loss of Fcp1 has widespread transcriptional effects.

Negative Elongation Factors

Two factors, DSIF and NELF, can confer sensitivity to the transcription inhibitor DRB, which inhibits mRNA synthesis and CTD phosphorylation (Yamaguchi et al., 2001). DSIF was isolated by its ability to induce pausing of the transcription complex in conjunction with the transcription inhibitors DRB and H8. The two subunits of DSIF are homologous to the Spt4 and Spt5 gene products of *S. cerevisiae* and genetic studies link the function of these genes to elongation. NELF also confers DRB sensitivity and functions cooperatively with DSIF in repressing transcription (Figure 3).

TRANSCRIPTION FACTORS AND INDUCIBLE GENE EXPRESSION

Inducible gene expression is often controlled by a complex regulatory network, in which specialized transcription factor relay the extracellular and/or intracellular signals to specific target genes, leading to appropriate physiological responses. Therefore, the

levels of the DNA-binding transcription factors, or rather their activities, are decisive as to whether their target genes are transcribed and to what extent. This implies that these regulators of gene expression in their turn must be tightly regulated. The question arises as to how this is accomplished without the need of an ever-increasing number of upstream regulatory genes. There are several ways by which cells extend the diversity of their regulatory repertoire. One way is to make use of the combinatorial action of a limited set of transcription factors. Another way is to modulate the activity of a transcription factor once it has been synthesized. The various ways by which transcription factor gene expression can be regulated are depicted in Figure 4.

Nucleocytoplasmic shuttling of transcription factors

Regulation of the nuclear import of transcription factors is an important control point for a variety of cellular processes including cell cycle regulation, transcriptional activation and repression, growth and development (Affolter et al., 1999; Macara, 2001; Moroianu, 1999). Depending on the size of the protein, nuclear import through the nuclear pore complex (NPC) can occur either through passive diffusion for small molecules of less than 50 KDa, or by an active process facilitated by the presence of a nuclear localization signal (NLS) (Gorlich and Kutay, 1999; Macara, 2001). Two types of classical NLSs are known; a monopartite NLS consisting of a small stretch of 4 to 6 basic amino acids, typified by the SV40 large T antigen's NLS (P**KKKRK**). A second more complex bipartite NLS consisting of two basic amino acids segments separated by a spacer region of 10 to 12 amino acids is represented by the nucleoplasmin NLS (**KR**PAATKKAGQA**KKKK**LD) (Macara, 2001). These classical NLSs are usually

recognized by the heterodimeric receptor karyopherin (Kap) α and β. Kap α recognizes and binds to classical NLS through its NLS-binding domain (arm repeats), and Kap β is responsible for the docking of the karyopherin α-cargo complex to the cytoplasmic side of the NPC and its subsequent translocation through the pore.

The general model of Kap α/β mediated nuclear transport cycle includes the following steps (Figure 5). (1) Import substrate binds to the Kap α/β heterodimer, in which the Kap α subunit provides the NLS binding site. (2) The resulting trimeric complex docks via Kap β to the cytoplasmic periphery of the NPC. (3) The complex translocates to the nuclear side where it meets an environment with a high RanGTP concentration. The mechanism of translocation is not yet understood. (4) The direct binding of nuclear RanGTP to Kap β terminates the translocation and disassembles the Kap α/β heterodimer. (5) The NLS protein is released from Kap α. (6) Kap α is incorporated into a trimeric RanGTP/CAS/Kap α complex. (7) CAS promotes export to the cytoplasm. (8) In the cytoplasm, RanBP1 causes the dissociation of RanGTP from the RanGTP/CAS/Kap α complex, which is subsequently made irreversible by RanGAP1-triggered GTP hydrolysis. (9) The Ran-free CAS is now in the low-affinity form for Kap α binding, Kap α is released. (10) Kap α can combine with Kap β to re-form the heterodimer and participate in another round of import. Kap β probably accounts for its own export as a RanGTP complex. The efficient cytoplasmic release of RanGTP from Kap β requires RanBP1, RanGAP1, and also the presence of Kap α. Two molecules of Ran appear to be exported per transport cycle.

The activation of nuclear factor κ B (NF-κB) is extensively studied as a classical example of transcriptional regulation by nucleocytoplasmic shuttling of transcription factors. NF-κB family members have been reported as playing a major role in the transcriptional control of many pro- and antiapoptotic genes such as tumor necrosis factor α (TNF-α), interleukin (IL)-2, granulocyte-macrophage colony-stimulating factor (GM-CSF), C-myc and p53 (Cartwright and Helin, 2000). NF-κB is maintained in an inactive form by sequestration in the cytoplasm through interaction with inhibitory proteins, the IκBs. The NLS of NF-κB is masked by I-κB and thus is inaccessible to karypopherins. Various extracellular and intracellular stimuli, such as TNF-α, IL-1, or LPS, trigger rapid degradation of the IκBs (especially IκBα). For IκBα, this degradation requires phosphorylation at serines 32 and 36 by IκB kinase (IKK). Phosphorylation leads to the polyubiquitinylation of IκBα, which targets IκBα for rapid degradation by the 26S proteasome. The degradation of its inhibitor exposes the NLS of NF-κB, resulting in the binding of NF-κB to karyopherins and translocation of NF-κB to the nucleus. This irreversible step in the signaling pathway constitutes a commitment to transcriptional activation. The signal is eventually terminated through cytoplasmic resequestration of NF-κB, which depends on IκBα synthesis, a process requiring NF-κB transcriptional activity, as well as on IκBα–dependent nuclear export (Figure 6).

Regulation of gene expression by controlling RNA polymerase II elongation

The elongation phase of transcription by RNA polymerase II is one of the many steps during the generation of mature mRNAs that is subject to regulation. Shortly after initiation, RNA polymerase II comes under the control of negative transcription

elongation factors, generally termed N-TEFs, and enters abortive elongation. During this postinitiation process, only short transcripts are generated that may be prematurely terminated. These short transcripts arise from transcription of many genes, including c-myb, c-myc, c-fos, HSP70, and the human immunodeficiency virus (HIV) long terminal repeat (LTR), and are normally subject to rapid degradation (Price, 2000). Escape from the action of N-TEF requires the action of at least one positive transcription elongation factor, eventually identified as P-TEFb. P-TEFb allows the transition into productive elongation, producing long transcripts from which mRNAs are derived. In this way, the fraction of initiating RNA polymerase II molecules that produce full-length transcripts is controlled by a selection process that occurs early in the elongation phase of the transcription cycle (Price, 2000).

The expression of viral genes encoded by HIV-1 provides the most striking demonstration of regulation at transcription elongation stage. Tat, a small protein encoded by HIV genome, is required to activate the promoter contained within the viral LTR. This strong transactivator is targeted to the viral promoter through interaction with a region of the nascent RNA transcript called TAR. Tat associates with a three-nucleotide bulge in the stem of a hairpin structure that forms spontaneously in TAR (Figure 7). In the absence of Tat, short abortive transcripts that encode no proteins are predominately produced. The enhancement of processivity brought about by Tat requires P-TEFb. Recent studies have shown that the cysteine residue at position of 261 of cyclin T1 is required for a zinc-dependent interaction between cyclin T1 and HIV-1 Tat (Garber et al., 1998). The targeting of cyclin T1 by Tat seems well suited to the viral goal of propagating during the activation of T cells and the differentiation of monocytes to

macrophages, since it is demonstrated that the ability of the peripheral blood lymphocytes to support HIV replication and productive infection directly correlated with the levels of induced cyclin T1 and CTD kinase activity (Garriga et al., 1998). Results obtained from numerous studies all support the notion that Tat transactivation and HIV replication are closely tied to the levels of P-TEFb (Cdk9-cyclin T1). This allows the virus to infect many cells but maintain its latency until the host cell is activated (Figure 7).

HEAT SHOCK RESPONSE

Background

The heat shock response, characterized by increased expression of heat shock proteins (Hsps) is induced by exposure of cells and tissues to extreme conditions that cause acute or chronic stress. Hsps function as molecular chaperones in regulating cellular homeostasis and promoting survival. If the stress is too severe, a signal that leads to programmed cell death, apoptosis, is activated, thereby providing a finely tuned balance between survival and death (Edwards, 1998). Considering the key role of Hsps in protection against stress-induced damage, it is of utmost importance to elucidate the regulatory mechanisms responsible for Hsp expression.

The inducible Hsp expression is regulated by the heat shock transcription factors (HSFs). In response to various inducers, such as elevated temperatures, oxidants, heavy metals, and bacterial and viral infections, most HSFs acquire DNA binding activity to the heat shock element (HSE), thereby mediating transcription of the heat shock genes, which results in accumulation of Hsps (Wu, 1995). Since the isolation of a single HSF gene from *Saccharomyces cerevisiae* and *Drosophila melanogaster*, several members of the

HSF family have been found in vertebrates (HSF1–4) and plants (Figure 8). The existence of multiple HSFs in vertebrates and plants suggests that different HSFs mediate the responses to various forms of physiological and environmental stimuli (Pirkkala et al., 2001).

Structural and functional features of HSFs

The importance of HSFs as regulators of the heat shock response is reflected by their high cross-species conservation in evolution. The amino-terminal helix-turn-helix DNA binding domain (DBD) is the most conserved functional domain of HSFs (Figure 8 and 9). In fact, DBD is the only domain where comparative structural data is available, since the crystal structure of *Kluyveromyces lactis* and the solution structures of *K. lactis*, *D. melanogaster*, and tomato HSF DBDs have been determined (Littlefield and Nelson, 1999).

Activation-induced trimerization of HSFs is mediated by three arrays of hydrophobic heptad repeats (HR-A/B) characteristic for helical coiled-coil structures, i.e., leucine zippers (Figure 8). The trimeric assembly of HSFs is unusual, as leucine zipper proteins typically are known to associate as homo- or heterodimers. The suppression of HSF trimerization is likely to be mediated by another region of hydrophobic heptad repeats (HR-C) adjacent to the carboxyl terminus of the protein. The HR-C is well conserved among the vertebrate HSFs but poorly conserved in plant and *Saccharomyces cerevisiae* HSFs, which may be a reason underlying constitutive trimerization of HSF in *S. cerevisiae*.

Apart from DBD and oligomerization domain, homology comparisons reveal no other significant conservation in the activation domains of HSFs. By deletion mapping and analysis of chimeric proteins containing HSF sequences fused to heterologous DNA-binding domains, three activation domains in *Saccharomyces cerevisiae* HSF were identified. The activation domain II (AII, residue 410-648) is essential for growth during heat shock but not for normal conditions. The roles of activation domain I (AI, residue 1-172) and activation domain III (AIII, residue 589-833) were not clear when they were first identified by Nieto-Sotelo *et. al* (Nieto-Sotelo et al., 1990), because AIII is dispensable for viability at both normal and stress temperatures. Later studies showed that the AI domain probably functions in non-shocked cells to allow viability and is responsible for transient activity, while the AIII domain is probably responsible for sustained activities at increased temperature (Sorger, 1990). All three activation domains are constitutively active when fused to yAP-1 DNA-binding domain (Nieto-Sotelo et al., 1990). Mechanisms of repression at normal growth temperatures remain unknown, however, some evidence suggests that activation domains are restrained by the DNA-binding domain and trimerization domain (Nieto-Sotelo et al., 1990). Deletions of certain residues in the DNA-binding domain increase the overall activity of HSF, indicating that DNA-binding domain may play an important regulatory role in addition to HSE recognition.

In the budding yeast, HSF is required for both growth at normal temperatures and viability upon stress. In fact, HSF is bound to DNA at all temperatures and heat shock converts the DNA-bound transcriptionally weak HSF into a highly active factor (Nieto-Sotelo et al., 1990). Although upon heat shock the ScHSF shows some increased binding

to secondary, low-affinity HSEs, the overall effect on DNA binding is insignificant and thus not likely to be an important regulatory step in ScHSF activation (Erkine et al., 1999; Jakobsen and Pelham, 1988). Other mechanisms, such as phosphorylation and (or) dephosphorylation, which modify the activity of the transcription activation domains, are more likely to be involved in the activation process (Hoj and Jakobsen, 1994; Shi et al., 1995; Sorger, 1990; Sorger and Pelham, 1988).

In contrast to HSF in budding yeast, *D. melanogaster* HSF and HSF1 of higher eukaryotes exist as monomers in unstressed cells. It has been of great interest to study how the monomeric status of HSF1 is retained under nonstressful conditions. Studies showing that HSF1 produced in bacteria and overexpressed in mammalian cells acquires DNA binding activity spontaneously in the absence of heat shock, leading to the conclusion that the intrinsic activity of endogenous HSF1 is negatively regulated by a titratable cellular factor in mammalian cells. However, when recombinant or overexpressed mammalian HSF1 was expressed at lower concentrations, the DNA binding activity did not occur spontaneously but was shown to be regulated by heat. Therefore, oligomerization of the factor could be repressed in the monomer, possibly by intramolecular interactions between the DBD and oligomerization domains (Pirkkala et al., 2001).

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 from a wide variety of organisms in addition to flies including mice, frogs, and sea urchins. Despite the fact that in the Drosophila embryo the HSF 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). Later, it is demonstrated that early embryo is deficient in Karyopherin α3, the nuclear transporter that specifically transports HSF into nucleus, through cycle 12. From cycle 13 onward the transport factor is present and the HSF is localized within the nucleus thus allowing the embryo to respond to heat shock (Fang et al., 2001).

Transcription regulation at the level of promoter escape: Drosophila Hsp 70 promoter is primed for rapid activation

The Drosophila Hsp70 gene is rapidly and vigorously activated by heat shock. An instantaneous heat shock triggers a 200-fold increase in the level of transcription in 3 minutes (Obrien et al., 1994). The framework for this rapid activation was first suggested by measurements demonstrating the DNase-I-hypersensitive structure of Hsp70 promoter (Lis, 1998). This open chromatin configuration could provide HSF and the general transcription machinery rapid access to specific sequence of the promoter of heat shock genes. At least two protein factors can bind heat shock promoter sequence under non-stress conditions: GAGA factor, which interacts with GAGA repeats in many Drosophila genes (Lis and Wu, 1993), and TATA-binding protein (TBP). The binding of GAGA factor is critical in generating accessibility of promoter sequences. Drosophila

transformants carrying mutation in upstream GAGA repeats show that this element is required for creating the nuclease sensitivity in Drosophila or yeast, and for the rapid recruitment of HSF.

In addition to GAGA factor and TBP, RNA polymerase II molecule is also bound to the unstressed heat shock promoter, further priming the transcription for immediate activation (figure 10). The polymerase is paused after initiating a short transcript. The number of paused RNA polymerase II molecules is estimated at about 1.0 per Hsp70 gene in Drosophila. Characterization of nuclear run-on transcripts and mapping of transcription bubbles in vivo with the single-stranded DNA probe $KMnO_4$ demonstrated that polymerase II is paused at sites covering the interval from +21 to +35, with two peaks of pausing within this interval that are separated by a turn of the DNA helix. The paused polymerase II is largely hypophosphorylated, and its associated short RNA is uncapped when polymerase II is at the start of the paused interval but is largely capped at the distal portion of the paused region.

Interestingly, the GAGA elements were also found to be important for establishing a paused polymerase II and bound TBP. While mutations in the HSE have little effect on generating the pause (but do dramatically affect heat shock inducibility), mutations in the GAGA element reduce the level of paused polymerase by several fold. Sequence around the start site also play a role in generating paused polymerase when the amount of Hsp70 upstream sequence is limiting. The alterations to hybrid Hsp70 promoter that reduce pausing also reduce the heat inducibility of the promoter, indicating that the formation of paused polymerase is a crucial intermediate in the pathway to full transcriptional activation (Lis and Wu, 1993).

Heat shock triggers the trimerization and highly specific binding of HSF to HSE, as a result, polymerase II molecules escape to productive elongation once every 4 seconds (vs. every 10 minutes in uninduced cells). The mechanism by which HSF stimulates this dramatic and rapid increase in transcription is not completely understood, but the most likely explanation is that in response to HSF activation, paused pol II is modified to an elongationally competent form. UV-crosslinking and immunoprecipitation studies show that the paused pol II at the start of uninduced heat shock genes is hypophosphorylated, whereas the pol II population on the body of the induced gene is composed of polymerases that contain both hyper- and hypo-phosphorylated CTD. Therefore, the paused pol II that lacks phosphorylation is likely the form that enters the promoter. In contrast, the activated body of the gene is covered with CTD-phosphorylated pol II. It is tempting to consider that the escape of paused pol II depends on its CTD phosphorylation by a CTD kinase that is either recruited or activated by HSF. P-TEFb, a heterodimer of the kinase Cdk9 and cyclin T, is likely the most promising candidate. P-TEFb was isolated as a factor that stimulates formation of productive transcription elongation complexes in vitro. It is reported that upon heat shock, P-TEFb, like the regulatory factor HSF, is rapidly recruited to heat shock loci, and this recruitment is blocked in an HSF mutant. Direct recruitment of a Gal4-binding domain P-TEFb hybrid to an *hsp70* promoter in *Drosophila* cells is sufficient to activate transcription in the absence of heat shock (Lis et al., 2000). Analyses of point mutants show this P-TEFb stimulation is dependent on Cdk9 kinase activity and on Cdk9's interaction with cyclin T (Lis et al., 2000). These results, coupled with the frequent colocalization of P-TEFb and the hypophosphorylated form of RNA polymerase II found at promoter-pause sites,

support a model in which P-TEFb acts to stimulate promoter-paused Pol II to enter into productive elongation.

RNA INTERFERENCE

The term "RNA interference" (RNAi) was coined after the ground-breaking discovery that injection of double-stranded RNA (dsRNA) into the nematode *Caenorhabditis elegans* leads to specific silencing of genes highly homologous in sequence to the delivered dsRNA (Tuschl, 2001; Zamore, 2001). The RNAi phenotype is either identical to the genetic null mutant or resembles an allelic series of mutants. The dsRNA can also be delivered by feeding bacteria that express dsRNA from recombinant plasmids to the worm or by soaking the worm in a solution containing the dsRNA. In rapid sequence, RNAi was observed in other animals including mice, and therefore this process possibly exists also in humans.

RNAi appears to be related to the post-transcriptional gene silencing (PTGS) mechanism of cosuppression in plants and quelling in fungi. Cosuppression is the ability of some transgenes to silence both themselves and homologous chromosomal loci simultaneously. The initiator molecule for cosuppression is believed to be aberrant RNA, possibly dsRNA, and some components of the RNAi machinery are required for post-transcriptional silencing by cosuppression (Barstead, 2001; Bastin et al., 2001).

The natural function of RNAi and cosuppression is thought to be protection of the genome against invasion by mobile genetic elements, such as transposons and viruses, which produce aberrant RNA or dsRNA in the host cell when they become active. Thus,

specific mRNA degradation is thought to prevent transposon and virus replication (Bosher and Labouesse, 2000; Hammond et al., 2001).

RNAi is a two-step process. First, the dsRNA is cleaved to yield siRNAs. Then, the siRNAs target the corresponding mRNA for destruction (Zamore, 2001). Since both steps require multidomain proteins, multiple proteins, or both, it is difficult to imagine how the pathway could have arisen unless each step operates independently in cellular functions separate from RNAi. In support of this idea, Dicer, the enzyme that cleaves siRNAs from long dsRNA, also functions in the production of small temporal RNAs (stRNAs) from precursor stem-loop transcripts. The stRNAs *lin-4* and *let-7* regulate the timing of development in *C. elegans*. *lin-4* appears to be restricted to worms, whereas *let-7* is found more widely among animals, but only those with bilateral symmetry. The presence of Dicer homologs in *Arabidopsis thaliana*, humans, and *Schizosaccharomyces pombe* suggest that a wide variety of small regulatory RNAs remains to be discovered. Furthermore, RNAi-like phenomena are found throughout eukaryotes, suggesting that the RNAi machinery is quite ancient, having evolved prior to the divergence of animals, plants, and fungi. Intriguingly, the genes thought to encode the RNAi machinery appear to have been lost in *Sacchromyces cerevisiae*. A key to understanding the evolutionary history of RNAi will be the discovery of widely conserved, post-transcriptional silencing phenomena that do not require dsRNA processing by Dicer.

A particularly fascinating aspect of RNAi is its extreme efficiency—a few trigger dsRNA molecules suffice to inactivate a continuously transcribed target mRNA for long periods of time; the inactivation persists through cell division, spreads to untreated cells and tissues of plants, and is even inherited by subsequent generations of nematodes.

Although conversion of the long trigger dsRNA (usually several hundred bp) into many 21–25 nt siRNA fragments results in some degree of amplification, it appears that an additional mechanism is necessary to explain the potency and self-sustaining nature of RNAi observed in *C. elegans*. Since RNA-directed RNA polymerase (RdRP) was one of the genes identified through genetic screenings for RNAi mutations, its involvement in the amplification process has been postulated. In plants, however, the RdRP gene (SDE-1) is required only for transgene-induced PTGS, but not for virus-induced PTGS. Similarly, EGO-1, the SDE-1 homolog of nematodes, is essential for RNAi but only in germline cells. In addition, sensitivity of the antisense but not the sense strand of the trigger dsRNA to chemical modification seemed to be at odds with the presumed role of RdRP in replication of trigger dsRNAs or siRNAs, since modification of the original trigger dsRNA, either sense or antisense strand, would be diluted during multiround replication by RdRP, such that the overall RNAi efficacy should not be affected. These previous studies cast doubt on the role played by RdRP in the general RNAi mechanism. Recently, new studies provide convincing biochemical and genetic evidence that RdRP indeed plays a critical role in amplifying the RNAi effect. In addition, characteristics of siRNA that may be critical for serving as a primer for RdRP have been revealed. A new model for RNAi emerges from these recent studies (Figure 11) (Nishikura, 2001).

Further evidence of RdRP involvement has been obtained by cleverly designed genetic studies in *C. elegans*. The polarity of the RdRP reaction predicts that the newly synthesized dsRNA may extend beyond the sequence complementary to the initial trigger dsRNA, into upstream regions of the target mRNA. Furthermore, a new population of secondary siRNA might be generated from the extended dsRNA (Figure 12). These

secondary siRNAs were also able to induce secondary RNA interference, a phenomenon termed "transitive RNAi" (Nishikura, 2001).

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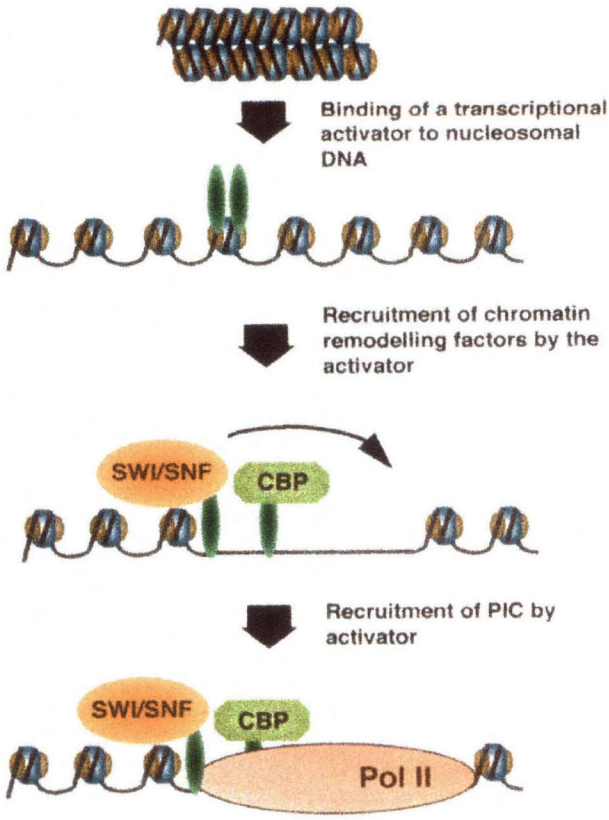

Figure1. Steps in transcriptional regulation. Cartoon of the steps required for transcriptional activation of a gene. SWI/SNF and CBP are given as examples of the different chromatin-remodeling activities. The activator is shown in green.

(Roberts, S. G. E., Cellular and Molecular Life Sciences, vol 57, 1149-1160, 2000)

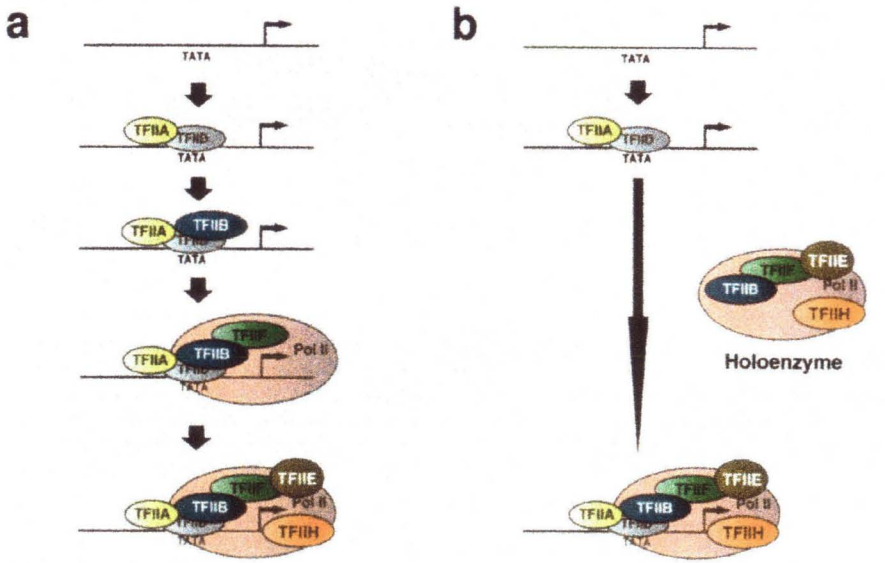

Figure 2. Preinitiation complex assembly.

(a) The ordered assembly model of the preinitiation complex.

(b) The holoenzyme model of pre-initiation complex assembly.

(Roberts, S. G. E., Cellular and Molecular Life Sciences, vol 57, 1149-1160, 2000)

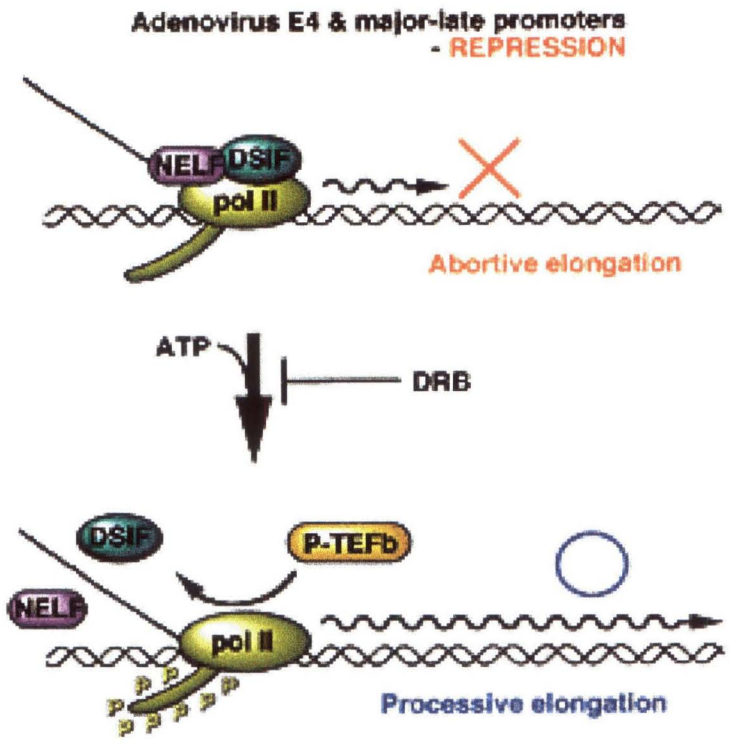

Figure 3. Positive and negative elongation factors. DSIF and NELF function together to induce the arrest of elongating RNA polymerase II. The inhibitory activities of DSIF and NELF can be overcome by P-TEFb, a kinase complex that phosphorylates CTD of pol II.

(Yamaguchi et. al., Journal of Biochemistry, vol 129, 185-191, 2001)

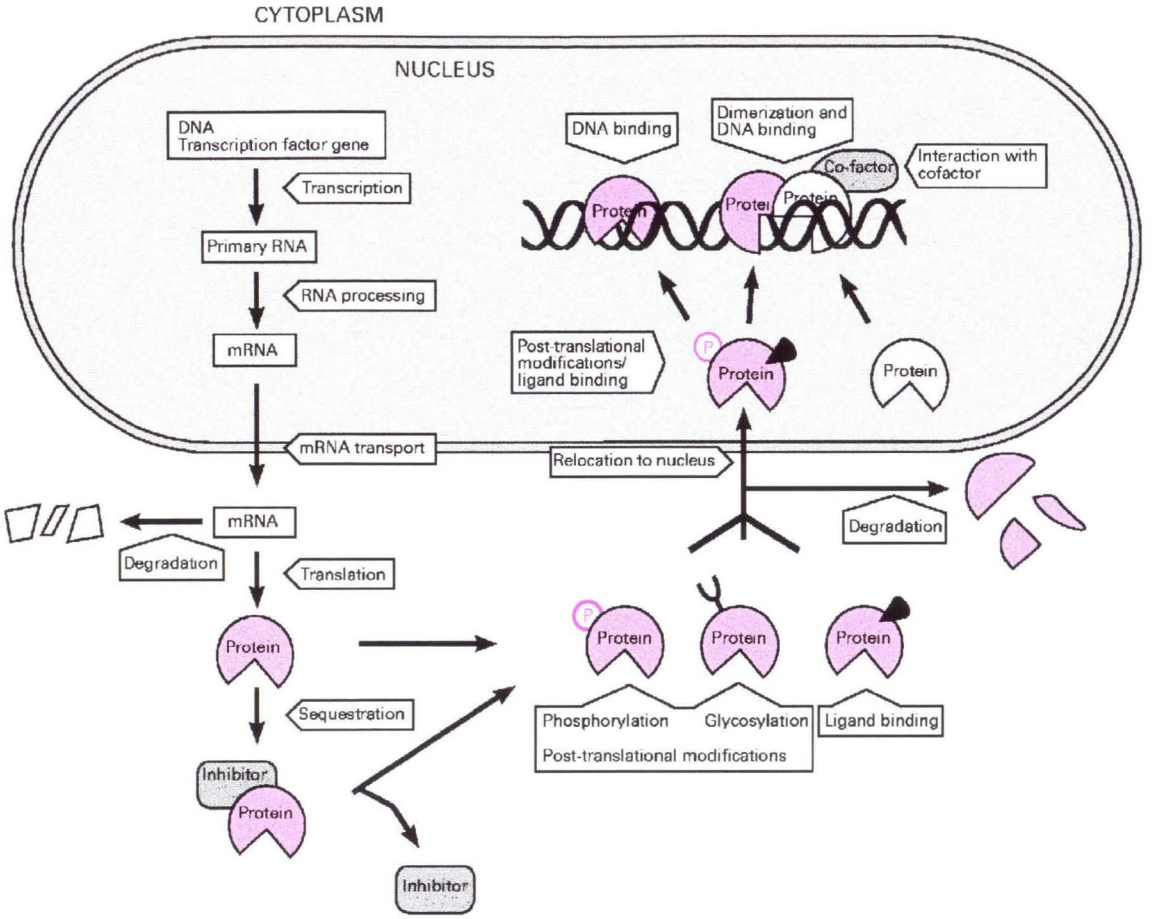

Figure 4. Control levels in the expression and activation of transcription factors. The scheme depicts crucial steps in the synthesis and activation transcription factors. Along the pathway potential regulatory points are indicated (white labels). The concentration and activity of a particular transcription factor may be regulated at several points down the pathway to its ultimate action in gene transcription.

(Calkhoven and AB, Biochemical Journal, vol 317, 329-342, 1996)

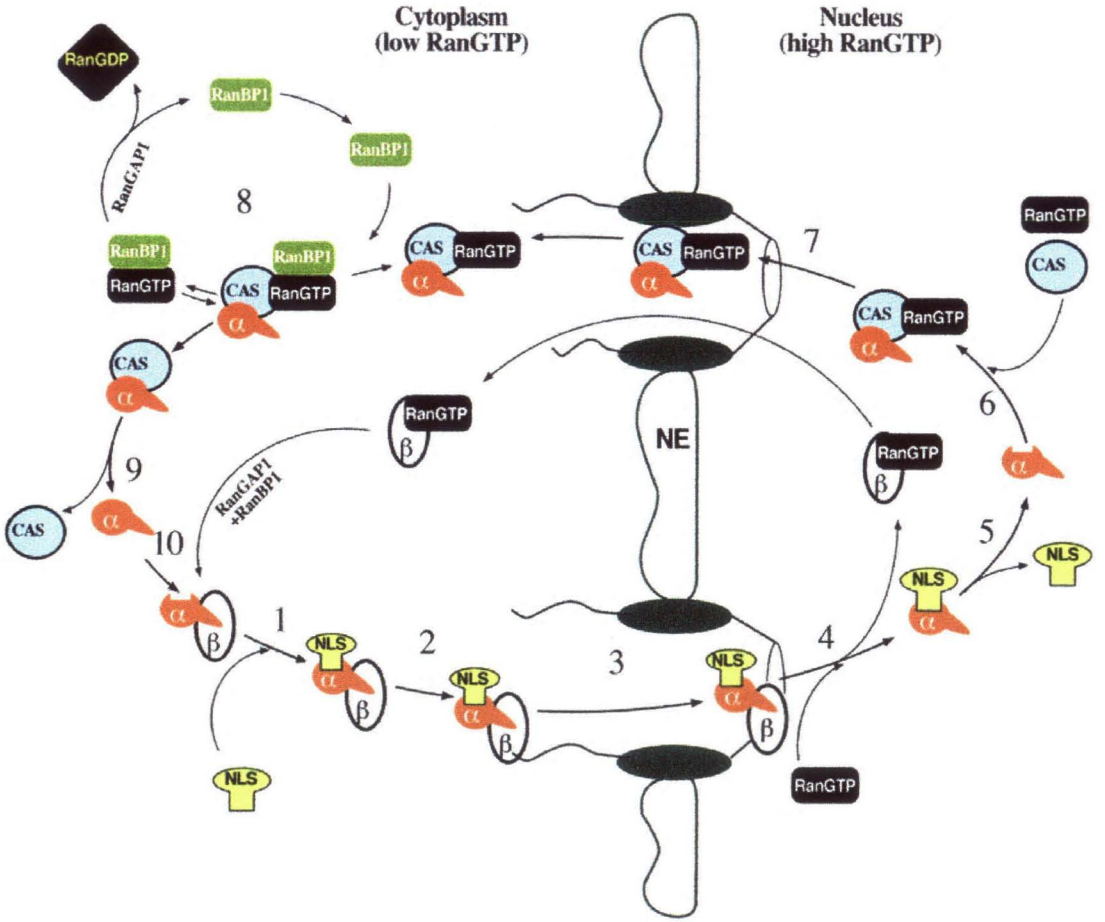

Figure 5. Transport cycles of the classical NLS-dependent import pathway. For details see text (page 13). Abbreviations: α and β, Karyopherin α and β, respectively; NLS (nuclear localization signal) indicates the import substrate.

(Gorlich and Kutay, Annual Review of Cell and Developmental Biology, vol 15, 607-660)

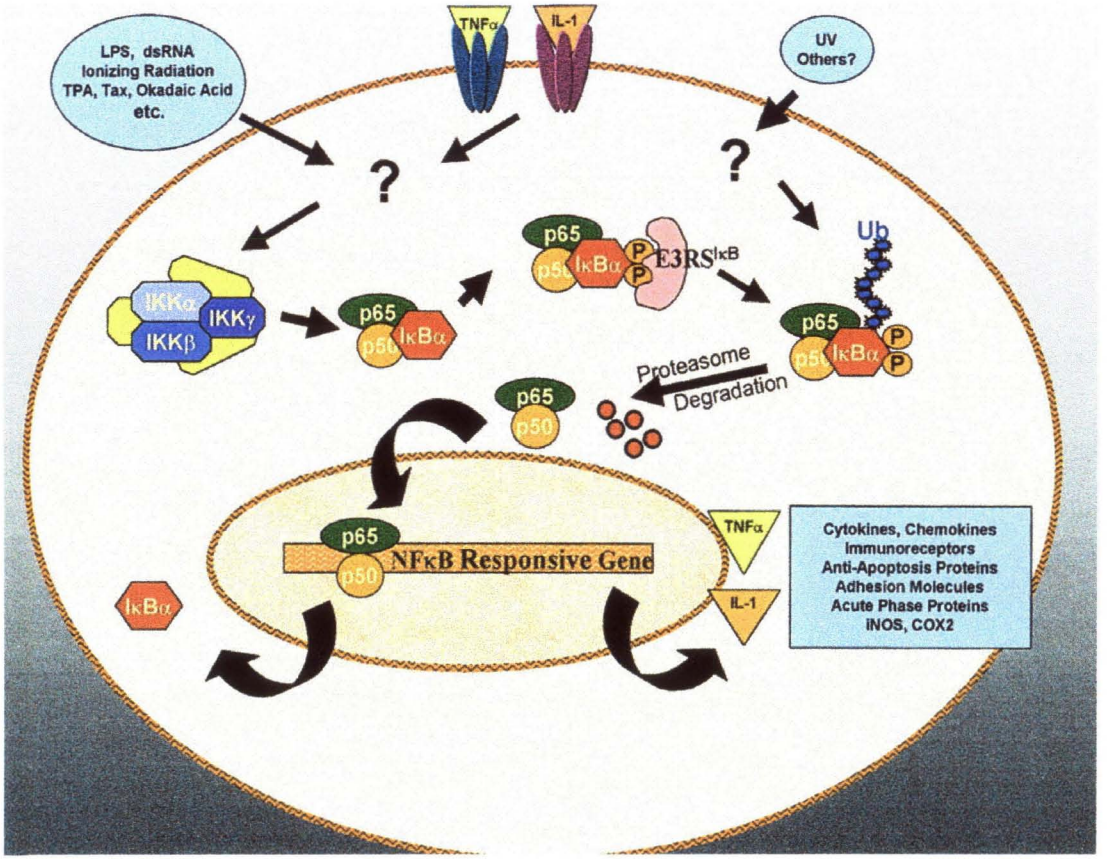

Figure 6. A schematic model of NF-κB activation. Various stimuli activate IKK through the action of as-yet-unidentified components. Once activated, IKK phosphorylates IκB , leading to the polyubiquitination of IκB . This then targets IκB for rapid degradation by the 26S proteasome. IκB degradation exposes the nuclear localization sequence on NF-κB resulting in its translocation to the nucleus. In the nucleus NF-κB regulates transcription of target genes, including IκB , which functions to terminate NF-κB activity. Some of the NF-κB target genes code for inflammatory mediators, such as TNFa and IL-1 and chemokines, which can lead to recruitment of additional cells to the inflammatory response.
(Karin and Ben-Neriah, Annual Review of Immunology, vol 18, 621-663)

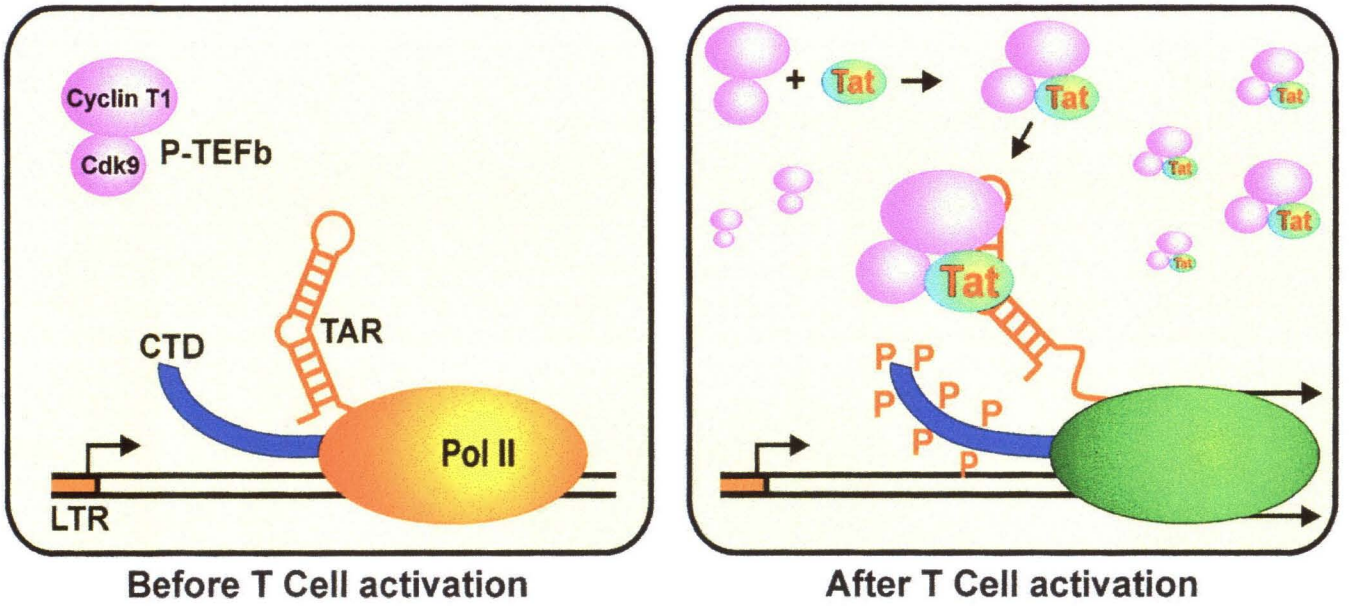

Figure 7. Model for recruitment of P-TEFb during T-cell activation. Before T-cell activation, the HIV LTR produces mostly short transcripts because of low levels of P-TEFb, comprised of Cdk9 and cyclin T1. As T cells are activated, cyclin T1 levels and then Tat levels rise. P-TEFb-Tat complexes form, associate with TAR, cause hyperphosphorylation of the CTD, and activate the LTR to produce full-length mRNAs.

(Price, Molecular and Cellular Biology, vol 20, 2629-2634)

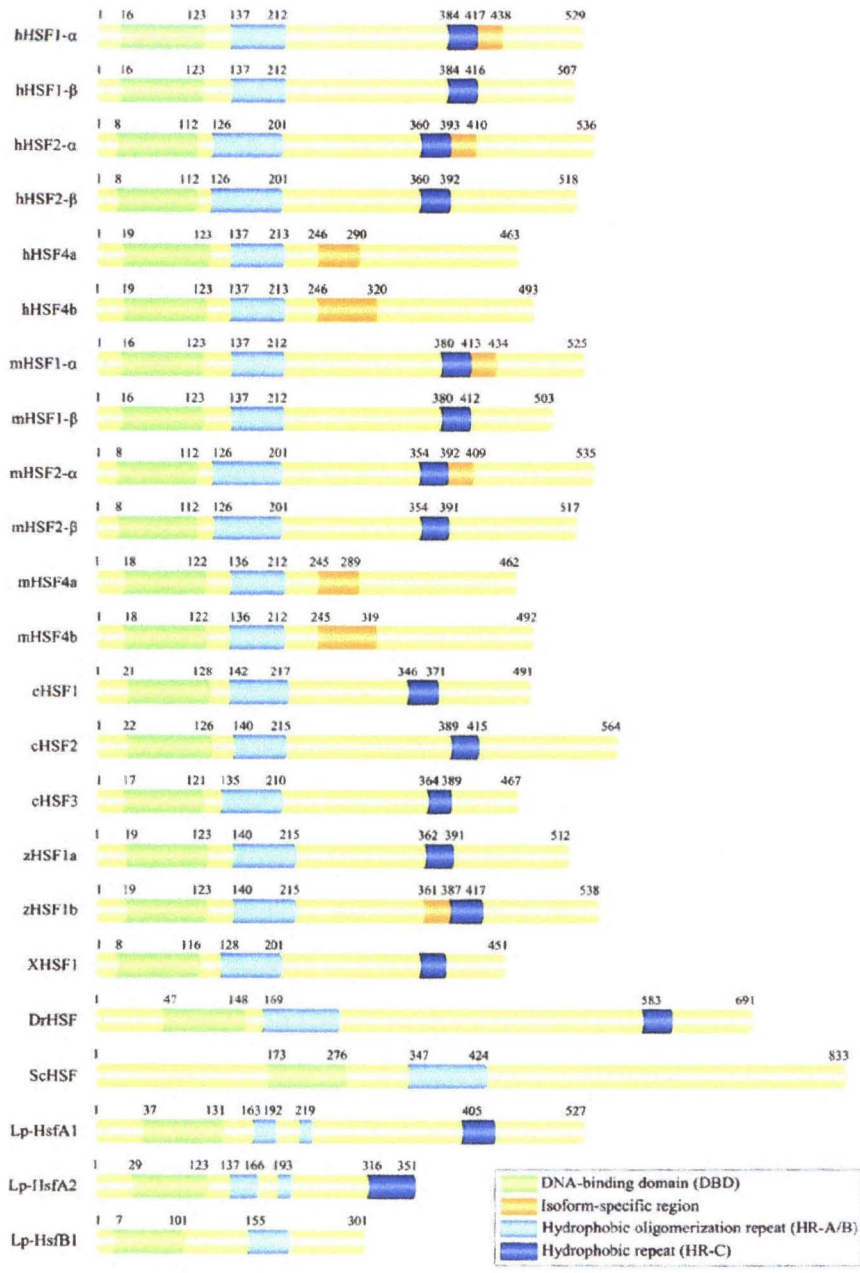

Figure 8. The HSF family: comparison of structural domains. The conserved domains of the distinct HSFs—i.e., the DNA binding domain (DBD), the oligomerization domain (HR-A/B), the carboxyl-terminal heptad repeat (HR-C), and the isoform-specific regions—are indicated.

(Pirkkala et. al., The FASEB Journal, vol 15, pp1118-1131, 2001)

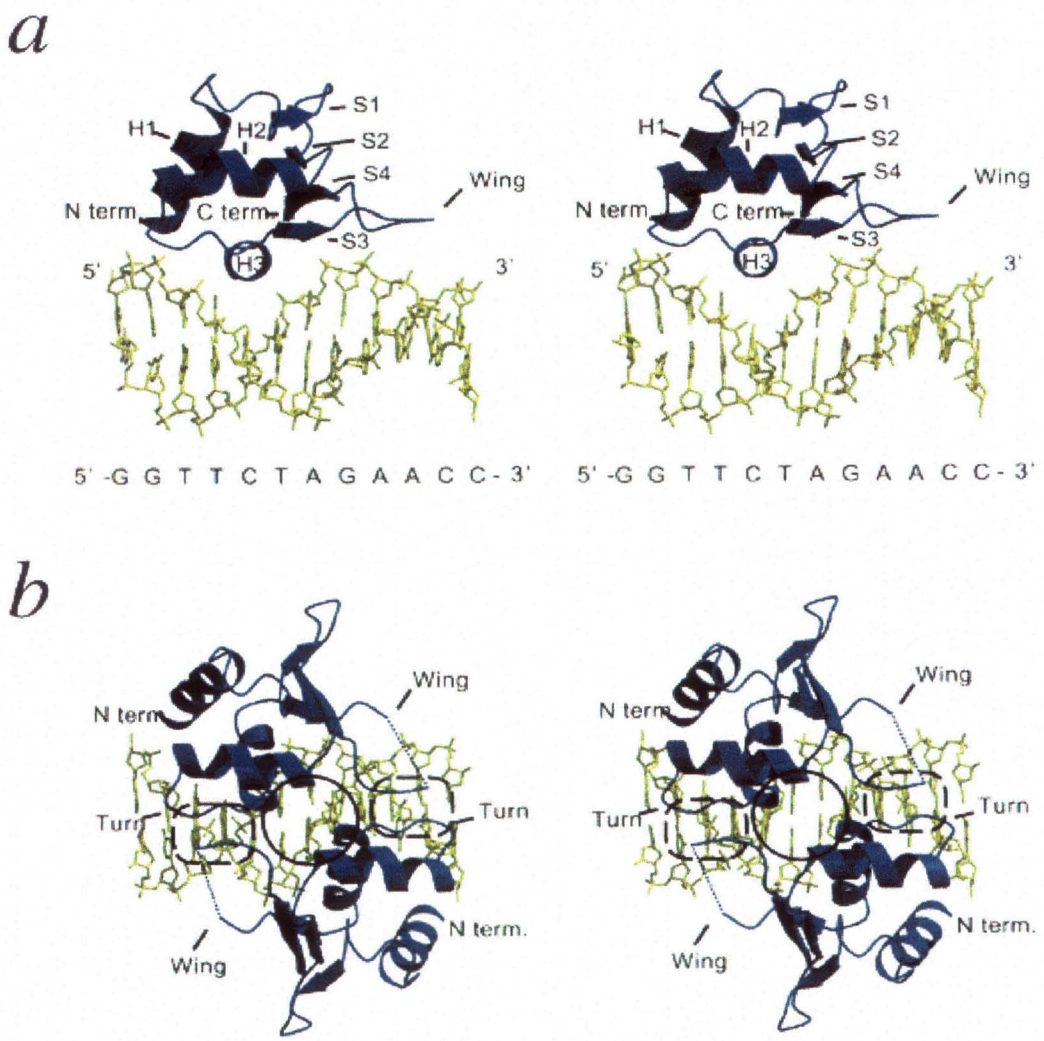

Figure 9. Overall structure of the HSF DBD–DNA complex. Two different stereo views of the complex were generated. The DNA is in yellow and the DBD is in blue. The helices are labeled H1, H2 and H3, respectively; the strands are labeled S1, S2, S3 and S4. The 'wing' and the turn of the helix-turn-helix are labeled as well.

(Littlefield and Nelson, Nature Structure Biology, vol 6, 464-470, 1999)

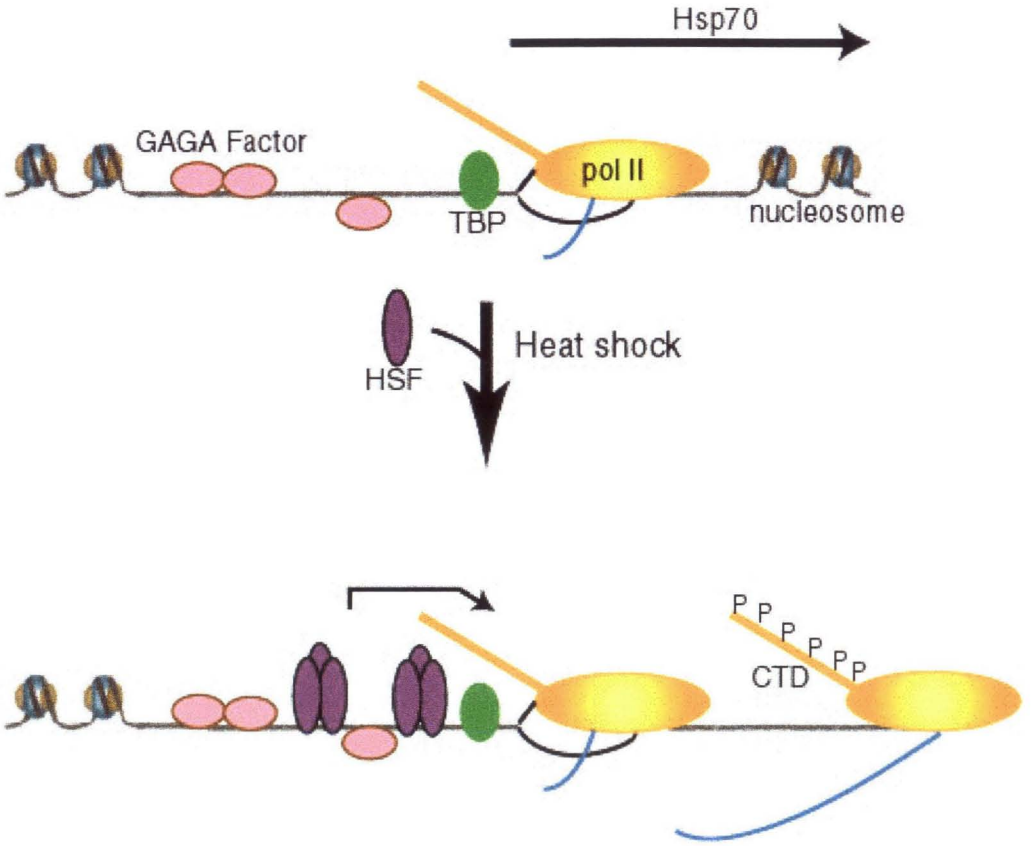

Figure 10. Model depicting the Hsp70 promoter architecture before and after heat shock activation. The "P" sign associated with CTD (C-terminal domain) of the largest subunit of pol II indicate the hyperphosphorylated state.

(Adapted from Lis., J., Mechanisms of Transcription, Cold Spring Harbor Symposium on Quantitative Biology, vol 63, 347-364, 1998)

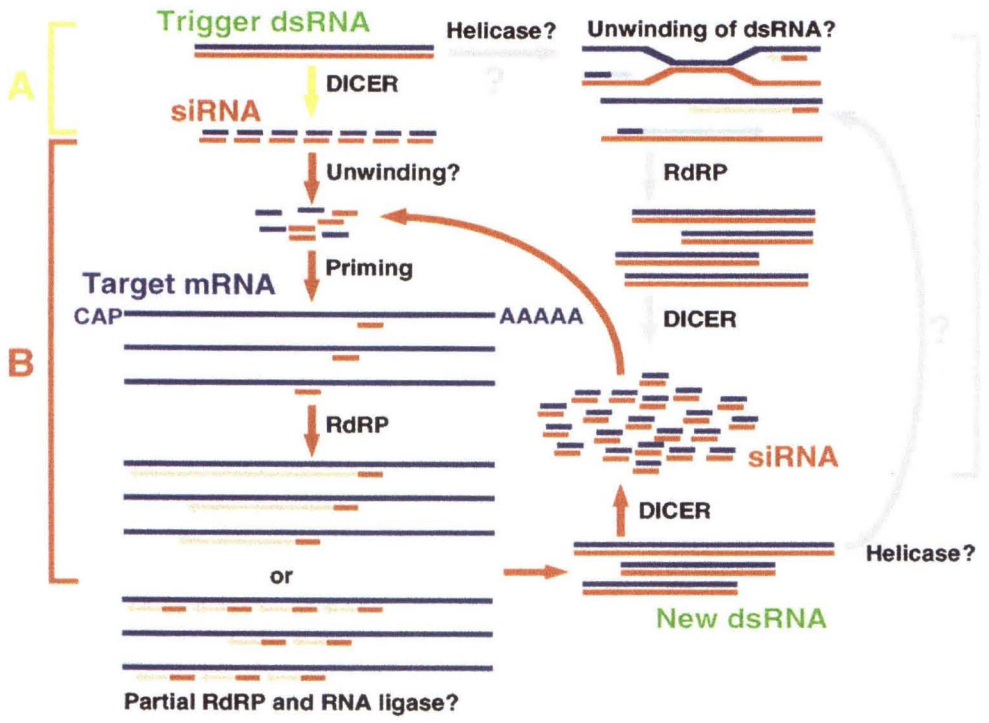

Figure 11. A Model for RNAi

(A) In the "initiation" stage of RNAi, a small amount of trigger dsRNA is processed into siRNA (yellow arrow), which is used as an RdRP primer.

(B) The RdRP reaction converts target mRNAs into new dsRNAs (next generation of trigger dsRNAs), which are then processed into new siRNAs, establishing a self-sustaining cycle of RNAi "maintenance" (red arrows).

(C) Replication of trigger- or newly synthesized-dsRNA by RdRP would amplify the potency of RNAi by further increasing the amount of siRNA, since both sense and antisense strands of trigger dsRNA and siRNA can be now utilized. However, the in vivo significance of this pathway (gray arrows) has not yet been established.

((Nishikura, K., Cell, vol 107, 415-418, 2001)

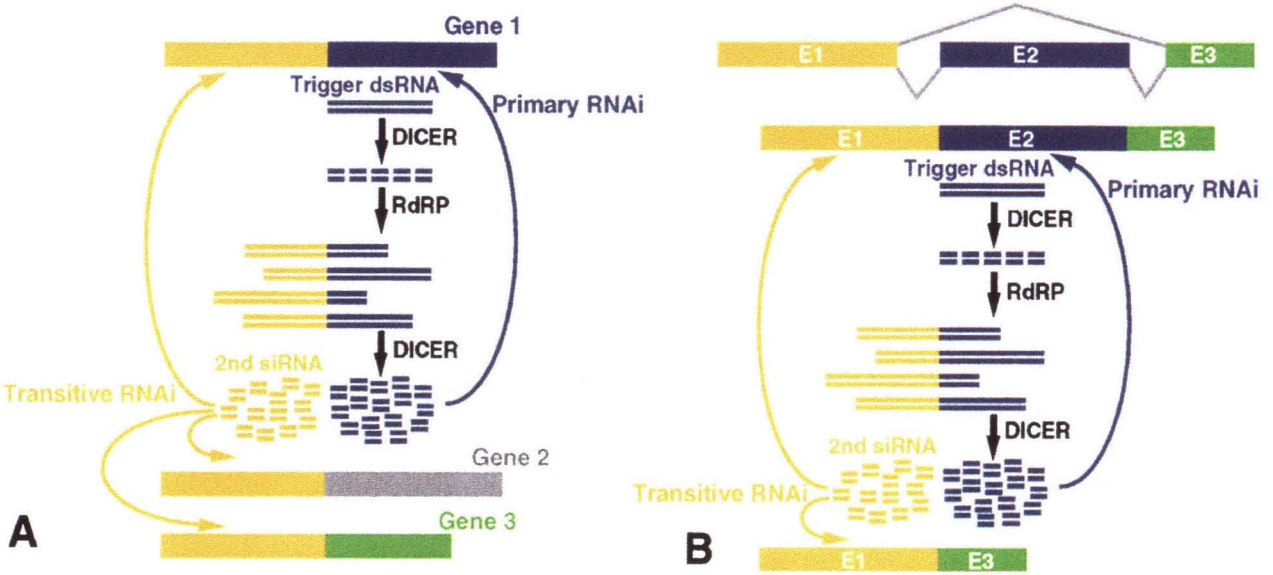

Figure 12. Transitive RNAi by Secondary siRNA. Secondary siRNAs, generated from the dsRNA extended (orange) upstream of the primary target region (blue) by RdRP, promote transitive RNAi against their sequence homologous gene family members (A) or alternatively spliced mRNAs (B).

(Nishikura, K., Cell, vol 107, 415-418, 2001)

CHAPTER TWO

DEVELOPMENTAL REGULATION OF THE HEAT SHOCK RESPONSE BY NUCLEAR TRANSPORT FACTOR KARYOPHERIN–α3

The work in this chapter was done in collaboration with Dr. Xiang-Dong Fang and Kim Tran.

Xiang-Dong Fang, Tianxin Chen, Kim Tran and Carl S.Parker
Developmental Regulation of the Heat Shock Response by Nuclear Transport Factor Karyopherin α3.
Development, vol 128, no. 17, 3349 -3358

ABSTRACT

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 nuclear transport protein Drosophila 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. Consistent 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.

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 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 from 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) which 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 129bp 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 BamHI and NdeI restriction sites. The fusion plasmid DNA was then amplified and purified from *E. coli*. A Drosophila embryonic c-DNA 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 (pH8). 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 (pH8), 150 mm NaCl and 2.5 mm Ca_2Cl.

Colony Lift β-galactosidase Filter Assay

The primary His^+ transformants were grown in 5ml selective SD medium at 30^0C to O.D. 0.5. Cells were then re-streaked onto a 150 mm SD/-His/-Trp/-Leu agar plate and incubated for 72 hr at 30°C. A sterile Whatman #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 min. A second sterile Whatman #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 2nd filter, colony side up. Both filters were incubated at room temperature for up to 8 hr until the appearance of blue colonies.

In-vitro Cross-linking Assay

A 191aa 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 154aa polypeptide with the NLS deleted (mini-ΔNLS probe) was also over-expressed in DE3 as were and 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, pH7.9, 100 mm KCl, 1 mm EDTA and 0.2% Triton-x 100) with 2 μl of γ^{32}P-Mini probes (1 ng/μl, ~80000cpm/μl,) for 20 min 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 min at 25°C. The reaction was quenched by the addition of 2 μl of 200 mM Lysine for 10 min. 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 GeneBank (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 poly-L-lysine-coated slides for 20 min on ice. Then the cells were permeabilized with 45 μg/ml digitonin in Buffer A (20mM Hepes (pH7.3), 110mM potassium acetate, 5mM sodium acetate, 2mM magnesium acetate, 1mM EGTA, 2mM DTT, 1mM PMSF, and "Complete Protease Inhibitors" from Boehringer for 20 min on ice. NLS-EGFP fusion proteins, dKap-α3 and dKap-β were pre-incubated on ice for 20 min, then were incubated with permeabilized cells at room temperature for 20 min. Cells were washed with Buffer A and fixed with Histochoice Tissue Fixative MB (Amresco). Slides were mounted in A Buffer/90% glycerol containing 1mg/ml o-phenylenediamine.

Immuno-fluorescence 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 reveals only a single strongly reactive protein species of the correct molecular weight.

18x18 mm #1 cover slips were coated in 1 mg/ml poly-L-lysine for 15 min and air-dried. 0.5 ml of Drosophila SL2 cells with a density of $4x10^5$ cells/ml were placed

onto each cover slide and incubated for 15 min at room temperature. Cells were then heat shocked at 37°C for various times, washed with PBST, and fixed with Histochoice 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 hr at room temperature. Cells were then washed with PBST 4 times for 10 min 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 fluorscent images were viewed and photographed with Zeiss Axioplan microscopy with UV irradiation and appropriate filters.

Fixed Drosophila embryos were rehydrated by a series methanol/PBST mixtures: 15 min each in 75%, 50%, 25% methanol/PBST and 30 min in PBST. Immuno-fluorescent staining was then carried out as described by (Patel, 1994). All antibodies were pre-incubated with 0-12 hr embryos overnight at 4° C. Anti-dKap α3 antibodies were diluted 1:500 and anti-dHSF antibodies were diluted at 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 min. Images were taken by 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

0-2 hr, 0-4 hr and 0-6 hr embryos were collected and washed with 0.03% Triton X-100/0.9% NaCl. Non-shocked or heat shocked (37°C for 15 min) embryos were rinsed twice with homogenization buffer (50mM Tris (pH7.5), 140mM NaCl, 5mM $MgCl_2$, 0.05% NP-40, 1mM PMSF, 1μg/ml pepstain A, 1-2μg/ml aprotinin, 1μg/ml leupeptin, and then homogenized in 2V 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% NFDM overnight. The blot was then probed with anti-dKap–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 min. All embryos were washed with NaCl-Triton (0.9% NaCl, 0.03% Triton-X 100), dechorionated in 50% bleach for 3 min and fixed in n-heptane/formaldehyde/PBS (5:1:5) for 30 min. 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 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 (Figure 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 $5x10^6$ cDNAs from a Drosophila embryonic library (see Experimental Procedures). All three clones contained overlapping amino acid sequences (Figure 1B). One of the three contained an apparent full-length cDNA sequence encoding a 514-residue protein. 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 carboxyl portion of the protein (Figure 1C) (Conti et al., 1998). At the amino acid level dKap-α3 shows 45% identity to Drosophila-α1, 44% identity to Drosophila-α2 and 67% identity to human 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* (Figure 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' (Figure 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-PAG electrophoresis (Figure 2 lane 1). A complex of approximately 90kD was observed 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 (Figure 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 Figure 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 Figure 2, those mutants, which 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, which are transported in vivo, do bind dKap α3 in vitro although one more weakly than wild type: Q399I/K400R/L404R, lane 2; E406P, lane 8. A constitutively localized mutant Q403L binds efficiently 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 Experimental Procedures). 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 (Figure 3A; compare lanes 1, 2 and 3). Interestingly, the molecular weight 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 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 to 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 Figure 3B, lanes 3 to 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 α binds to its target NLS-peptide cargo within the arm repeats. Indeed, an N-terminal β-binding domain-deleted form of the yeast α 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 (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 Figure 4, wild type NLS fused to EGFP was effectively docked on the nuclear periphery in the presence of both α and β proteins (Figure 4, panel B). The NLS-EGFP protein alone was not able to dock on the nuclear membrane (Figure 4, panel A), 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 (Figure 4 panel C). 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 (Figure 4, panel D).

Drosophila Karyopherin–α3 Is Excluded from the Nucleus After Heat Shock

Biochemical and immuno-cytochemical studies of Hela and Drosophila SL2 cells have shown that vertebrate HSF1 and dHSF are distributed in a diffused 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 immunofluorescence 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 (Figure 5). However, after 15 minutes of heat shock, dKap α3 is localized exclusively in the cytoplasm around the nuclear periphery. This peripheral nuclear staining increased 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 Figure 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 (Figure 6; lanes 1 and 2). At 0-4 and 0-6 hours, dKap α3 is abundantly expressed (Figure 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 (Figure 6B).

In situ immunostaining of Drosophila embryos was used to assess the temporal and spatial expression of both dHSF and dKap α3 proteins. In Figure 7A-7L, non-heat shocked embryos are shown on the left (NS) and heat shocked embryos on the right (HS). The embryonic nuclei are stained with DAPI to facilitate nuclear positioning and determination of the division cycle of the embryo. Figure 7A shows a representative

cycle 11 embryo and 7E shows a cycle 13 embryo. Figures 7C and 7D show cycle 12 and 13 heat shocked embryos, respectively. The distribution of dHSF is revealed by immunostaining with an anti-dHSF monoclonal antibody. At cycle 11 dHSF is clearly seen in the cytosol of the embryo and excluded from the nucleus (Figure. 7E). At cycle 13 dHSF is localized within the nucleus in the absence of heat shock (Figure. 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 (Figure. 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 (Figure. 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 punctuate/granular pattern to the nuclear staining (Figure. 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 (Figure. 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 Figure 7M (panel 1), where a cycle 10-11 embryo is compared against 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, 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. Further analysis 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 (Figure 7M, panel 2). These data further strengthen the correlation between the presence of dKap-α3 and the nuclear localization of dHSF.

DISCUSSION

Early embryos 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 which 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 was 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 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 coincide 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 to 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 the 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 dKap α3 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 not 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 likely 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 for future studies 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.

ACKNOWLEDGEMENTS

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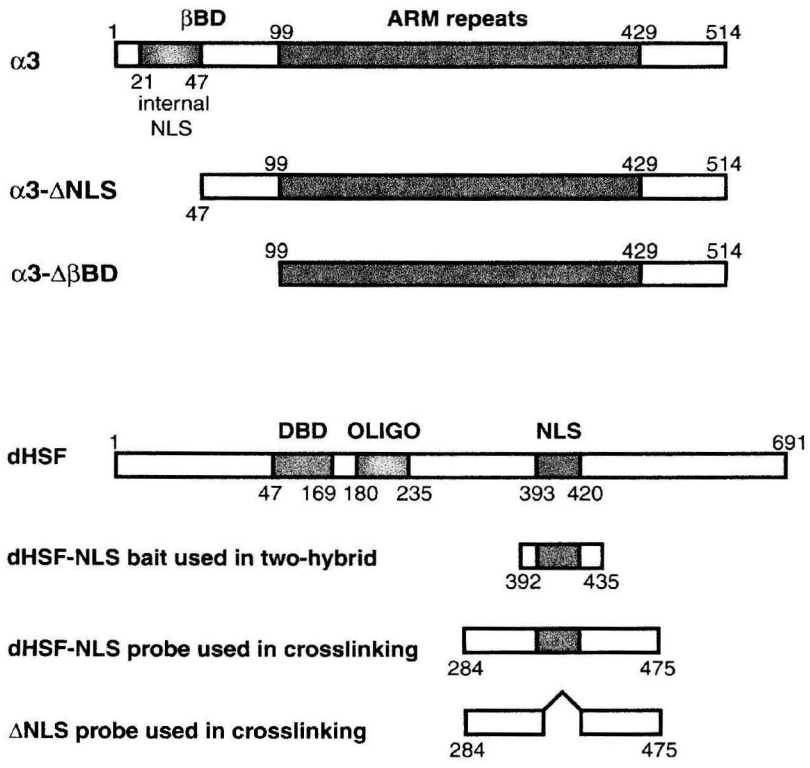

Figure 1A

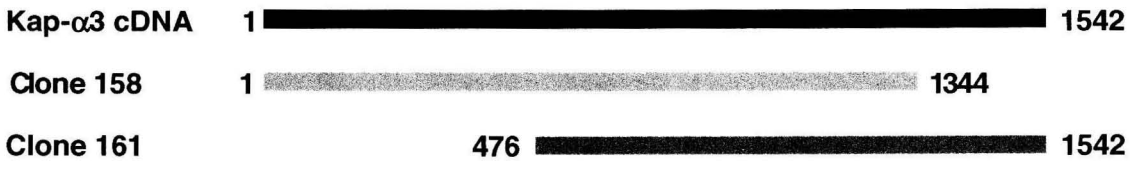

Figure 1B

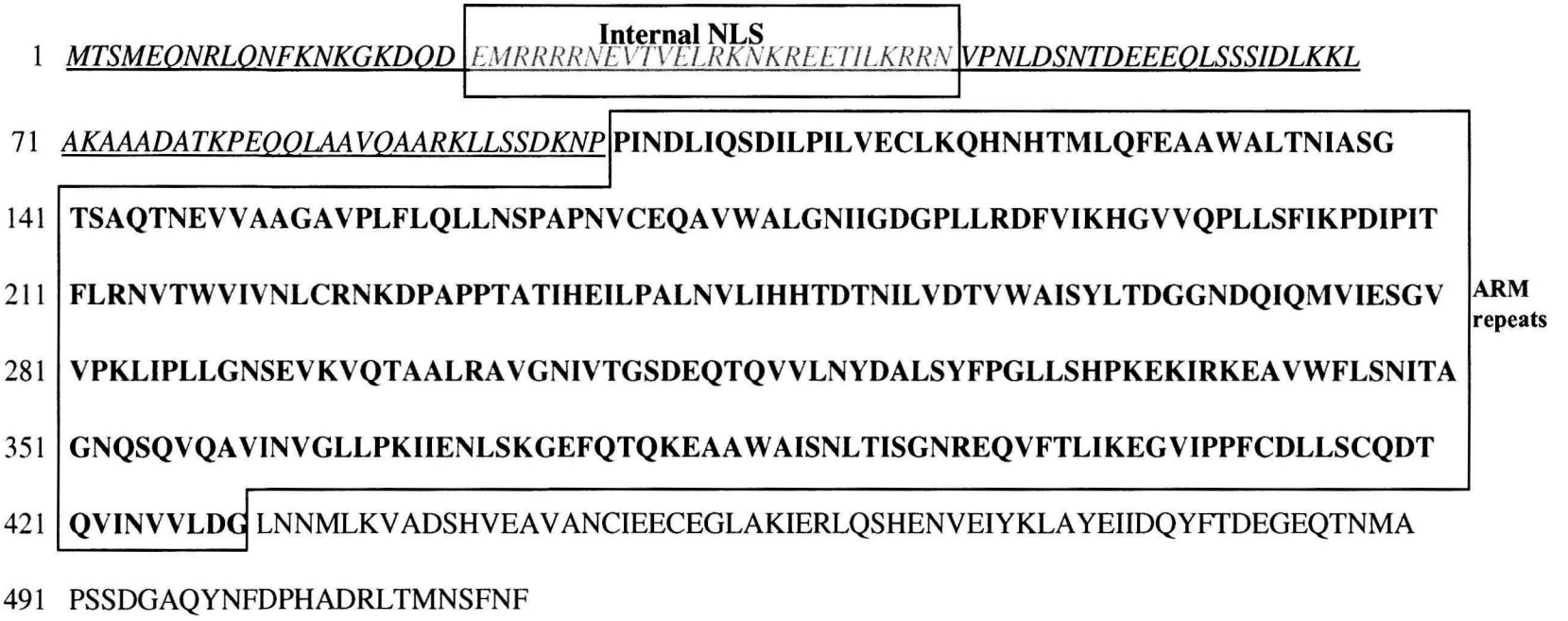
Internal NLS
1 MTSMEQNRLQNFKNKGKDQD EMRRRRNEVTVELRKNKREETILKRRN VPNLDSNTDEEEQLSSSIDLKKL
71 AKAAADATKPEQQLAAVQAARKLLSSDKNP PINDLIQSDILPILVECLKQHNHTMLQFEAAWALTNIASG
141 TSAQTNEVVAAGAVPLFLQLLNSPAPNVCEQAVWALGNIIGDGPLLRDFVIKHGVVQPLLSFIKPDIPIT
211 FLRNVTWVIVNLCRNKDPAPPTATIHEILPALNVLIHHTDTNILVDTVWAISYLTDGGNDQIQMVIESGV
281 VPKLIPLLGNSEVKVQTAALRAVGNIVTGSDEQTQVVLNYDALSYFPGLLSHPKEKIRKEAVWFLSNITA
351 GNQSQVQAVINVGLLPKIIENLSKGEFQTQKEAAWAISNLTISGNREQVFTLIKEGVIPPFCDLLSCQDT
421 QVINVVLDG LNNMLKVADSHVEAVANCIEECEGLAKIERLQSHENVEIYKLAYEIIDQYFTDEGEQTNMA
491 PSSDGAQYNFDPHADRLTMNSFNF
ARM repeats

Figure 1C

FIGURES AND FIGURE LEGENDS

Figure 1A. 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.

Figure 1B. 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.

Figure 1C. 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

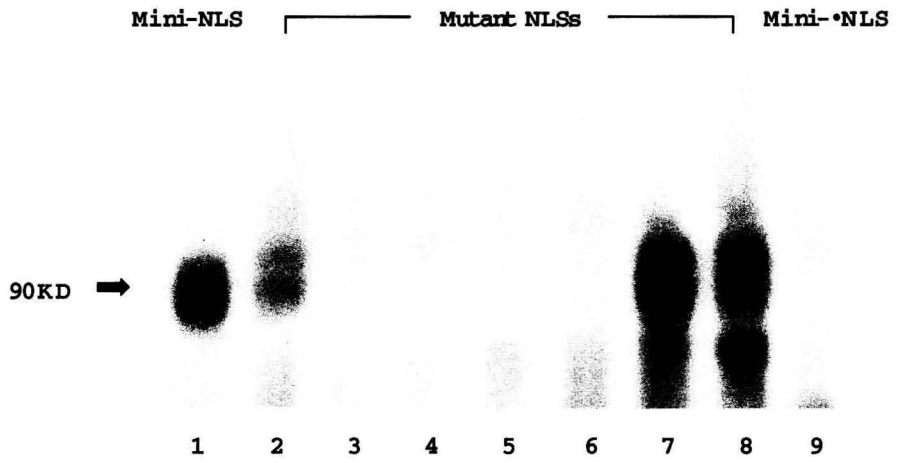

Figure 2

Figure 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 immuno-staining (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 (lane2); 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 (lane8) 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 (~90KD) 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 weight complexes seen are dimers of probe and are mini-NLS probe specific.

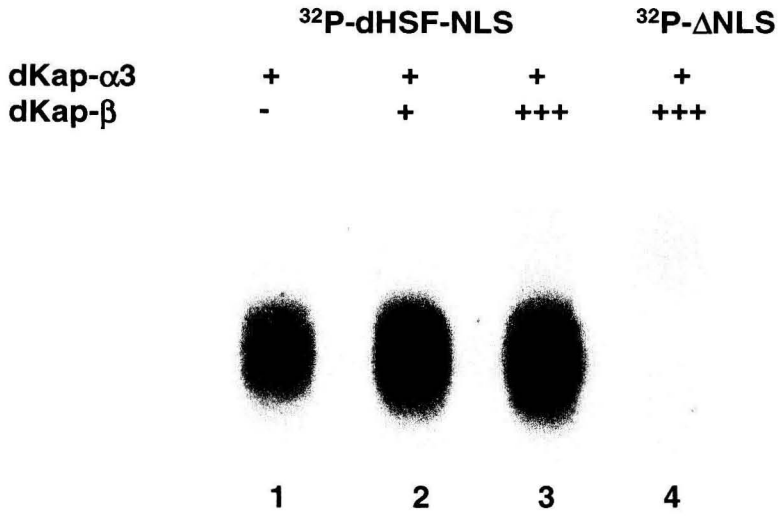

Figure 3A

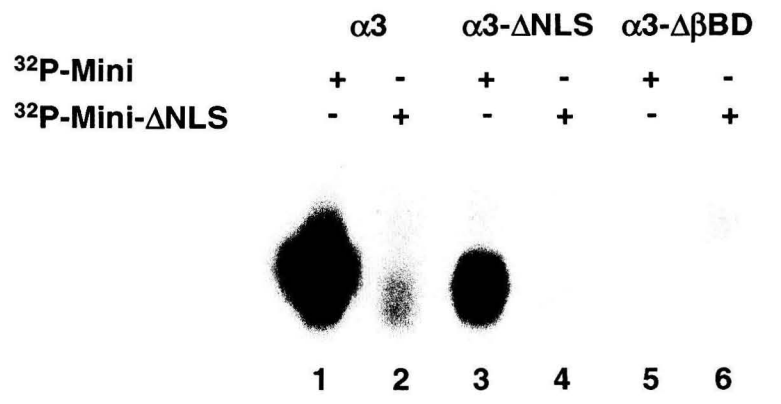

Figure 3B

Figure 3A. The effect of dKapβ on dKap-α3 binding to Drosophila HSF's NLS.

200ng 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, 200ng dKap-β; lane 3, 1μg dKap-β. No enhancement of dKap-α3 binding to the mini-ΔNLS reaction was observed even with the addition of 1μg of dKap-β (lane 4).

Figure 3B. Domains of dKap-α3 required for Drosophila HSF NLS binding.

Recombinant wild type or mutant dKap–α3 protein (1μg) was cross-linked to either mini-NLS or mini-ΔNLS probes, which were labeled with γ^{32}P-ATP using MAP kinase. 2ng of probe (150,000cpm) 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).

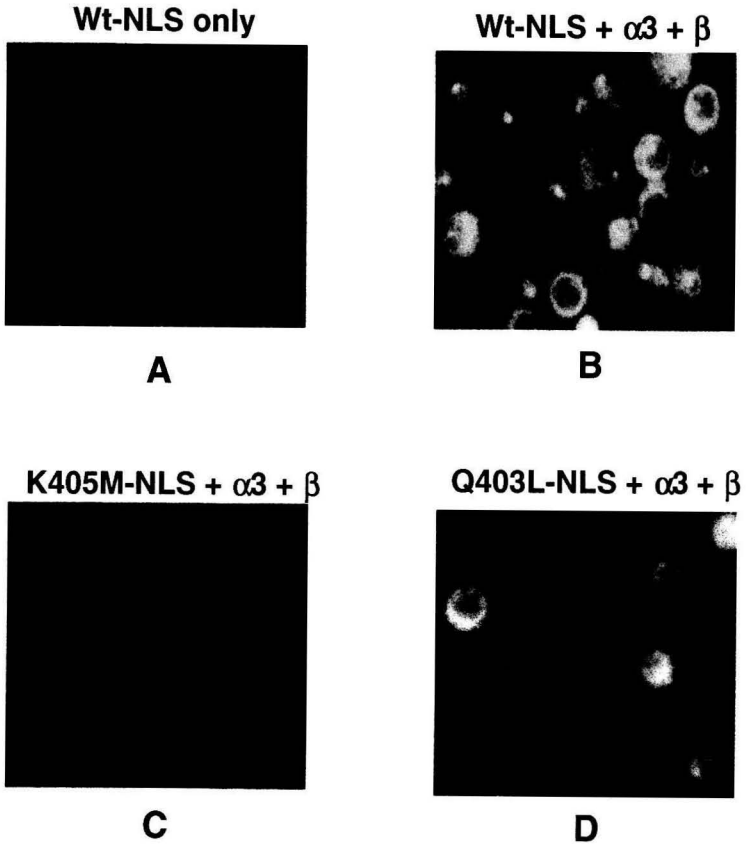

Figure 4

Figure 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. Panel A: wild type NLS-EGFP fusion protein only; Panel B: wild type NLS-EGFP fusion protein, kap α3 and kap-β; Panel C: K405M mutant NLS-EGFP fusion protein, dKap -α3 and dKap-β; Panel D: Q403L mutant NLS-EGFP fusion protein, dKap-α3 and Kap β.

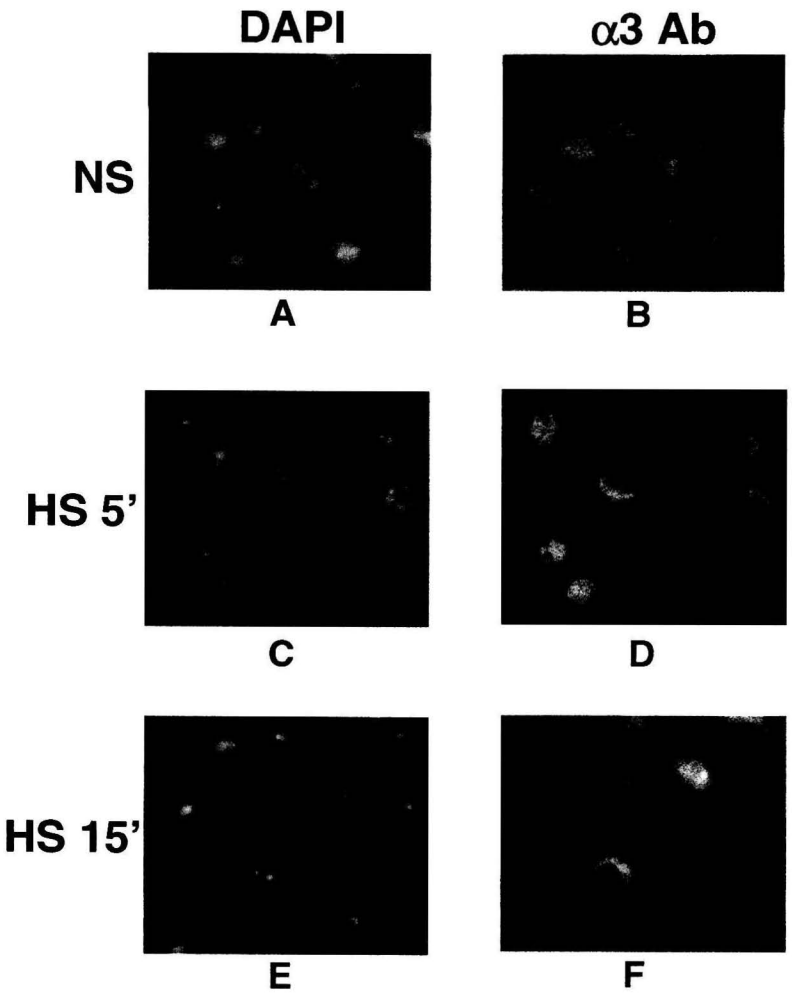

Figure 5

Figure 5. Effect of heat shock on the sub-cellular distribution of dKap-α3 in SL2 cells. Schneider cells stained with DAPI are displayed 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) Heat-shocked cells at 37°C.

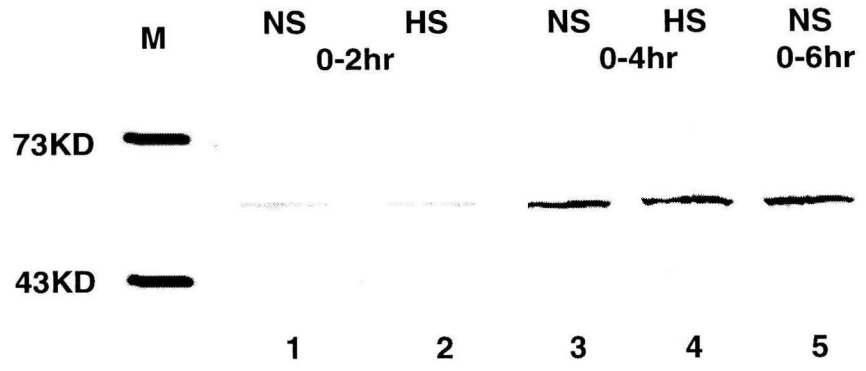

Figure 6A

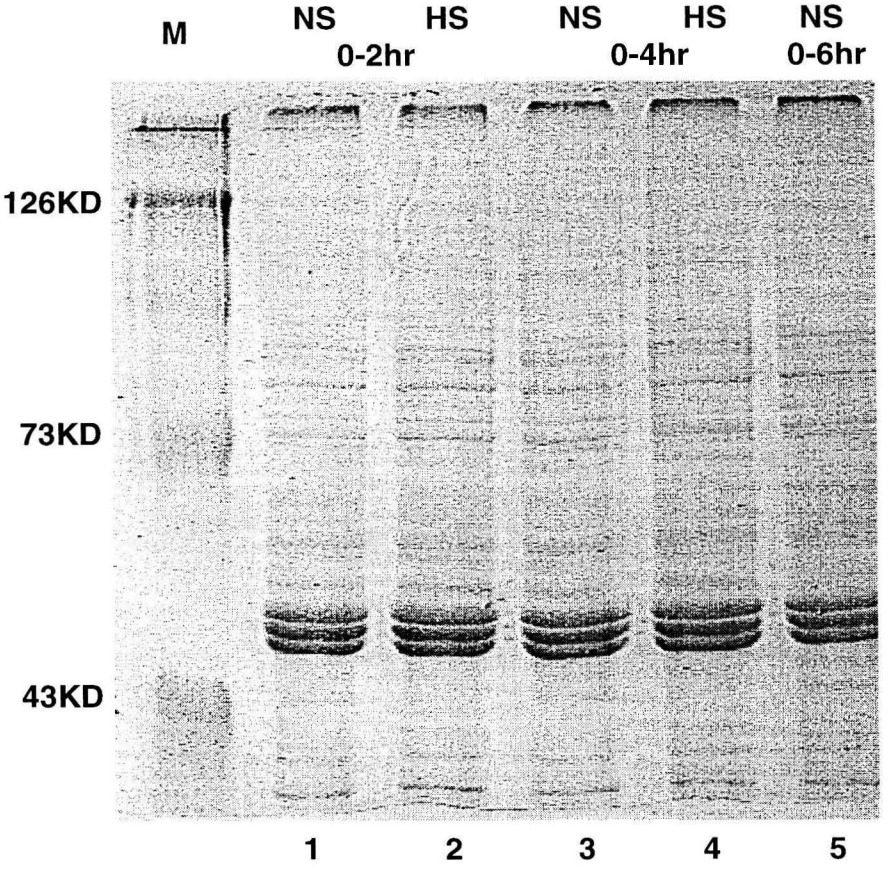

Figure 6B

Figure 6: Developmental western analysis of dKpa-α3 expression in Drosophila embryos.

0-2 hr, 0-4 hr and 0-6 hr (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 (**Figure 4A**) or stained with coomassie blue (**Figure 4B**).

M: molecular weight standards; NS: non-shock embryos; HS: heat-shocked embryos. Lane 1: NS 0-2hr embryos, lane 2: HS 0-2 hr embryos, lane 3: NS 0-4 hr embryos, lane 4: HS 0-4 hr embryos, lane 5: NS 0-6 hr embryos.

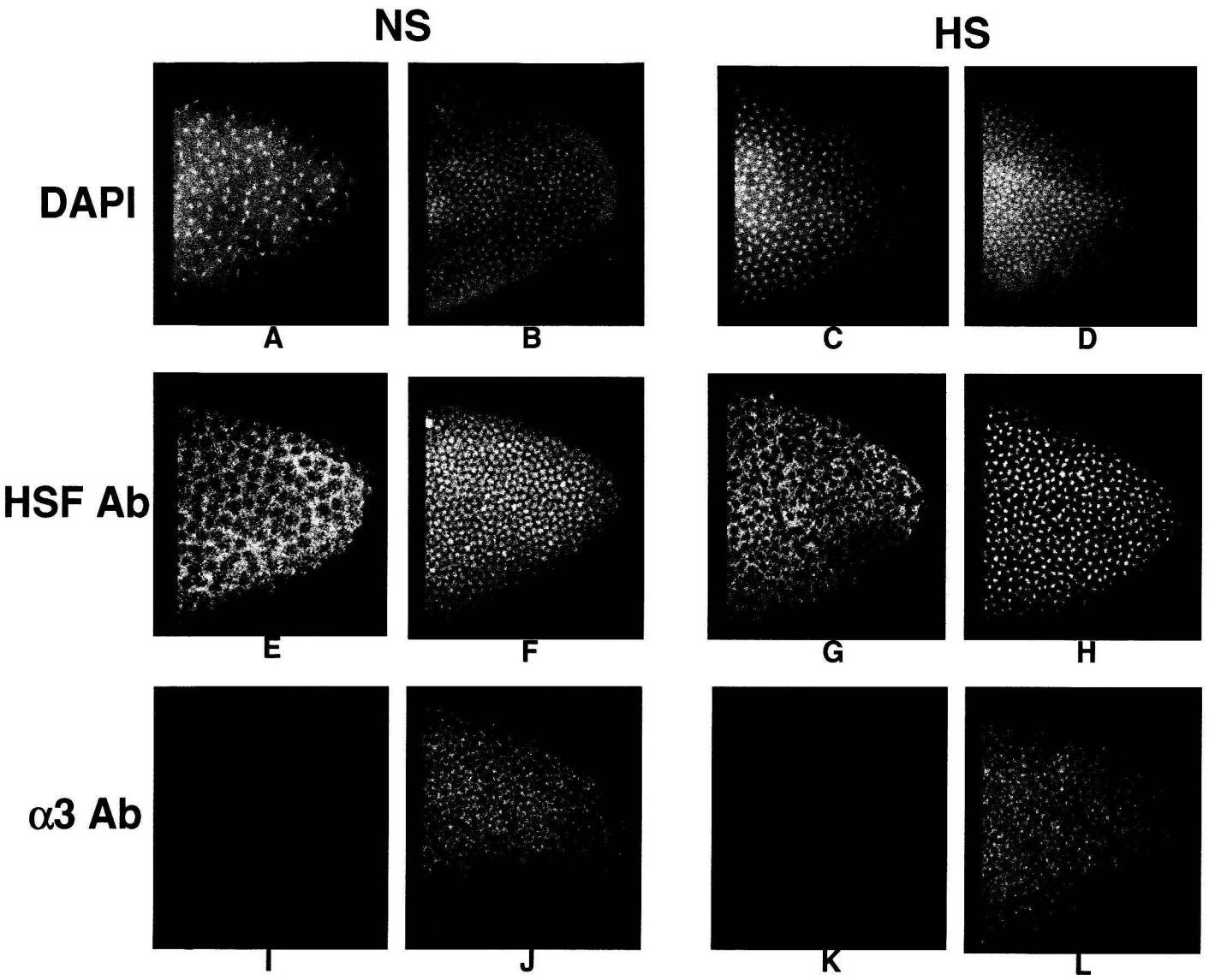

Figure 7 A-L

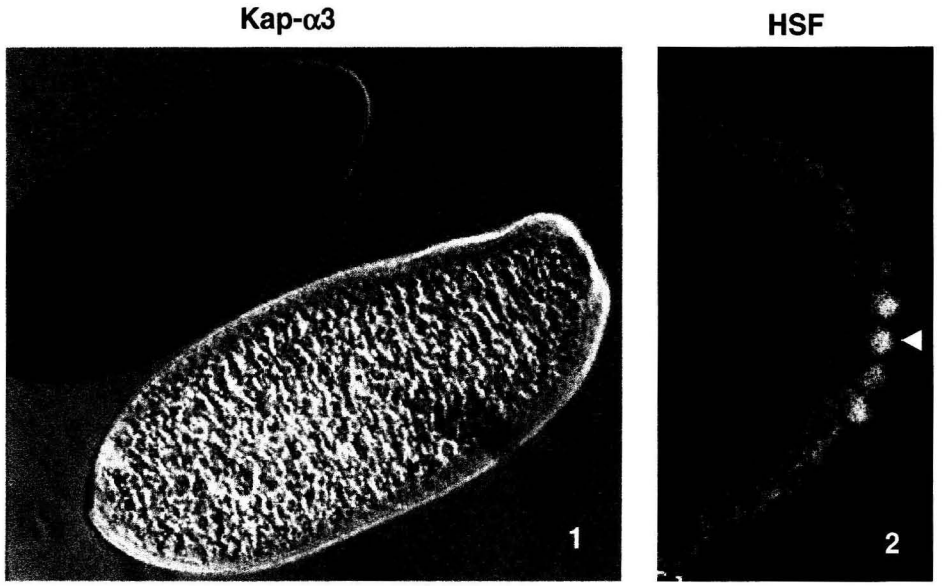

Figure 7M

Figure 7. Developmental timing of dKapα3 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, and HS; heat-shocked embryos. Cycle 11 embryos are shown on the left under NS and cycle 12 embryos shown on the left under HS, respectively. Cycle 13 embryos are shown under both the NS and HS on the right side. Clearly present but excluded from the nucleus is dHSF in both non-shocked cycle 11 embryos (panel E) and heat-shocked cycle 12 embryos (panel G). Similarly staged embryos display essentially no dKap-α3 staining (panels 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 (panel J). In heat-shocked cycle 12 embryos, the dHSF remains excluded from the nucleus 9 panel G) and no dKap-α3 is observed as in non-shocked embryos (panel K). At cycle 13 in heat-shocked embryos the characteristic punctuated pattern of dHSF nuclear staining is observed (panel H) and dKap-α3 remains excluded from the nucleus (panel 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 arrow, 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 (panel 2), corresponding to the presence of Kap-α3 mRNA in this region of the pre-cycle 13 embryo (panel 1).

CHAPTER THREE

DETERMINATION OF THE FUNCTIONAL DOMAIN ORGANIZATION OF NUCLEAR TRANSPORT FACTOR KARYOPHERIN α3

ABSTRACT

Preliminary domain mapping of Karyopherin α3 (Kap α3) by in vitro crosslinking showed that the N-terminal β-binding domain of Kap α3 is required for its binding to the Nuclear Localization Signal (NLS) of the Drosophila Heat Shock Transcription Factor (dHSF). To further understand the domain organization of Kap α3, binding affinities of Kap α3 to Kap β, dSHF-NLS, and CAS were measured using yeast two-hybrid system, and the detailed domain map of Kap α3 is shown here.

INTRODUCTION

Nucleocytoplamsic transport through nuclear pore complexes (NPCs) is mediated by transport receptors that shuttle between the nucleus and cytoplasm. Depending on the size of the protein, nuclear import through NPCs can occur either through passive diffusion for small molecules of less than 50 kDa, or by an active process facilitated by the nuclear localization signal (NLS) present in nuclear proteins (Gorlich and Kutay, 1999), (Macara, 2001). Two types of classical NLSs have been well characterized. Monopartite NLS consists of a single stretch of 4-6 basic amino acids, such as SV40 large T antigen (P**KKKRK**), and a bipartite NLS consists of two basic amino acids, a spacer region of 10-12 amino acids and a cluster of 3-5 basic resides, such as that found in nucleoplasmin (**KR**PAATKKAGQA**KKKK**LD) (Macara, 2001). Classical NLSs are usually recognized by the heterodimeric receptor karyopherin (Kap) α and β. Kap α is the NLS receptor and Kap β is responsible for the docking of the karyopherin-cargo

complex to the cytoplasmic side of the NPC and its subsequent translocation through the pore. Once inside the nucleus, Ran-GTP binding to Kap β causes the dissociation of the import complex and the release of the cargo (Gorlich and Kutay, 1999), (Mattaj and Englmeier, 1998). Kap α and Kap β are then separately recycled back to the cytoplasm. The export of Kap α requires CAS, an export factor that belongs to Kap β family (Kutay et al., 1997).

According to the structural studies of *Saccharomyces cerevisiae* Kap α (SRP1) and the alignment with other known human Kap α proteins, the putative functional domains of Drosophila Kap α3, a nuclear import factor identified by this lab are proposed (Fang et al., 2001). In brief, the core consists of eight degenerate repeats of about 42 amino acids, relatively hydrophobic sequence termed the armadillo (Arm) motif. Arm repeats extends from 99 aa to 429 aa, and is thought to be the binding domain for NLS-containing cargos (Figure 1). The N-terminal hydrophilic domain (1-99) is important for Kap β binding (βBD domain), and within this domain, a stretch of basic amino acid resembling classical NLS is found (defined as internal NLS, Figure 1) that can also bind to Arm repeats and plays an autoinhibitory role (Kobe, 1999).

Preliminary in vitro crosslinking studies using either full length or βBD-deleted recombinant Kap α3 showed that 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 α binds to its target NLS-peptide cargo within the arm repeats. Indeed, an N-terminal β-binding domain-deleted form of the yeast Kap α protein was sufficient for crystallization with an SV-40 NLS peptide (Conti et al., 1998). It is likely that the significant size difference

between the dHSF-NLS probe used in our crosslinking experiments (192 aa) and the SV-40 peptide (8 aa) may explain why other domains of the Kap α3 protein are needed for binding.

In order to better define the functional domains of Kap α3, we used the yeast two-hybrid system to study the interactions between Kap α3, Kap β, dHSF-NLS and CAS. By measuring the β-galatosidase activities, we were able to obtain a detailed functional domain map of Kap α3.

MATERIALS AND METHODS

Construction of the Plasmids

Drosophila embryonic cDNA library (Clontech) was amplified according to the manufacturer's instructions. Briefly, 150 LB+Amp plates (150mm diameter) were used to amplify the library in order to obtain 2-3X the number of independent clones, and plamids were obtained using Bio-Rad Maxiprep kit. Using cDNA library as the template, two fragments of Drosophila CAS cDNA were obtained using PCR, one fragment from 1 to 1324 bp, the other from 1325 to 3928 bp. Full length CAS was obtained by ligating the two pieces together and cloned into pGBKT7 vector (Clontech). Kap β and Kap β-ΔN288 cDNA were amplified by PCR using pfu polymerase (Stratagene) and cloned into pGBKT7. A fragment of dHSF cDNA containing wild type NLS (340 aa to 461 aa) or a mutated from (K405M) were cloned into pBGKT7 as well. Various deletions of Kap α3 cDNA were amplified by PCR and cloned into pGADT7 vector (clontech).

For transfection, Kap α3-ΔC449 was cloned into pAc5.1/V5-His A vector using EcoRI and BamHI site.

Yeast Two-hybrid Analysis

The interactions between Kap α3 (and various deleted forms), Kap β and CAS was assayed using the yeast two-hybrid protein interaction system (protocols provided by Clontech). First, reconstructed pGBKT7 (encoding GAL4 1-147 DNA binding domain fused to Kap β, NLS or CAS) and pGADT7 (encoding GAL4 activation domain fused to Kap α3 and deleted forms) were transformed into yeast strain AH109 (Clontech) using ALKALI-CATION Yeast Transformation Kit from BIO 101 Inc. (The protocols are provided by the manufacturer and are based on lithium acetate procedure.) The β-galactosidase activities of the transformants were measured by liquid culture assay using CPRG as substrate (protocols provided by Clontech, *Yeast Protocols Handbook*, PT3024-1).

Transfection and Immuno-fluorescence Staining

1μg of Kap α3-ΔC449/pAc was tranfected into S2 cells following the protocol of Effectene Transfection Kit (Qiagen). 72 hours after the transfection, cells were fixed and stained with anti-V5 monoclonal antibody (Invitrogen). Detailed protocol for immunostaining was described before (Fang et al., 2001).

RESULTS

NLS-binding domain of Drosophila Karyopherin α3

Figure 1 shows the predicted domains of Drosophila Karyopherin α3 according to the alignment of other known Kap αs. In order to determine the NLS-binding domain of Kap α3, a series of deletion mutants of Kap α3 were constructed and fused with GAL4 Activation Domain. A fragment of dHSF cDNA containing wild type NLS (340 aa to 461 aa) was fused to GAL4 DNA Binding Domain. A mutated NLS, K405M, which abolished nucleus entry when fused to GFP (Zandi et al., 1997), and was unable to cross-link to Kap α3 (Fang et al., 2001), was also used as the negative control. After both plasmids were transformed into yeast strain AH109, the β-gal activities of the transformants were measured using CPRG as substrate and shown in Table 1.

Table 1. Interactions of Kap α3 deletion mutants and NLS

	Wt-NLS	K405M-NLS
α3(1-514)	3.45	0
α3(48-514)	5.90	0
α3(70-514)	5.22	ND
α3(92-514)	5.21	0
α3(48-390)	3.16	0
α3(48-350)	2.87	ND
α3(48-306)	2.82	ND
α3(1-222)	0.07	ND

Data are given as β-gal activity units measured according to Clontech protocols. ND: not determined

Deletion of the internal NLS (21- 47 aa) enhanced the interaction between Kap α3 and wtNLS, which is consistent with the previous structural study that internal NLS auto-inhibits the binding of Kap α to its NLS cargos. In addition, deleting first 92 aa does not

affect NLS binding affinity (Table 1, and Kobe, 1999). Combining this result with the previous crosslinking data (Fang et al., 2001), it seems that residues from 92 to 99 are import for NLS binding. Deleting Arm repeat 6, 7, 8 reduced NLS binding affinity by 50%, but did not completely abolished the binding. Structure studies of mammalian Kap α complexed with a bipartite NLS showed that there are two binding pockets within the Kap α Arm repeats. The N-terminal major pocket binds to the downstream, longer cluster of the basic residues, and the C-terminal minor pocket binds to the upstream short KR sequence (Conti and Kuriyan, 2000; Fontes et al., 2000). Consistent with this result, deleting the Kap α3 C-terminal minor pockets only reduced but not abolished the NLS binding.

Karyopherin β-Binding Domain of Kap α3

Table 2 showed the relative β-galatosidase activities of the strains carrying Kap β and Kap α3 deletion mutant. Full-length Kap β interacts with full-length Kap α very weakly. This is expected, as the interaction of Kap β with RAN-GTP inside the nucleus is predicted to cause the dissociation of Kap α and Kap β heterodimer (Chook and Blobel, 2001). Structural studies showed that for Kap β, RAN-GTP binding and Kap α binding are mutually exclusive (Conti and Izaurralde, 2001). Therefore, the first 288 amino acid of Drosophila Kap β, which, according to the alignment to human Kap β, includes most of the RAN-GTP-binding domain (Herold et al., 1998), was deleted. As expected, the N-terminal region of Kap α3 is very important for Kap β binding. Deleting the first 48 residues completely abolished the interaction.

Table 2. Interactions of Kap α3 deletion mutants and Kap β

	Kap β	Kap β-ΔN288
α3(1-514)	0.11	3.28
α3(48-514)	0.06	0.11
α3(70-514)	ND	0.07
α3(92-514)	ND	0.09
α3(1-449)	ND	3.73

Data are given as β-gal activity units measured according to Clontech protocols. ND: not determined

CAS-Binding Domain of Kap α3

As shown in Table 3, CAS-binding domain of Kap α3 is located at its C-terminus. Deletions beyond 449 abolished the interaction of Kap α3 and CAS. Since the crystal

Table 3. Interactions of Kap α3 deletion mutants and CAS

	CAS
–	0
α3(1-514)	2.72
α3(1-222)	0.03
α3(223-514)	2.43
α3(48-514)	2.82
α3(92-514)	2.85
α3(1-449)	0.05
α3(48-390)	0
α3(48-350)	0
α3(48-306)	0.02

Data are given as β-gal activity units measured according to Clontech protocols.

structure of CAS bound by Kap α has not been solved, immuno-fluorescence staining was performed in order to confirm the results obtained from yeast two-hybrid analysis. V5-epitope-tagged recombinant Kap α3 (1-449) was transfected into Drosophila Schneider S2 cells, and stained with anti-V5 antibody 72 hours after the transfection. While full length Kap α3 showed a whole cell staining (Fang et al., 2001), Kap α3 (1-449) was completely nuclear (Figure 2), indicating that the C-terminal-deleted form of Kap α3 cannot be recycled back to cytoplasm by CAS. This result independently confirmed that the C-terminal acidic region of Kap α3 is important for CAS binding.

CONCLUSION

The functional domain organization of Drosophila Kap α3 is summarized in Figure 1.

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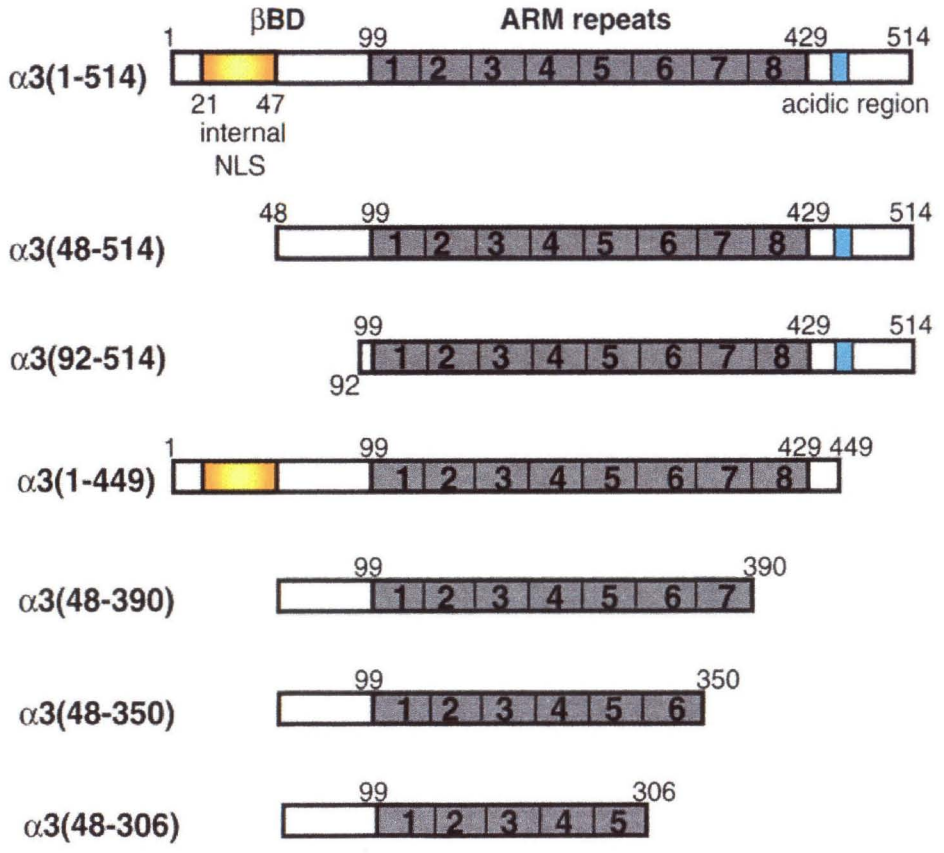

	Kap β-ΔN288	CAS	wtNLS
α3(1-514)	++	++	+
α3(48-514)	-	++	++
α3(92-514)	-	++	++
α3(1-449)	++	-	ND
α3(48-390)	ND	-	+
α3(48-350)	ND	-	+
α3(48-306)	ND	-	+

Figure 1

FIGURES AND FIGURE LEGENDS

Figure 1. Schematic diagram of the domain organization of Karyopherin α3. Amino acid endpoints for each region, as well as their proposed functions, are indicated. Kap-α3 deletion mutants used in the yeast two-hybrid analysis are also shown. The acidic region of the C-terminus is proposed to be CAS-Binding domain. ++: strong interaction. +: moderate interaction. -: no interaction. ND: not determined.

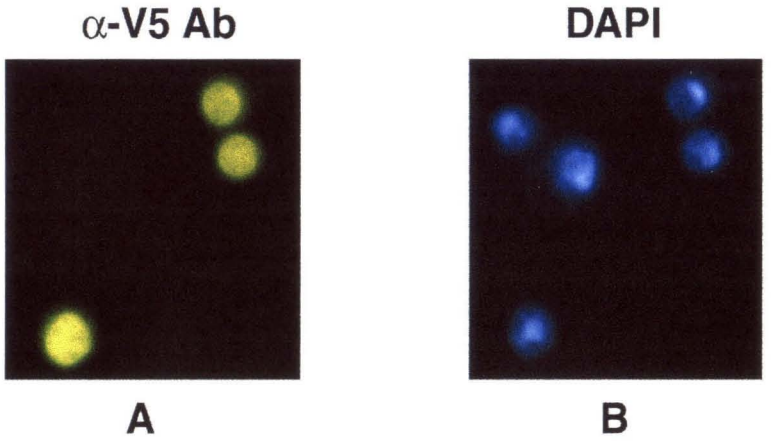

Figure 2

Figure 2. C-terminal acidic region of Kap α3 is important for CAS binding. S2 cells were transiently transfected with V5-epitope-tagged recombinant Kap α3(1-449), and 72 hours after transfection, stained with anti-V5 antibody (panel A). DAPI staining was performed at the same time to show nucleus (panel B). Note that Kap α3 (1-449) is located exclusively in nucleus.

CHAPTER FOUR

TRANSCRIPTION FACTORS ENTER THE NUCLEUS BY UNIQUE AND SPECIFIC PATHWAYS INVOLVING THE KARYOPHERIN α FAMILY OF NUCLEAR TRANSPORT FACTORS IN DROSOPHILA

The work in this chapter is submitted to **Genes & Development**.

Tianxin Chen and Carl S. Parker

Transcription Factors Enter the Nucleus by Unique and Specific Pathways Involving the Karyopherin α Family of Nuclear Transport Factors in Drosophila

ABSTRACT

Protein trafficking into and out of the nucleus is a key process in eukaryotic cells. A specific nuclear localization signal (NLS) is required for the nuclear entry of most proteins. Transport factors that specifically recognize the NLS and carry the NLS-containing protein into the nucleus belong to the karyopherin α (Kap α, or importin α) family of nuclear transporters. While *Saccharomyces cerevisiae* has only one Kap α; SRP1, higher eukaryotes contain multiple Kap αs. The roles of these multiple transporters and their specificities in nuclear import are not understood. Drosophila has three Kap α proteins (Kap α1, 2 and 3). To determine whether the Drosophila karyopherins have unique roles in nuclear protein trafficking, we used RNA interference to knock out each Kap α protein individually in Drosophila Schneider 2 cells. The nuclear entry of two general and one specific transcription factors was then monitored by indirect immuno-fluorescence. We found that RNA polymerase II, TATA binding protein (TBP) and heat shock transcription factor (HSF) were transported into the nucleus by different karyopherins. These observations suggest that unique and non-overlapping transport pathways exist in higher eukaryotes.

INTRODUCTION

In eukaryotes, the segregation of the cell into nuclear and cytoplasmic compartments dictates the necessity of selective and regulated trafficking of proteins and RNA between the two compartments. Regulation of the nuclear import of proteins is an important control point for a variety of cellular processes including: cell cycle regulation, transcriptional activation and repression, growth and development (Affolter et al., 1999; Macara, 2001; Moroianu, 1999). Depending on the size of the protein, nuclear import through the nuclear pore complex (NPC) can occur either through passive diffusion for small molecules of less than 50 KDa, or by an active process facilitated by the presence of a nuclear localization signal (NLS) (Gorlich and Kutay, 1999; Macara, 2001). Two types of classical NLSs are known; a monopartite NLS consisting of a small stretch of 4-6 basic amino acids, typified by the SV40 large T antigen's NLS (P**KKKRK**). A second more complex bipartite NLS consisting of two basic amino acids segments separated by a spacer region of 10-12 amino acids is represented by the nucleoplasmin NLS (**KR**PAATKKAGQA**KKKK**LD) (Macara, 2001).

The classical NLSs are usually recognized by the heterodimeric receptor karyopherin (Kap) α and β. Kap α is the NLS receptor and Kap β is responsible for the docking of the karyopherin α-cargo complex to the cytoplasmic side of the NPC and its subsequent translocation through the pore. Once inside the nucleus, Ran-GTP binding to Kap β causes the dissociation of the import complex and the release of the cargo (Gorlich and Kutay, 1999; Mattaj and Englmeier, 1998). In addition, ribosomal proteins and a few transcription factors (such as Pho4p) contain non-classical NLSs, which have diverse

amino acid sequences, and are believed to be recognized by Kap β homologues directly without Kap α (Chook and Blobel, 2001).

While *Saccharomyces cerevisiae* only has one karyopherin α (SRP1), higher eukaryotes possess more than one form of Kap α, with humans and mouse possessing at least six isoforms (Malik et al., 1997). The number of distinct Kap α isoforms in higher eukaryotes indicates that there may be specialization in their cellular roles, and that different isoforms could bind uniquely to particular target proteins (Jans et al., 2000). Consistent with this view is the fact that any single human karyopherin α cannot complement a *Saccharomyces cerevisiae* SRP1 mutant. Direct evidence, although limited, indicates that the respective karyopherin α subunits may have distinct binding properties. Human Karyopherin α1 but not α2 recognizes the transcriptional factor STAT1 (Sekimoto et al., 1997), while in contrast to Kap α1 and α2, human Kap α3 requires additional flanking residues either side of conventional T-antigen-like NLSs to mediate binding and nuclear import (Miyamoto et al., 1997).

The most commonly used techniques to identify the substrates for specific karyopherins include the yeast two-hybrid assay, immunoprecipitation and pull down assays (Jans et al., 2000). While valuable information concerning the binding properties of karyopherins has been obtained using these techniques, there are distinct limitations. Yeast two-hybrid assay tends to produce false positives and false negatives, while immunoprecipitation using recombinant proteins does not necessarily recapitulate the cellular environment. Moreover, multiple Kap αs are co-expressed within the normal context of the cell, but the current analysis has been limited to individual karyopherin proteins in isolation. Thus the redundancy and uniqueness of their functions cannot be

conclusively determined using these techniques. In this study, the substrate specificity of three Kap αs found in the Drosophila genomic sequence was analyzed by RNA interference (RNAi). We showed that RNAi can effectively and specifically inhibit the expression of the targeted Kap α in cultured Drosophila Schneider line 2 cells. By knocking out individual Kap αs and examining the nuclear import of various transcription factors we learned that Kap αs specifically and uniquely transport individual transcription factors.

MATERIALS AND METHODS

Establishment of the Permanent Cell Lines. cDNA sequences corresponding to Drosophila Karyopherin α1, α2, and α3 were obtained by PCR using Drosophila Embryonic cDNA library (Clontech, amplified according to the manufacturer's instructions) as the template, and pfu polymerase (Stratagene). The cDNAs were then cloned into pDS47/V5-His A vector (Invitrogen) in-frame with the C-terminal V5 epitope and 6 His tag. The recombinant plasmids were mixed with pCoHYGRO (Invitrogen) with 3:1 ratio, and transformed into Schneider 2 cells using Effectene Transfection Kit (Qiagen). Stable transformants were selected by adding hygromycin-B to 300 μg 72 hours after transfection (detailed protocols provided by Invitrogen).

dsRNA Synthesis. DNA sequences (approximately 700 bp in length) to be used for dsRNA synthesis were generated by PCR using Taq polymerase (Qiagen) with a Drosophila Embryonic cDNA library (Clontech, amplified according to the manufacturer's instructions) as the template, the primers each contained a T7 promoter

sequence on its 5' end (GAA TTA ATA CGA CTC ACT ATA GGG AGA), followed by sequences specific for the targeted genes. The purified PCR products were used as templates for dsRNA synthesis using recombinant T7 RNA polymerase (gift of Dr. R. Roberts). The dsRNA products were ethanol precipitated and resuspended in RNA storage buffer (Ambion). The dsRNAs were annealed by incubation at 70°C for 10 min followed by slow cooling to room temperature right before use.

RNA Interference in Drosophila Cell Culture. For RNA pol II staining, Drosophila Schneider 2 cells were diluted to a final concentration of 2 x 10^6 cells/ml in Drosophila Expression System serum-medium (Invitrogen). 1 ml of cells was plated per well of a six-well tissue culture dish. About 40 μg of dsRNA was added directly to the media and followed immediately by vigorous agitation. The cells were incubated for 30 min at room temperature followed by addition of 2 ml Schneider's media containing 10% FBS (GibcoBRL). The cells were incubated for an additional 70 hours to allow for turnover of the target protein. For HSF staining and Kap α1, α2, α3 staining, established permanent cell lines expressing V5-tagged Kap α1, α2, α3 were used instead of regular S2 cells. For TBP staining, first, Drosophila TBP coding region was amplified by PCR using pfu polymerase (Stratagene) and Drosophila embryonic cDNA library. TBP cDNA was then cloned in-frame into pAc5.1/V5-His A vector (Invitrogen). 2μg of the plasmid only (control) or 2 μg of the plasmid mixed with 1μg of Kap alpha dsRNA (RNAi) were transfected into S2 cells using Effectene Transfections Kit (Qiagen). Cells were harvest 70 hours after RNAi treatment.

Antibodies. Monoclonal mouse anti-V5 antibody was from Invitrogen. Monoclonal anti dKap α3 antibody has been described previously (Fang et al., 2001). PCR-amplified coding region of RNA polymerase II C-terminal domain (CTD) was cloned into pGEX-2t expression vector and the GST-tagged CTD was purified using GST-affinity resin (Stratagen). Recombinant GST-CTD was then used to generate anti-RNA pol II CTD antibody. Monoclonal antibody was selected by western blotting against S2 cell nuclear extract yielding a single band corresponding to the size of RNA pol II.

Immuno-fluorescence Staining. 18x18 mm #1 cover slips were coated in 1 mg/ml poly-L-lysine for 15 min and air-dried. 0.5ml of Drosophila S2 cells were placed onto each cover slide and incubated for 15 min at room temperature to allow attachment. For RNA pol II and TBP staining, cells were then washed with PBST after attachment and fixed with Histochoice Tissue Fixative MB (Amresco) for 15 min on ice. For HSF and Kap α1, α2, α3 staining, cells were heat shocked at 37°C for 20 minutes after the attachment, washed with PBST, and then fixed. After several washes to remove the fixative, the fixed cells were incubated with 1:1000 dilution of monoclonal anti-V5 antibody (Invitrogen), or monoclonal anti-HSF antibody, or monoclonal anti-pol II CTD antibody in PBS buffer containing 1% bovine serum albumin (BSA) at 4°C overnight. Cells were then washed with PBST 4 times for 10 min each to remove unbound first antibodies. Alexa Fluor® 488 goat anti-mouse IgG (H+L) secondary antibody (Molecular Probes) was then added at 1:500 dilution in 1% BSA in PBS and incubated at room temperature for 2 hours. 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 (Sekimoto et al., 1997) octane (Sigma)). The fluorescence images were viewed and photographed with Zeiss Axioplan microscopy with UV irradiation and appropriate filters.

RESULTS

The Effectiveness of RNA Interference

A search of Drosophila genomic sequence database (flybase.org) revealed three Karyopherin α homologues, Kap α1, Kap α2 (pendulin or Oho31), and Kap α3. To determine whether RNAi can effectively inhibit the expression of Drosophila Karyopherin αs in Schneider S2 cells, double-stranded RNA (dsRNA) corresponding to the first 700 bp of the coding region of the three Kap αs was synthesized. Since we do not have the antibodies against Kap α1 and Kap α2, C-terminal V5-epitope tagged recombinant Kap α1, α2 and α3 under Drosophila DS47 promoter (for moderate-level constitutive expression) were transfected into S2 cells. Cell lines that stably express the recombinant proteins were established by Hygromycin selection. Western blotting (data not shown) and immuno-fluorescence staining confirmed the expression of recombinant Kap αs (Figure 1, Panel A, E, I). dsRNA representing each Kap α was then delivered to these three established cells lines. As shown in Figure 1 (panel C, G, K), the addition of dsRNA effectively inhibited the expression of the corresponding recombinant (and presumably, endogenous) Kap αs. Expression of exogenous Kap α proteins was essentially undetectable after dsRNA treatment. S2 cells with Kap α1 or Kap α2

expression repressed are viable, with no obvious growth defects during the 72-hour time period that the cells are maintained after RNAi treatment. In fact, Kap α2 knockout cells grow at a slightly higher rate than control cells, this may reflect the fact that Kap α2 (pendulin) is a cell cycle-regulated protein (Kussel and Frasch, 1995; Torok et al., 1995). However, when Kap α3 was knocked out, a small fraction cells showed enlarged nuclei and some cell death was observed 72 hours after RNAi treatment.

Drosophila Heat Shock Factor is Transported into the Nucleus by Karyopherin α3

Previous studies have showed that heat shock induces stable nuclear localization of Drosophila Heat Shock Factor (HSF) (Zandi et al., 1997). The nuclear localization sequence (NLS) of Drosophila HSF is found between amino acids 393-423 (Zandi et al., 1997), and belongs to the bipartite NLS family. Protein crosslinking and nuclear docking experiments previously showed that dHSF NLS binds specifically to Kap α3 (Fang et al., 2001). To determine the substrate specificity of the three Drosophila Kap αs and whether others are involved in the nuclear transport of dHSF, we examined the effects of knocking out the individual Kap α proteins. 70 hours after the addition of dsRNA, cells were heat-shocked at 37°C for 20 minutes and fixed immediately for immuno-fluorescence staining. As shown in Figure 2, without RNAi treatment, dHSF is localized within the nucleus upon heat shock in all three of the cell lines (Figure 2, control, panel A, E, I). Knocking out Kap α1 or α2 did not affect the nuclear localization of dHSF (compare panel A and C, panel E and G). However, knocking out Kap α3 caused nuclear exclusion of dHSF in all cells examined (compare panel I and K). These observations

clearly demonstrate that only Kap α3, not Kap α1 or α2, mediates the nuclear localization of dHSF.

Kap α2 is involved in the transport of RNA polymerase II

To identify the potential transporter(s) for RNA polymerase II, RNA interference was used to knock out specific Kap αs. S2 cells were treated with dsRNA corresponding to the three Kap αs separately, incubated for 70 hours and stained with a monoclonal antibody recognizing the C-terminal domain repeats (CTD) of RNA polymerase II. As shown in figure 3, knocking out Kap α1 and α3 did not affect nuclear localization of RNA polymerase II (panel C and G). When Kap α2 was knocked out, a small population of cells, about 5% to 10%, showed whole cell staining instead of nuclear staining (Panel E). Even though RNA polymerase II was still nuclear in the majority of the Kap α2 knockout cells, the same phenomenon was not observed when Kap α1 or α3 was knocked out. It is possible that RNA polymerase II enters the nucleus through multiple pathways simultaneously, using non-classical transporters such as Karyopherin β homologues as well, so eliminating Kap α2 would not completely eliminate RNA polymerase II nuclear entry. Scanning the sequence of RNA polymerase II revealed one basic amino acid stretch, G**KR**IPYGF**RKR**TLP (787-800), but does not quite fit into the consensus of either monopartite or bipartite NLSs. Considering that RNA polymerase II is extremely important for the expression of almost all the genes, it is not surprising that polymerase II can be imported into the nucleus by multiple Karyopherins.

We note that Kap α2 was previously identified as the *pendulin* gene and *oho31*gene (Kussel and Frasch, 1995; Torok et al., 1995). Analysis of mutations in the

pen gene reveals that it is required for normal cell proliferation and in hematopoeitic cells leads to melanotic tumors (Kussel and Frasch, 1995; Torok et al., 1995). Indeed we observe that in S2 cells where RNAi is used to eliminate Kap α2 the cells divide more rapidly suggesting similar effects on the cell cycle in cultured cells. It is curious however that cytosolic localization of RNA polymerase II is seen in only a small population of cells and not in all cells in which Kap α2 is eliminated. This result suggests that Kap α2 may be required for RNA polymerase II nuclear entry at specific times during the cell cycle, and perhaps only a small fraction of cells were in this stage when they were fixed for immunofluoresence. Indeed in Drosophila embryos, Pendulin rapidly translocates into the nuclei at the transition between G2 and M-phase, suggesting that it may transport proteins into the nucleus during this stage of the cell cycle (Kussel and Frasch, 1995).

TATA-binding protein is transported by Kap α1

A similar strategy was used to study the nuclear import of TBP. Since we do not have an effective antibody against Drosophila TBP, a C-terminal V5-epitope tagged recombinant TBP was used. S2 cells were either transfected with TBP-V5 DNA alone (control), or TBP-V5 DNA, mixed with dsRNA for each Kap α (knockouts), and incubated for 70 hours prior to fixation. As shown in Figure 4, the exogenous TBP was localized within the nucleus of the control cells (Panel A) and those cells treated with RNAi against Kap α2 (panel E), or Kap α3 (panel G). However, in Kap-α1 knockouts, about 60% of the transfected cells showed cytoplasmic localization of TBP (Panel C), indicating that Kap α1 is involved in the nuclear import of TBP. The cells in which nuclear entry of TBP was still observed may be the result of alternative pathways

transporting TBP in some cells but not all cells. A more likely alternative, however, is that the RNAi treatment was not fully effective in eliminating Kap α1 from those cells where TBP is nuclear.

In *Saccharomyces cerevisiae* nuclear import of TBP is mediated by Kap 114p, a Kap β homologue, with Kap α not involved in this process (Morehouse et al., 1999; Pemberton et al., 1999). The difference between Drosophila TBP and yeast TBP nuclear transport could be explained by the absence of a classic NLS in the yeast TBP. The Drosophila TBP has a basic amino acid stretch, PIL**KK**F**KK**QS at the C-terminus, which may constitute the NLS of Drosophila TBP. A similar functional NLS can be found in the proto-oncogene c-myc (PAAKRVKLD), where the proline and aspartic acid residues found on either side of the central basic cluster are important for recognition and nuclear targeting by Kap α1 and α2 (Makkerh et al., 1996). So the putative NLS could make Drosophila TBP a suitable substrate for Kap αs.

DISCUSSION

The existence of multiple karyopherins in higher eukaryotic cells means that the cell is able to modulate the nuclear import of particular proteins by regulating different nuclear transport pathways in response to various extra-cellular signals and developmental stages. One can envision that regulation of the levels of individual Kap α proteins might effectively control a particular transport pathway, without altering the nuclear entry of other proteins. Thus the spatial and temporal distributions of the karyopherins may well be an important component of developmental programming. In

addition, different karyopherin αs can compete for the same protein cargo, and different protein cargos can compete for the same karyopherin (Jans et al., 2000), adding one more layer of complexity for the regulation gene expression.

It is conceivable that most housing keeping proteins, such as RNA polymerase II and TATA-binding protein, are transported into nucleus by multiple pathways, to ensure the continuous nuclear presence of these important proteins. Gene-specific transcriptional factors are perhaps more likely to adopt a specific import pathway to ensure proper regulation. Our results, however, indicate that even the housekeeping genes do not share the same import pathway, at least as far as the Kap αs are concerned. Kap α1 participates in TBP nucleus import, while Kap α2 plays a role in the nuclear import of RNA polymerase II. These observations help to explain the differential timing of nuclear localization observed for these two general transcription factors and HSF during embyogenesis (Wang and Lindquist, 1998). It has been shown that RNA polymerase subunit IIc was nuclear in about half of cycle 7 embryos, and nuclear in all cycle 8 embryos. TBP was cytoplasmic prior to cycle 8. At cycle 9, most embryos exhibited nuclear localization of TBP(Wang and Lindquist, 1998). dHSF did not enter nuclei until cycle 13, correlating with Hsp70 inducibility (Wang and Lindquist, 1998). Although Kap α3's presence in the embryo is directly correlated with dHSF nuclear entry (Fang et al., 2001), this has not yet been established for Kap α1 or α2 and their potential import substrates, TBP or RNA polymerase II. This will be of interest for future studies.

RNA Interference has proven to be a simple and rapid method for inhibiting specific gene function in Drosophila, and can be further used as a tool in the analysis of nucleus transporters of specific proteins and mRNAs during development. Targeted

karyopherin(s) can be knocked out by simply micro-injecting dsRNA into Drosophila embryos. Furthermore, RNAi also makes it possible to study essential genes whose knockout is lethal. In this study, knocking out Kap α3 resulted in significant cell death 72 hours after RNAi treatment. However, cells harvested prior to this time were sufficiently viable to assess the nuclear localization of the transcription factors. Similar strategies can be envisioned with mammalian systems as well. Although mammalian cells induce global changes in gene expression when challenged with classic dsRNA, recent studies showed that Small Interfering RNAs (siRNA) effectively repressed gene expression in cultured mammalian cells (Caplen et al., 2001; Elbashir et al., 2001; Lipardi et al., 2001). It would be interesting to study the substrate specificities of mammalian karyopherins using siRNA and compare the similarities and differences with the Drosophila system.

ACKNOWLEDGEMENTS

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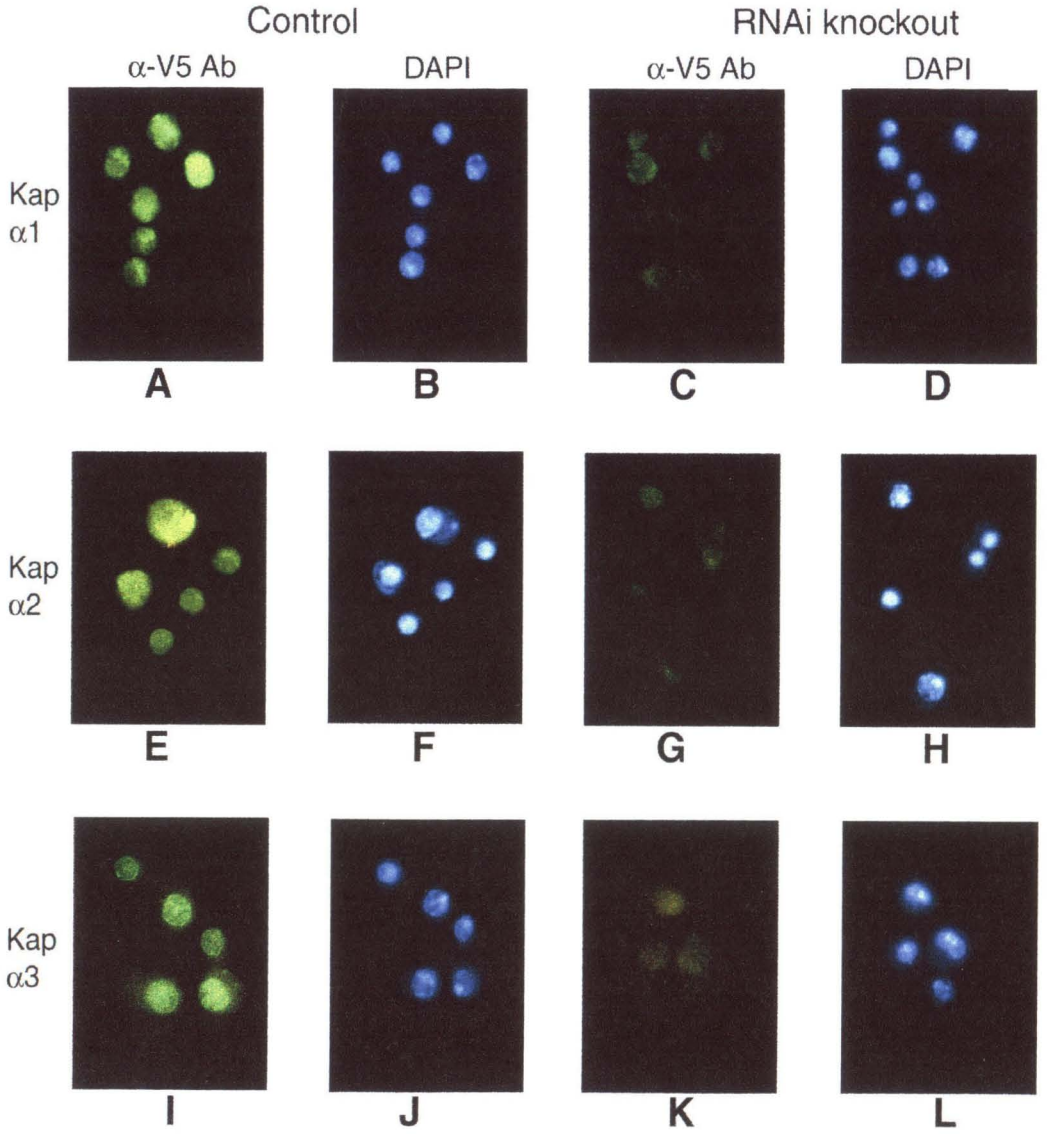
Control
RNAi knockout
α-V5 Ab
DAPI
α-V5 Ab
DAPI
Kap α1
A
B
C
D
Kap α2
E
F
G
H
Kap α3
I
J
K
L

Figure 1

FIGURES AND FIGURE LEGENDS

Figure 1. Effect of RNAi knockouts on Kap α1, α2 and α3 expression. Stably transformed S2 cells expressing V5 epitope-tagged (C-terminal) Kap α1, α2 or α3 under DS47 promoter were treated with 0 μg (Control, Panel A, E, I) or 40 μg of dsRNA (RNAi knockout, panel C, G, K), and stained with anti-V5 antibody 72 hours after dsRNA treatment. DAPI staining was performed at the same time to show the nuclei. Panel C, G, K demonstrate the absence of Kap αs after RNAi treatment.

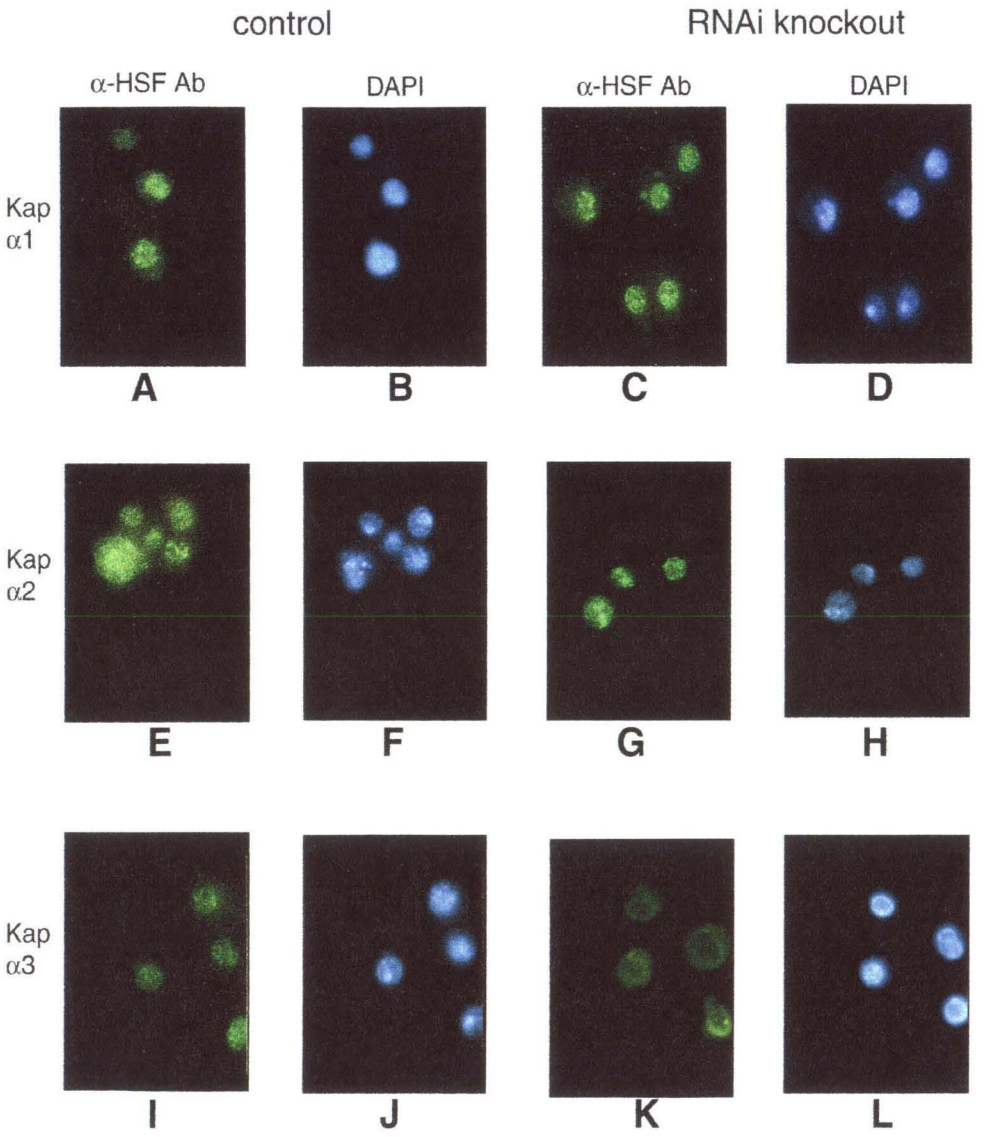
control
RNAi knockout
α-HSF Ab
DAPI
α-HSF Ab
DAPI
Kap α1
A
B
C
D
Kap α2
E
F
G
H
Kap α3
I
J
K
L

Figure 2

Figure 2. HSF is excluded from nucleus when Kap- α3 expression is repressed by RNAi. Stably transformed S2 cells expressing V5 epitope-tagged (C-terminal) Kap α1, α2 or α3 under DS47 promoter were treated with 0 μg (Control, Panel A, E, I) or 40 μg of dsRNA (RNAi knockout, panel C, G, K). 72 hours after dsRNA treatment, cells were heat shocked at 37°C for 20 minutes and then stained with monoclonal anti-HSF antibody. DAPI staining was performed at the same time to show the nuclei. Knocking out Kap α1 and α2 does not affect the nuclear localization of HSF upon heat shock (compare Panel A and C, E and G), while HSF is completely excluded from nucleus if Kap α3 is knocked out (compare panel I and K).

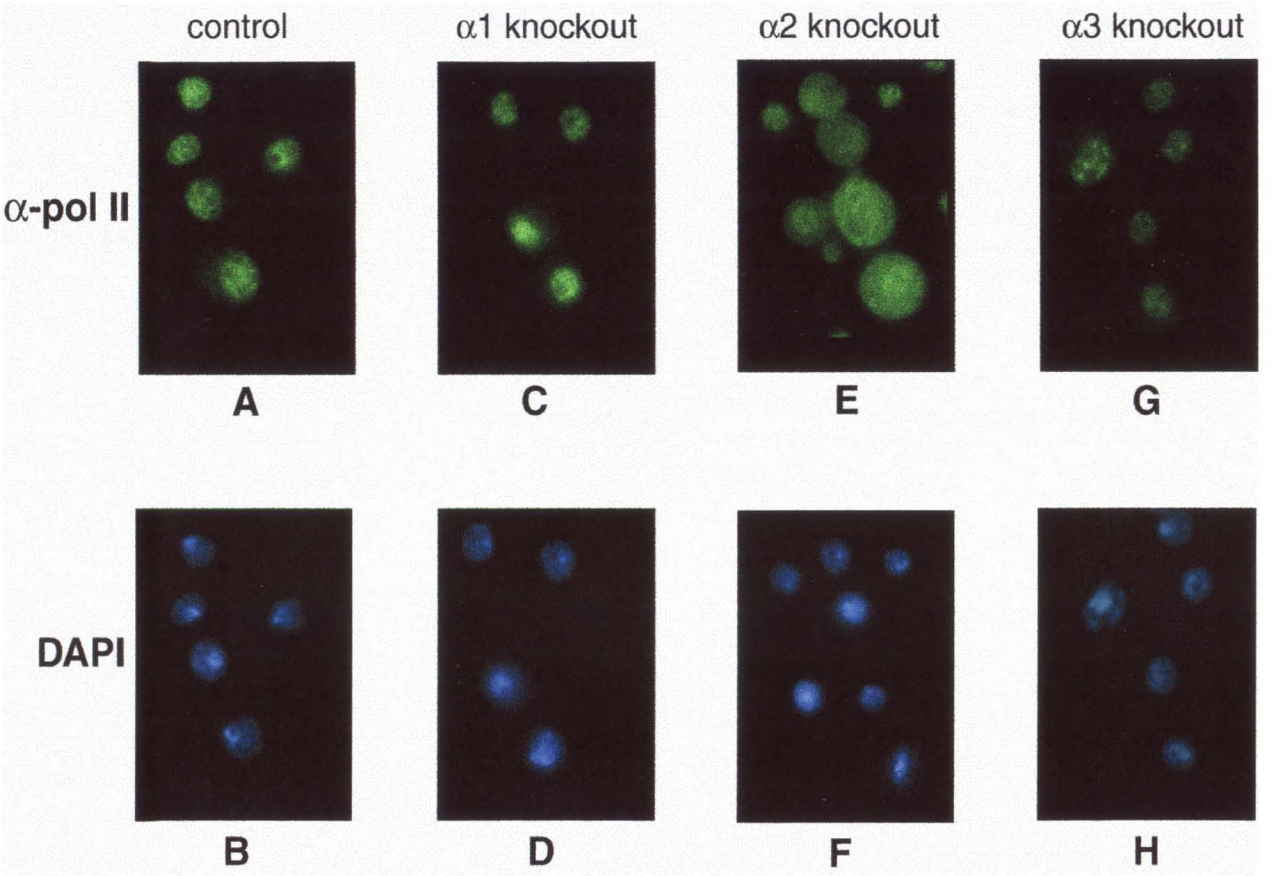
control
α1 knockout
α2 knockout
α3 knockout
α-pol II
DAPI
A
B
C
D
E
F
G
H

Figure 3

Figure 3. RNA polymerase II is localized both in cytoplasm and nucleus in a small population of cells when Kap α2 expression is repressed by RNAi. S2 cells were treated with 0 μg (Control, Panel A) or 40 μg of Kap α1, α2 or α3 dsRNA (knockout, panel C, E, G). 72 hours after dsRNA treatment, cells were fixed and stained with monoclonal anti-polymerase II CTD antibody. DAPI staining was performed at the same time to show the nuclei. Knocking out Kap α1 and α3 does not affect the nuclear localization of RNA polymerase II (compare Panel A, C, G). When Kap α2 is knocked out, abut 5-10% of the cells show whole cell staining (Panel E).

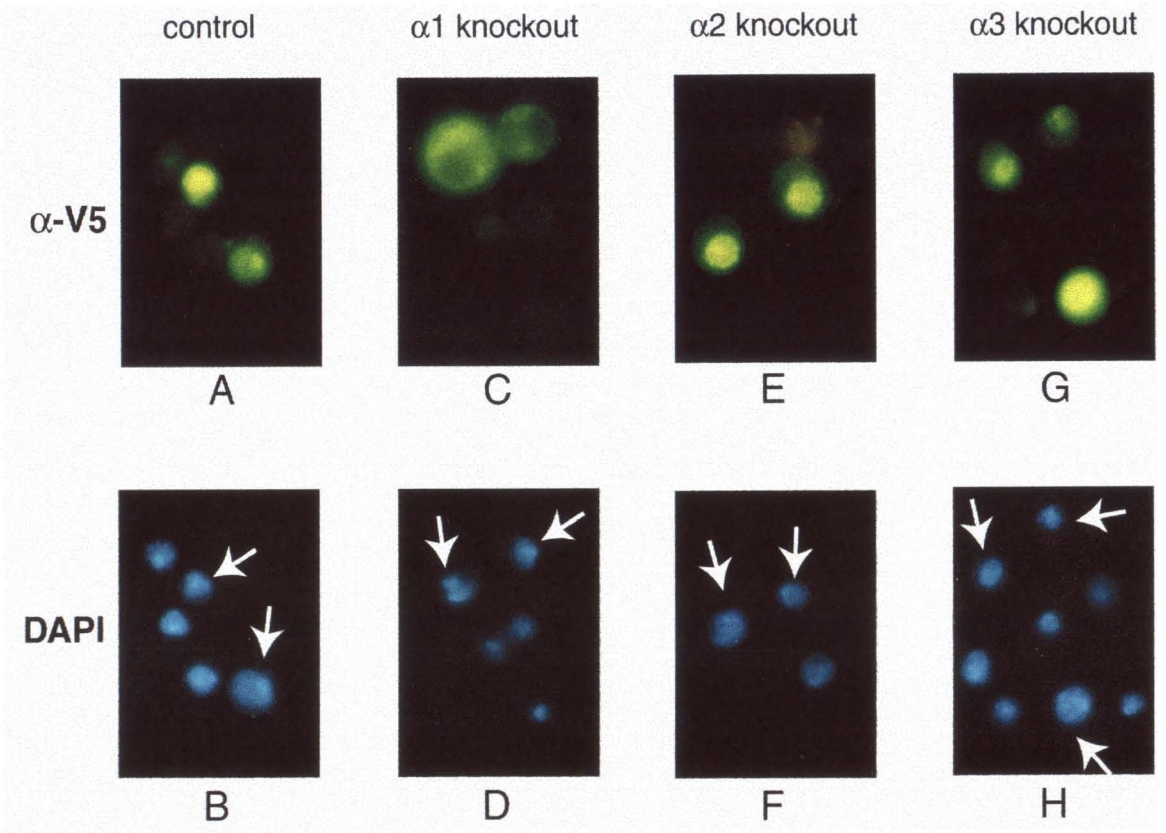
control
α1 knockout
α2 knockout
α3 knockout
α-V5
DAPI
A
B
C
D
E
F
G
H

Figure 4

Figure 4. Transfected TATA-Binding Protein cannot be imported into nucleus efficiently when Kap α1 expression is repressed by RNAi. S2 cells were transfected with C-terminal V5-epitope tagged TBP either alone (Control, Panel A) or together with dsRNA targeting Kap α1, α2 or α3 (knockout, panel C, E, G)). 72 hours after transfection and RNAi treatment, cells were fixed and stained with monoclonal anti-V5 antibody. DAPI staining was performed at the same time to show the nuclei. The nuclei of the cells expressing recombinant TBP are pointed out by the arrows. Knocking out Kap α2 and α3 does not affect the nuclear localization of TBP (compare Panel A, E, G). When Kap α1 is knocked out, recombinant TBP is predominantly localized in cytoplasm in about 60% transfected cells (compare panel A and C).

CHAPTER FIVE

DYNAMIC ASSOCIATION OF TRANSCRIPTIONAL ACTIVATION DOMAINS AND REGULATORY REGIONS IN *SACCHAROMYCES CEREVISIAE* HEAT SHOCK FACTOR

Tianxin Chen and Carl S. Parker
Dynamic Association of Transcriptional Activation Domains and Regulatory Regions in *Saccharomyces cerevisiae* Heat Shock Factor
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ABSTRACT

In *Saccharomyces cerevisiae*, the heat shock transcription factor (HSF) is thought to be a homotypic trimer that is bound to the promoters of heat shock protein (HSP) genes at both normal and heat shock temperatures. Exposure to heat shock greatly and rapidly induces HSF transcriptional activity without further increasing DNA-binding affinity. It is believed that HSF is under negative regulation at normal growth temperatures but the detailed mechanism by which HSF is activated is still not clear. We report the analysis of mutations in a conserved Arginine (residue 274) at the C-terminal end of the DNA-binding domain (DBD). Two mutations significantly increase both basal activity of HSF at normal temperatures and induced activity upon heat shock. We demonstrate by co-immunoprecipitation experiments that the mutations reduce the association between the DNA-binding domain/oligomerization domain and the transcription activation domains. Our studies suggest that the DNA-binding domain of HSF can interact with activation domains directly, and this interaction is important for the repression of HSF activity under normal growth conditions. Destabilizing this interaction by heat or by mutations results in HSF transcriptional activation. We propose that Arg 274 is critical for intramolecular repression of HSF activity in normally growing cells.

INTRODUCTION

Eukaryotic cells respond to heat shock and other physiological stresses by dramatically increasing the expression of a specific set of genes encoding the heat shock proteins (HSPs, for reviews, see (Mager and Dekruijff, 1995; Wu, 1995). The promoters of these genes possess a cis-acting heat shock element (HSE) that consists of multiple inverted, tandem repeats of the consensus sequence AGAAn, where n can be any nucleotide (Amin et al., 1988; Perisic et al., 1989). The heat shock transcription factor (HSF), which binds specifically to HSEs and regulates the heat shock response, has been identified in yeast (Sorger and Pelham, 1988; Wiederrecht et al., 1988; Jakobsen and Pelham, 1991), Drosophila (Clos et al., 1990), human (Kingston et al., 1987), and many other organisms (Wu, 1995).

In the budding yeast *Saccharomyces cerevisiae*, ScHSF is required for both growth at normal temperatures and viability upon stress. In fact, ScHSF is bound to DNA at all temperatures and heat shock converts the DNA-bound transcriptionally weak ScHSF into a highly active factor (Nieto-Sotelo et al., 1990; Sorger, 1990). Although upon heat shock the ScHSF shows some increased binding to secondary, low-affinity HSEs, the overall effect on DNA binding is insignificant and thus not likely to be an important regulatory step in ScHSF activation (Jakobsen and Pelham, 1988; Erkine et al., 1999). Other mechanisms, such as phosphorylation and (or) dephosphorylation, which modify the activity of the transcription activation domains, are more likely to be involved in the activation process (Sorger and Pelham, 1988; Sorger, 1990; Hoj and Jakobsen, 1994; Shi et al., 1995). Point mutation and deletion studies show that most mutations in

ScHSF cause an increase in the constitutive transcriptional activity (Nieto-Sotelo et al., 1990; Bonner et al., 1992; Morano et al., 1998; Morimoto, 1998), indicating that ScHSF is probably under negative regulatory control in the absence of stress.

The functional domains of HSFs have been well characterized. All HSFs from different species contain two conserved domains: a winged helix-turn-helix DNA binding domain (DBD) (Vuister et al., 1994) and a trimerization domain consisting of α-helical coiled-coil motif (Sorger and Nelson, 1989; Peteranderl and Nelson, 1992). Apart from these two domains, homology comparisons reveal no other significant conservation in the activation domains of HSFs. By deletion mapping and analysis of chimeric proteins containing HSF sequences fused to heterologous DNA-binding domains, three ScHSF activation domains were identified. The activation domain II (AII, residue 410-648) is essential for growth during heat shock but not for normal conditions. The roles of activation domain I (AI, residue 1-172) and activation domain III (AIII, residue 589-833) were not clear when they were first identified by Nieto-Sotelo et. al (Nieto-Sotelo et al., 1990), because AIII is dispensable for viability at both normal and stress-temperatures. Later studies showed that the AI domain probably functions in non-shocked cells to allow viability and is responsible for transient activity, while the AIII domain is probably responsible for sustained activities at increased temperature (Sorger, 1990). All three activation domains are constitutively active when fused to yAP-1 DNA-binding domain (Nieto-Sotelo et al., 1990). Mechanisms of repression at normal growth temperatures remain unknown, however, some evidence suggests that activation domains are restrained by the DNA-binding domain and trimerization domain (Nieto-Sotelo et al., 1990). Deletions of certain residues in the DNA-binding domain increase the overall activity of

HSF, indicating that DNA-binding domain may play an important regulatory role in addition to HSE recognition. However, it is not clear that the DNA-binding domain represses ScHSF activity through direct intramolecular interactions with activation domains, or perhaps indirectly with the association of another member of heat shock response circuitry, such as HSP70 or HSP90.

In this paper, we report experiments dealing with a key arginine residue (R274) and its role in HSF regulation. Arg274 is a highly conserved residue located at the C-terminal end of the HSF DNA-binding domain (Wu, 1995). We show that two mutations significantly increase both constitutive activity of HSF at normal temperatures and induced activity upon heat shock. In addition, co-immunoprecipitation experiments reveal that the DBD will bind to all three activation domains and mutations in Arg274 reduce this association. Our studies suggest that DNA-binding domain of HSF can interact with activation domains directly, and this interaction is important for the repression of HSF activity under normal conditions.

MATERIALS AND METHODS

Yeast Strains. The haploid yeast strain, pYES-URA, in which chromosomal loci of HSF was disrupted, was used. This hsf^- strain contains an episomal copy of HSF whose transcription is controlled by the GAL1,10 promoter (Wiederrecht et al., 1988).

Plasmid Constructions and Protein Expression. The shuttle vector used in this work, pYES2-FHC, is a 2μ-based high copy plasmid, with FLAG epitope and six-His tag at C-terminal, and with either TRP1 or URA3 selection (Sikorski and Hieter, 1989).

Expression vector pGEX-2t was used to make GST-fusion recombinant proteins. The fusion proteins were expressed in *E. coli* strain BL21(DE3), and purified with GST affinity resin (Stratagene). The GST-fusion proteins were eluted with 100mM reduced glutathione in 50mM Tris (pH 8.0), 0.5M NaCl, and 1% Triton X-100. Expression vector pCAL-n was used to make Calmudulin-Binding-Peptide (CBP)-fusion proteins (Stratagene), and the recombinant proteins were purified by Calmodulin affinity resin (protocols provided by Stratagene).

Mutagenesis. Desired mutant oligos were annealed to single-stranded dU DNA. The mixture was heated to 70°C, and slowly cooled to room temperature. Second strand was synthesized by adding T4 DNA polymerase and T4 DNA ligase. Sequencing of the plasmids confirmed that only the desired mutations were introduced. All the mutant HSFs were cloned into pYES2-FHC/TRP plasmid with BamHI and XhoI sites, and were selected by SG-TRP+URA+ 5-FOA plate.

Transformations. Yeast cells were transformed using ALKALI-CATION Yeast Transformation Kit from BIO 101 Inc. The protocols are based on lithium acetate procedure.

Liquid Culture β-galactosidase Assay. β-galactosidase enzyme assays were performed to quantify lacZ expression according to the protocols provided by CLONTECH Laboratories, Inc. (Palo Alto, CA) 1.5 ml mid-log cell culture (O.D. $_{600}$ = 0.5~ 0.8) was washed once and resuspended in Z buffer (60 mM sodium phosphate, pH7.0, 10 mM

KCl, 1 mM $MgSO_4$). Concentration factor V was noted. 100 µl of cell suspension was frozen in liquid nitrogen, and then thawed in 37°C for 1 minute. 0.7 ml Z buffer with 40 mM β-mercaptoethanol was added both to reaction and blank (100 µl Z buffer only) tubes. The reactions were started by adding 0.16 ml ONPG (prepared fresh before each use, 4 mg/ml in Z buffer) to reaction and blank tubes, and incubated at 30°C. After yellow color developed, 0.4 ml of 1M Na_2CO_3 was added to terminate the reaction. Start and Stop times were noted, the cells were spun down, and A420 of the supernatant was determined by spectrophotometrically.

$$\beta\text{-galactosidase units} = 1000 \times O.D._{420} / (tV \times O.D._{600})$$

Yeast Cell Extract Preparation and Western Blotting. Yeast whole cell extracts were prepared according to Scultz et. al (Schultz et al., 1991). Western blotting analysis was performed with about 100 µg of whole cell extract and anti-FLAG M2 antibody (Eastman Kodak Company, New Haven, CT). The immune complexes were visualized by AP (alkaline phosphatase)-based chromogenic method.

Total RNA Isolation and Primer Extension Analysis. Total RNA from control (25°C) and heat-shocked cells (40°C) was isolated using "RNAqueous Total RNA Isolation Kit" from Ambion Inc. (Austin, Texas). 5 ng of 5' end-labeled oligonucleotides (described below) were hybridized to 5~8 µg of total RNA in TE pH7.4 containing 200 mM KCl. Total final volume was adjusted to 12 µl. The oligonucleotides were annealed by heating the reaction mixture at 65°C for 30 minutes, followed by gradual cooling to room

temperature. Then AMV reverse transcriptase was added to the annealing product, and the reaction mixture was incubated at 42°C for 1 hour. The samples were electrophoresed on denaturing 8M urea-6% polyacrylamide gels. Dried gels were exposed to Molecular Dynamics PhosphoImager plates, which were then scanned on the Molecular Dynamics ImageQuant.

The oligonucleotides used for primer extension analysis are as follows:

SSA4: 5'- AGCATCGTTCGTCACTTCTGGATCA-3'

Actin: 5'- CCGGCTTTACACATACCAGA- 3'

In-vitro Cross-linking Assay. To investigate the oligomerization of wild type and mutant DBDs, 1μg of GST-tagged DBDs were cross-linked by addition of 2mM DSS to D buffer (25 mm Hepes, pH7.4, 100 mm KCl, 1 mm EDTA and 0.2% Triton X-100) and incubation for 30 min at 25° C. The reactions were quenched by the addition of Lysine to 20 mM, and analyzed by 8% SDS-PAGE. Western blotting was then performed using anti-GST antibody (Pharmacia), and molecular weight markers (Bio-Rad) were used to determine the approximate sizes of the complexes.

Mobility-shift Assay. Mobility-shift assay was performed using the HSE-containing fragment as the follows:

Wild type HSE: GCGCGCCTC**GAA**TG**TTC**GC**GAA**AAGA

Mutant HSE: GCGCGCCTC**GAA**TG**GG**CGC**GAA**AAGA

GAA repeats are shown in bold, and mutated nucleotides in mutant HSE are underlined. Binding assay buffer contains 250 μg/ml BSA, 100 μg/ml poly dI•dC, and 4% Ficoll.

γ^{32}P labeled HSEs or unlabeled HSE were incubated with 1μg of wild type or mutant DBDs at room temperature for 20 minutes and the reaction complexes were analyzed by 5% acrylamide, 0.5X TBE gel.

Co-immunoprecipitation Assay. To investigate the interactions between DBDs and activation domains, CBP-tagged activation domains were labeled with γ^{32}P-ATP by PKA. GST-tagged wild type and mutant DBDs were immobilized by anti-GST antibody coupled Protein G Sepharose (Pharmacia), then incubated with γ^{32}P-AI, AII or AIII (~50000 cpm/μl) for 30 min at 25°C. After 3 washes with D buffer containing 0.5M NaCl and 0.5% Triton X-100, the precipitates were resuspended in SDS loading buffer and analyzed by 12% SDS-PAGE and autoradiography.

RESULTS

Effect of a DNA-binding domain mutation on Heat Shock Factor activity

By mutagenesis of ScHSF, we isolated a temperature sensitive mutant that is only viable at room temperature and 30°C, but not at 37°C (Table 1). Sequence analysis showed that a mutation in the DBD of HSF had occurred. The original sequence Arg-Gln-Lys (amino acid 274-276) was altered to Ser-Gln-Gln-His-Ala. This mutant was designated *dbd-mut*.

To compare the transcriptional activities of wild type and *dbd-mut* HSFs, the levels of specific HSP gene transcription were analyzed. Transcriptional activities of wild type and *dbd-mut* HSFs were determined by directly measuring the level of mRNA transcription from SSA4, the major HSP70 inducible gene, by primer extension. Actin

mRNA was monitored simultaneously as an internal control. Surprisingly, although the transcription of SSA4 in wild type cells was not detectable under non-shock conditions, in the *dbd-mut* cells, SSA4 was actively transcribed at control temperature (25°C) (Figure 1A, lane 4). In addition, the activity of this mutant ScHSF can be further induced by heat shock: the transcription level of SSA4 in *dbd-mut* cells is about three times higher than that of wild type cells at heat shock temperature (40°C) (determined by phosphoimage analysis, Figure 1, compare lane 2 and 5). During recovery from heat shock, wild type cells recover significantly quicker than the *dbd-mut* cells (data not shown). While in wild type cells, SSA4 is not detectable 60 minutes after heat shock, in *dbd-mut* cells, SSA4 transcription returns to pre-heat shock levels.

The drastically increased HSP70 expression might account for the lethality of this mutant at heat shock temperatures. It has been reported before that elevated level of Hsp70 can impede cell growth and division (Edwards, 1998). With activation of the HS response, normal protein synthesis is suspended and protective HSPs are produced which rescue the heat-denatured proteins. But cell survival is achieved at the expenses of normal growth and development, presumably because HS response takes precedence over other developmental events and alters the normal programs of gene activity (Edwards, 1998). The hyperactive *dbd-mut* HSF could potentially disrupt the expression of essential housekeeping genes and cause cell death.

Arginine 274 is important for negative regulation of Heat Shock Factor's transcription activity

Among the amino acids altered in *dbd-mut* ScHSF, Arginine 274 is a highly conserved residue present in HSFs from many different species (Wu, 1995). The crystal structure of *Drosophila melanogaster* HSF's DNA-binding domain shows that Arg 274 is located in a flexible linker region at the very end of the C-terminus. Arg274 is about 20 residues downstream of the β4 strand, and probably not directly involved in DNA binding. To analyze the specific functional significance of Arg274, three site-directed mutants were created: R274K, R274G, and R274E, in which the Arg was changed to positive, neutral and negatively charged residues respectively. To study the properties of these mutant HSFs, we used a yeast strain carrying a disrupted chromosomal HSF locus. HSF is an essential gene in yeast, so the strain with a disrupted chromosomal HSF was kept viable with a HSF gene carried on an URA3-containing vector. This strain was transformed with mutant ScHSFs cloned in a TRP1-based plasmid. Selection on 5-fluoroorotic acid allowed the loss of the URA3-containing wild type ScHSF plasmid, leaving cells with only mutant ScHSF on the TRP1 vector.

R274E mutant is completely lethal, all the cells died after plating onto 5-FOA plates. R274G mutant grows normally at 30°C but has a slightly slower growth rate at 37°C, while R274K mutant exhibit wild type phenotype (Table 1). Primer extension analysis of HSP70 shows that the transcriptional activity of the R274K mutant HSF is properly regulated by temperature (Figure 1A, lane 7-9). On the other hand, although R274G mutant does not show growth defects, the activity of R274G-HSF is higher than that of wild type both at non-shock (25°C) and heat shock (39°C) temperatures (Figure 1A, lane 9-12). The abnormal behavior of *dbd-mut* and R274G HSF demonstrate that Arg274 is very important for the negative regulation of HSF activity. Without the

positive charge at this position, the activity of ScHSF is significantly elevated at control temperatures.

The viability of the mutant strains correlates with the severity of the mutations. The presence of a negatively charged residue probably destablizes the conformation of HSF significantly, causing cell death. On the other hand, R274G is a relatively mild alteration. Phosphoimager quantitation shows that SSA4 mRNA level in heat-shocked R274G mutant cells is about half as much as in heat-shocked *dbd-mut* cells. Yeast cells may be able to tolerate moderately high level of HSP70 expression, resulting in a slightly slower growth rate.

To rule out the possibility that different levels of HSP70 transcription are due to different levels of HSF expression, Western blotting analysis was used to detect the expression of HSF in wild and mutant cells. Equal levels of HSF were expressed in all these cells (Figure 1B), indicating that it is the Arginine 274 mutation that activates HSF under non-shock temperatures.

The transcriptional activities of mutant HSFs were also inspected by β-galactosidase enzyme activity assays using a yeast strain transformed with (HSE)4/LacZ reporter gene (Supplemental Figure). The results confirmed that *dbd-mut* and R274G HSFs have higher activity both at non-shock and heat shock temperatures, while the R274K HSF mutant was very similar to wild type.

Mutation of arginine 274 causes a reduction in DNA binding affinity

One possible explanation for the hyperactivity of *dbd-mut* and R274G HSF is increased DNA-binding affinity. Although several earlier studies have shown that the

level of DNA binding does not correlate with transcription levels before or after heat shock (Mager and Dekruijff, 1995), it was important to measure the DNA binding affinities of mutant HSFs. Wild type and mutant HSFs from residues 167 to 424 (including the DNA-binding domain and the helical oligomerization domain, Figure 4A) were fused to a GST-tag and the recombinant proteins were purified by GST-affinity chromatography (Stratagene). As determined by gel mobility shift assays, all four recombinant DBDs bind to ^{32}P labeled-HSE sequence (Figure 2, lane 1, 6, 11, 16), but none of the examined recombinant DBDs bound to ^{32}P labeled-mutant HSE, in which the nGAAnnTTCn consensus was mutated to nGGAnn**GG**Cn (lane 5, 10, 15, 20). In addition, binding to ^{32}P-HSE is effectively competed away by unlabeled HSE (Figure 2, lane 4, 9, 14. 19), demonstrating the specificity of wild type HSE recognition by recombinant DBDs. Interestingly, we observed that the wild type DBD has the highest affinity for HSE (lane 1-3), R274G also exhibits high DNA-binding affinity, but is slightly lower than wild type. Both the *dbd-mut* and R274E show a significant decrease in HSE binding affinity. This result clearly demonstrates that the derepression observed by the mutant HSFs is not due to an increased DNA binding activity. Furthermore, from the structural information available on the *Drosophila* HSF and *K. lactis* HSF, R274 does not interact with the DNA directly (Littlefield and Nelson, 1999; Wu, 1995), therefore, the decreased DNA-binding affinity must result from secondary effects of R274 mutations.

In addition to the major DNA- protein complex, mutant DBDs and HSE also form higher molecular weight complexes (Figure 2, lane 6, 11, 16, upper arrow). This high molecular weight complex is not seen in wild type DBD-DNA complex. To examine the

molecular interaction causing the higher molecular weight complex, wild type and mutant DBDs were crosslinked in the absence of DNA and analyzed by SDS-PAGE. Western blotting analysis of cross-linking products of DBDs shows that wild type DBD exists in an equilibrium of monomers, dimers and trimers (Figure 3, lane 1), whereas *dbd-mut* and R274E exist predominantly as trimers and hexamers (lane 2 and 4, based on their apparent molecular weight). Monomer forms of these two DBDs are almost absent. The oligomerization state of R274G is in between, with all four forms present (lane 3). This result provides additional evidence that Arg 274 mutation causes the overall conformational changes of DBD and oligomerization domain, and this conformational change leads to a greater tendency to form high molecular weight oligomers. It is likely that mutation of the Arginine perturbs the organization of oligomerization domain, increasing the tendency of HSF to form either high molecular weight oligomers or an alternate trimer conformation, perhaps reflecting an "activated" state.

DNA-binding domains of mutant ScHSFs have reduced affinity to the activation domains

Previous deletion studies of ScHSF have revealed the presence of certain functional domains (Figure 4A) (Nieto-Sotelo et al., 1990), including a DNA-binding domain, a helical oligomerization domain, and three transcription activation domains, AI, AII and AIII, whose activities are repressed at normal temperatures. It has been reported that DNA-binding domain may play an important regulatory role of repressing transcriptional activation in addition to its obvious role in mediating DNA binding (Bonner et al., 1992; Hardy et al., 2000). Many mutations in DNA binding domain

increase HSF's activity without enhancing the DNA binding properties. For example, a M232V mutation of ScHSF has a constitutively activating phenotype (Bonner et al., 1992).

The hyperactivity exhibited by *dbd-mut* and R274G ScHSFs suggest that under normal conditions, activation domains of HSF are probably masked by the DNA-binding and oligomerization domains. In case of these two mutated ScHSFs, loss of the positively charged Arg causes the derepression of the activation domains. To study the physical interactions between activation domains and DNA-binding domain, we separately cloned the AI (residue 1-172), AII (residue 410-648), AIII (residue 589-833), and DNA-binding and oligomerization domains (DBD, residue 167-424) into different prokaryotic expression vectors, and examined the interactions between them by immunoprecipitation.

Wild type and mutant DBDs were fused to GST-tag and purified as described before, and then immobilized by anti-GST antibody coupled to Protein G Sepharose. AI, AII and AIII were fused to calmodulin-binding-peptide (CBP)-tag, and the recombinant AI, AII and AIII were purified through calmodulin-coupled chromatography. Purified activation domains were labeled in vitro with PKA (protein kinase A) and γ^{32}P-ATP at a PKA target sequence within the CBP. The AI, AII and AIII were incubated with immobilized wild type and mutant DBDs, and the precipitated products were analyzed by SDS-PAGE. Figure 4B shows that HSF DBD binds to all three activation domains. The wild type DBD showed the highest level of association with the activation domains compared to the mutant DBDs (Figure 4B, lane 2, 7, 12). The binding properties of the mutant DBDs to the activation domains correspond well with their phenotypes and

transcription activities. The DBD derived from the lethal mutant R274E demonstrated the lowest level of binding to all three activation domains, while R274G is only moderately less able to bind AII and AIII than is wild type DBD. Figure 4C shows that same amount of DBDs were used in these experiments. These co-immunoprecipitation results confirm the idea that DBD interacts with the activation domains, and these interactions may be important for the repression of HSF activity at non-shock temperatures.

Considering the fact that three Arg274 mutations lowered the binding affinities for all three activation domains, this residue may be important for maintaining the overall structure of the DBD and oligomerization domain, but probably does not mediate specific interactions between the DBD and the activation domains. Circular dichroism studies of DBD shows that Arg 274 is within a flexible linker region without any ordered secondary structure (Flick et al., 1994). It has been suggested that this linker region might be important to adjust the 3-fold symmetric HSF trimers to the 180° symmetry between inverted HSE repeats. So it is likely that loss of the positive charged Arg274 causes some overall conformational changes of DBD and oligomerization domain, resulting in the decreased association with the activation domains.

dbd-mut HSF is activated at a slower rate than wild type HSF and may be analogous to partially activated HSF

The kinetics of ScHSF activation suggests that *dbd-mut* ScHSF may already be in the active conformation mimicking activated ScHSF. Figure 5 shows the transcription of SSA4 when wild type and *dbd-mut* cells were heat shocked for 0 minute, 5 minutes, 10

minutes, 15 minutes, 30 minutes, and 3 hours respectively. The induction of SSA4 transcription is very rapid in wild type cells. The amount of SSA4 mRNA is greatly increased after 5 minutes of heat shock, and reaches peak level only after 15 minutes of heat shock. Beyond 15 minutes, SSA4 transcription is maintained at a stable level (Figure 5A, lane 1-4). Long periods of heat shock (3 hours, lane 5) decrease SSA4 transcription, probably because expression of HSP70 negatively regulates HS response (Morimoto et al., 1992). In contrast, 5 minutes of heat shock did not induce further activation of *dbd-mut* HSF at all (lane 7). mRNA level of SSA4 remains unchanged. This observation suggests that *dbd-mut* mimics an activated HSF, so short-term heat shock will not further increase its transcriptional activity. Longer periods of heat shock (15 and 30 minutes, lane 8, 9) probably thermally destabilizes the structure of *dbd-mut* HSF, causing further exposure of activation domains, and in turn the further activation of mutant HSF. Three hours of heat shock causes massive death of mutant cells, which can be observed from the obviously decreased transcription of actin (lane 10). The kinetics of transcriptional activation was further examined by phospho-imaging and the results were plotted in Figure 5B. The ratios of SSA4 transcription to actin are displayed as a function of time. Clearly, the wild type responds rapidly and peaks at 15 minutes. The *dbd-mut* does not show the initial increase in transcription, rather a delayed stimulation is observed at 10 minutes.

DISCUSSION

The activation domains of ScHSF are repressed in non-stressed cells. It is known that sequences present in the DBD, oligomerization domain and a short conserved

element, CE2 are required to maintain repression (Wu, 1995). Within the DNA binding domain of ScHSF, mutation of residue M232 to V results in constitutive activation at non-stress temperatures (Bonner et al., 1992). In this report, we show that mutation of R274, located in a solvent-exposed position of the linker region between DNA binding domain and oligomerization domain, leads to derepression at normal growth temperature. These conserved residues must play an important role in the maintenance of the inactive state of the transcription factor.

Although not directly involved in DNA binding, Arginine 274 is highly conserved and found in *K. lactis* HSF, *S. pombe* HSF, *Arabidopsis thaliana* HSF, *Drosophila* HSF, mouse HSF1/2, and human HSF1/2 (Wu, 1995). Our results demonstrate that a conservative change of Arg274 to lysine has little effect on ScHSF transcriptional activity. In contrast a mutation that reverses that charge of R274 to glutamic acid results in lethality. These observations suggest that the positive charge at this position is important for normal activity of ScHSF. The DBDs from *dbd-mut* and R274G mutants showed reduced affinities for all three activation domains and tended to form higher order structures. Overall these observations suggest that Arg274 is critical for maintaining the inactive structure of ScHSF and mutations in this residue result in significant conformational changes. The portion of the DBD where Arg274 is located has no ordered secondary structure as determined by circular dichroism (Flick et al., 1994). Therefore, despite the fact Arg274 is a highly conserved residue it may be in a flexible portion of ScHSF. The value of a flexible linker between the DBD and oligomerization domain may be to assist DNA binding. If ScHSF binds to DNA as a trimer, three DBDs would be rotated 120° relative to each another. The HSEs however,

are palindromic repeats with 180° symmetry. The linker region perhaps maintains the correct positioning of the DBD and oligomerization domain, so that a trimer can fit into HSEs (Hardy et al., 2000). Without a positive charge at residue 274, as seen with the mutants described in this paper, the correct positioning of DBD and oligomerization domain might be disturbed, and the mutants can not bind to DNA with high affinity.

The structural integrity of the DBD and the oligomerization domain seems to be very important for their negative regulatory functions. Recent work from Hardy *et. al* (Hardy et al., 2000) showed that deletions of conserved residues within an α-helical bulge in DBD increases the overall activity of ScHSF, but when these conserved residues were mutated, there was little effect observed. This result demonstrates that the presence of a bulge in DBD, rather than specific residues, is critical for the negative regulation. Similarly, structural disturbance resulted from Arg274 mutation can account for the reduced affinities for activation domains and increased HSF transcriptional activities. Unfortunately, without the structural information of the full length HSF trimer complexed with DNA, we do not know at this time the detailed function of this arginine.

It has been shown that human HSF1 purified from Hela cell extract can be activated by heat shock (Larson et al., 1995), and recombinant mouse HSF1 can acquire DNA-binding upon in vitro heat shock (Goodson and Sarge, 1995). All these studies suggest that HSF may be capable of sensing heat shock directly. Perhaps heat shock induces structural changes of HSF, especially within DBD and oligomerization domain, destabilizing the interactions between DBD and activation domains and thus increasing transcriptional activity. The fact that the transcriptional activities of wild type, *dbd-mut* and R274G ScHSFs correspond very well with their respective affinities for activation

domains supports this idea. *dbd-mut* and R274G HSFs are activated even at 25°C. We suggest that this is the result of a less tight association between the DBD and activation domains. In other words, structural changes caused by the Arg 274 mutation might mimic the structural changes induced by heat shock.

We also demonstrated here that increased transcriptional activity of mutant ScHSFs are not due to increased DNA-binding affinity. A substantial amount of data already indicates that the degree of DNA-binding does not correlate with transcriptional activities of ScHSF. For example, SSA4, the major HSP70 in S. *cerevisiae*, is expressed at extremely low level during steady-state growth at 23° C, but its expression is greatly enhanced upon upshift to 39°C (Lindquist and Craig, 1988). However, no further increase in DNA-binding at the SSA4 promoter was observed (Sorger et al., 1987). In our case, although *dbd-mut* showed reduced DNA-binding affinity compared with wild type, its transcriptional activity, both at non-shock and heat shock temperatures, was actually higher. This observation further confirms that, at least for strong HSEs, like those present in the SSA4 promoter, increased DNA-binding is not the major regulatory process involving in HSF activation. In fact, conformational changes and destabilization of certain intramolecular interactions can even overcome the reduced DNA-binding affinity and activate HSF. For some promoters with weaker, secondary HSEs, such as the HSP 82 promoter, heat shock does lead to an increased occupancy of HSF, and the increased DNA-binding contributes to the overall activation process. However, it is not likely that the moderate increase in DNA-binding fully accounts for the very significant increase in transcriptional activity. Therefore, derepression of the activation domains is probably the most important mechanism involved in the activation process. Our results

support the hypothesis that DNA-binding domain of ScHSF has an important role in negative regulation, and that the activation process involves a dynamic dissociation of DBD/oligomerization domain from the activation domains. While this manuscript was being evaluated, related studies employing alanine-scanning mutations in the DBD of ScHSF also demonstrate the interactions between the DBD and the activation domains (Bulman et al., 2001). Structural studies of full length HSF will provide insights that should demonstrate how mutation of Arg274 causes conformational changes of the DBD and oligomerization domain.

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FIGURES AND FIGURE LEGENDS.

Phenotypes of yeast cells expressing mutant ScHSFs:

	Wild type	*dbd-mut*	R274 K	R274 G	R274 E
25°C	+++	++	+++	+++	-
30°C	+++	+	+++	+++	-
37°C	+++	-	+++	++	-

Table 1: Phenotypes of yeast cells expressing mutant ScHSFs. Yeast strain in which the chromosomal locus of ScHSF has been deleted was transformed with plasmids expressing wild type and mutant ScHSFs. Cell growth is scored by visually examining the growth properties of the cells on plates. +++ wild type growth, ++ good growth but slower than wild type, + slow growth, - lethal.

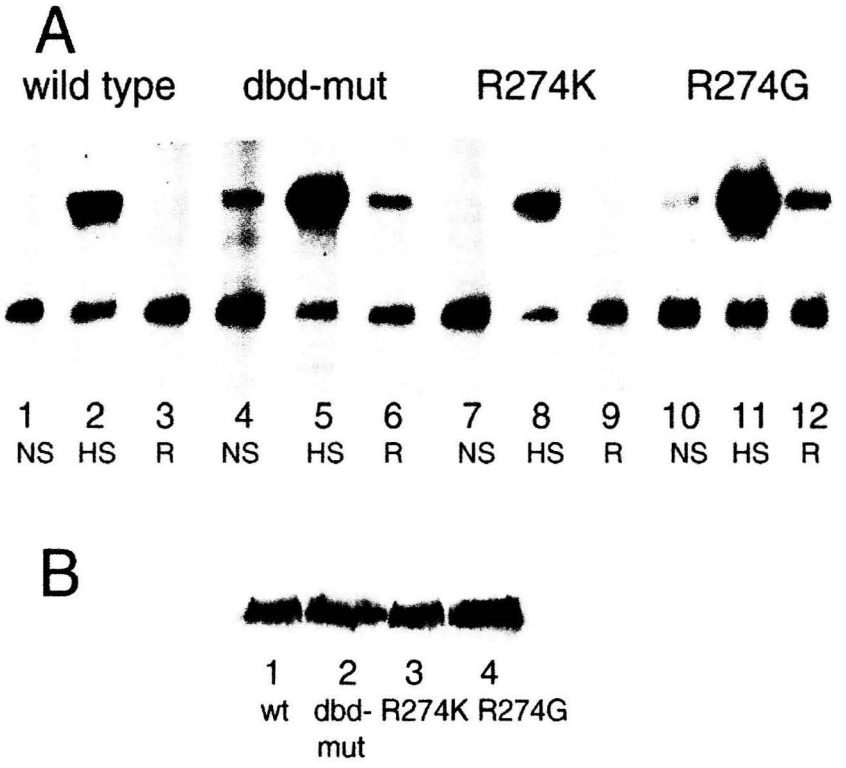

A
wild type
dbd-mut
R274K
R274G
1 2 3 4 5 6 7 8 9 10 11 12
NS HS R NS HS R NS HS R NS HS R
B
1 2 3 4
wt dbd- R274K R274G
mut

Figure 1

Figure 1. Transcriptional activities of mutant HSFs. A, Transcriptional activities of wild type and mutant ScHSFs are measured by levels of HSP70 mRNA using primer extension. Primer extension products are indicated (HSP70: 309-313 nucleotides, actin: 198-200 nucleotides). Lane 1-3: wild type HSF, lane 4-6: *dbd-mut* HSF, lane 7-9, R274K HSF, lane 10-12 R274G HSF. NS: non-shock, cells grown at 25°C. HS: heat shock, cells subjected to heat shock at 40°C for 30 minutes. R: recovery, cells put back to 25° C for 1 hour after heat shock to allow recovery. **B,** Western blotting analysis of wild type and mutant HSF proteins. Yeast cell extracts were subjected to SDS-PAGE and transferred to nitrocellulose filter. The protein blot was probed with anti-FLAG antibody (Sigma) fused to the C-terminus of HSFs.

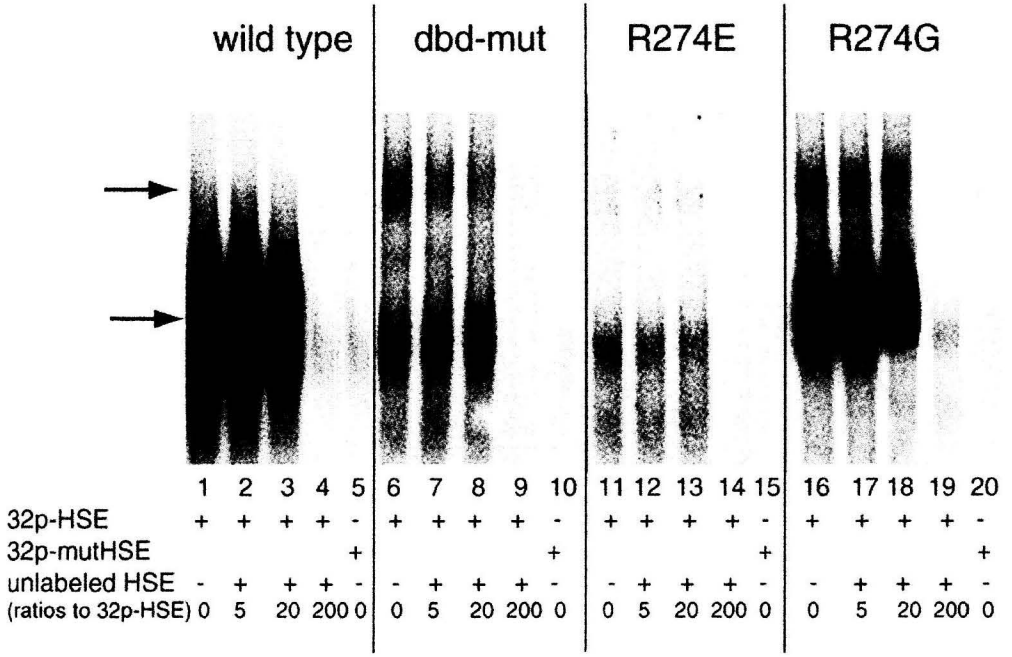

wild type
dbd-mut
R274E
R274G
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
32p-HSE + + + + - + + + + - + + + + - + + + + -
32p-mutHSE + + +
unlabeled HSE - + + + - - + + + - - + + + - - + + + -
(ratios to 32p-HSE) 0 5 20 200 0 0 5 20 200 0 0 5 20 200 0 0 5 20 200 0

Figure 2

Figure 2. DNA-binding assay. Gel mobility shift assay of wild type and mutant DBDs to labeled HSE is shown. A mutant HSE with nGAAnnTTCn repeats changed to nGAAnnGGCn was also used to ensure binding specificity (lane 5, 10, 15, 20). Increasing amount of unlabeled HSE was added for competition assay. The numbers shown at the bottom of each lane are the ratios of unlabeled HSE to ^{32}P-HSE. Major DBD-HSE complexes are shown by lower the arrow. Mutant DBDs also form higher-order complexes (upper arrow). Note that microgram quantities of HSFs are used in these experiments because the recombinant proteins bind to the HSE less well than the native HSF.

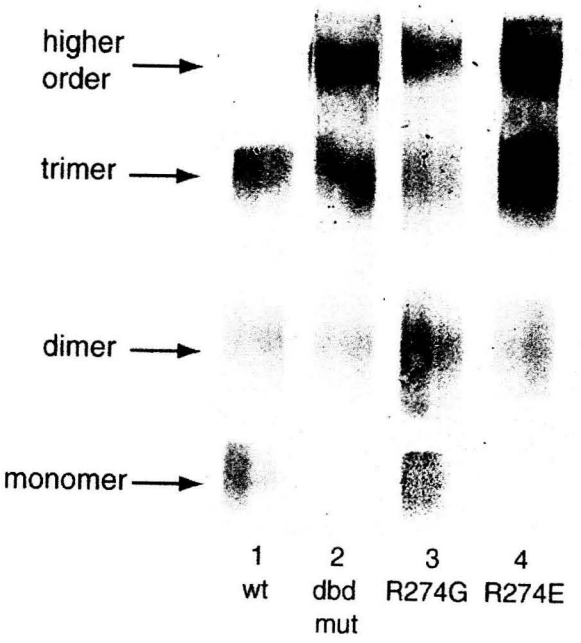

higher
order
trimer
dimer
monomer
1
wt
2
dbd
mut
3
R274G
4
R274E

Figure 3

Figure 3: Oligomerization state of wild type and mutant HSFs. 1 μg of recombinant wild type and mutant DBDs were cross-linked by DSS and analyzed by western blotting analysis. Wild type DBD exited as an equilibrium of monomer, dimer and trimer (lane 1). Mutant DBDs also formed hexamer (lane 2, 3, 4).

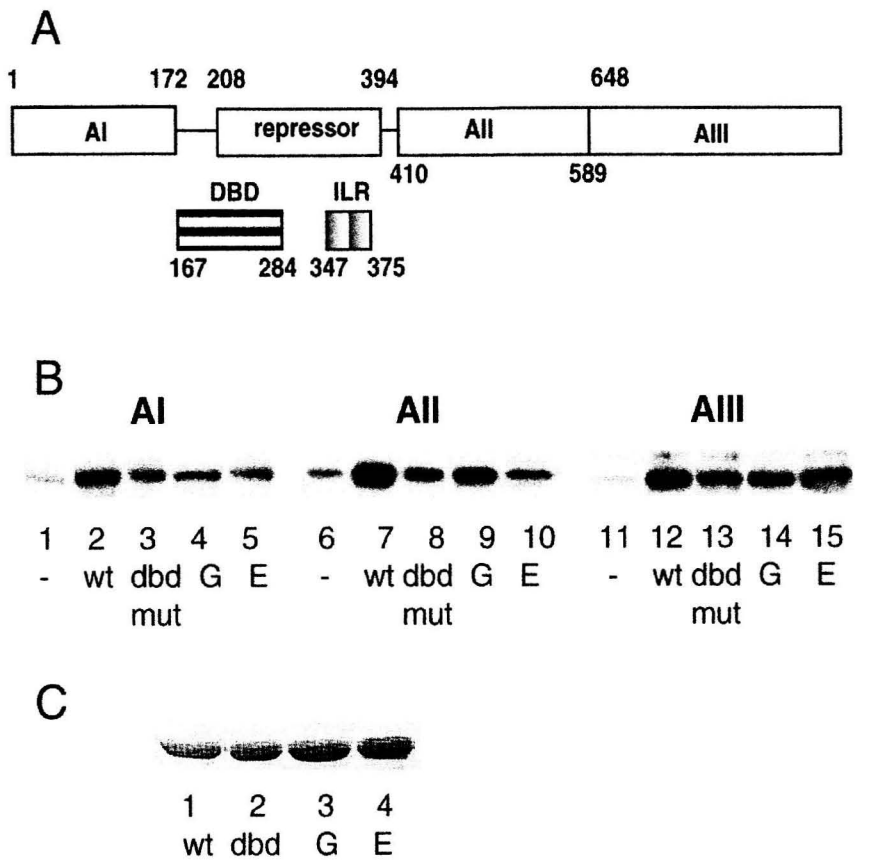

A
1
172
208
394
648
AI
repressor
AII
AIII
410
589
DBD
ILR
167
284
347
375
B
AI
AII
AIII
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
- wt dbd G E - wt dbd G E - wt dbd G E
mut
mut
C
1 2 3 4
wt dbd G E

Figure 4

Figure 4. Binding affinities between DNA-binding domain and activation domains correlate with HSF transcriptional activities. **A,** Schematic diagram of domain organization of *S. cerevisiae* HSF. Amino acid endpoints for each region, as well as their proposed functions are indicated. (AI, AII and AIII represent three constitutive activation domains. DBD, DNA binding domain, IRL, isoleusine repeat (oligomerization domain). This diagram is adapted from the work of Nieto-Sotelo et. al (10). **B,** Co-immunoprecipitation of activation domains with wild type and mutant DBDs. Recombinant wild type and mutant DBDs were immobilized by anti-GST antibody coupled Portein G Sepharose. Recombinant activation domain I (AI), activation domain II (AII) and activation domain III (AIII) were labeled with γ^{32}P-ATP and PKA catalytic subunit. ^{32}P-labeled AI (lane 1-5), AII (lane 6-10), and AIII (lane 11-15) were precipitated by immobilized DBDs. Equal amount of anti-GST antibody coupled Protein G Sepharose without DBD was used as control to asses non-specific interactions (lane 1, 6, 11). **C,** western blotting analysis of immobilized wild type and mutant DBDs used in co-immunoprecipitation.

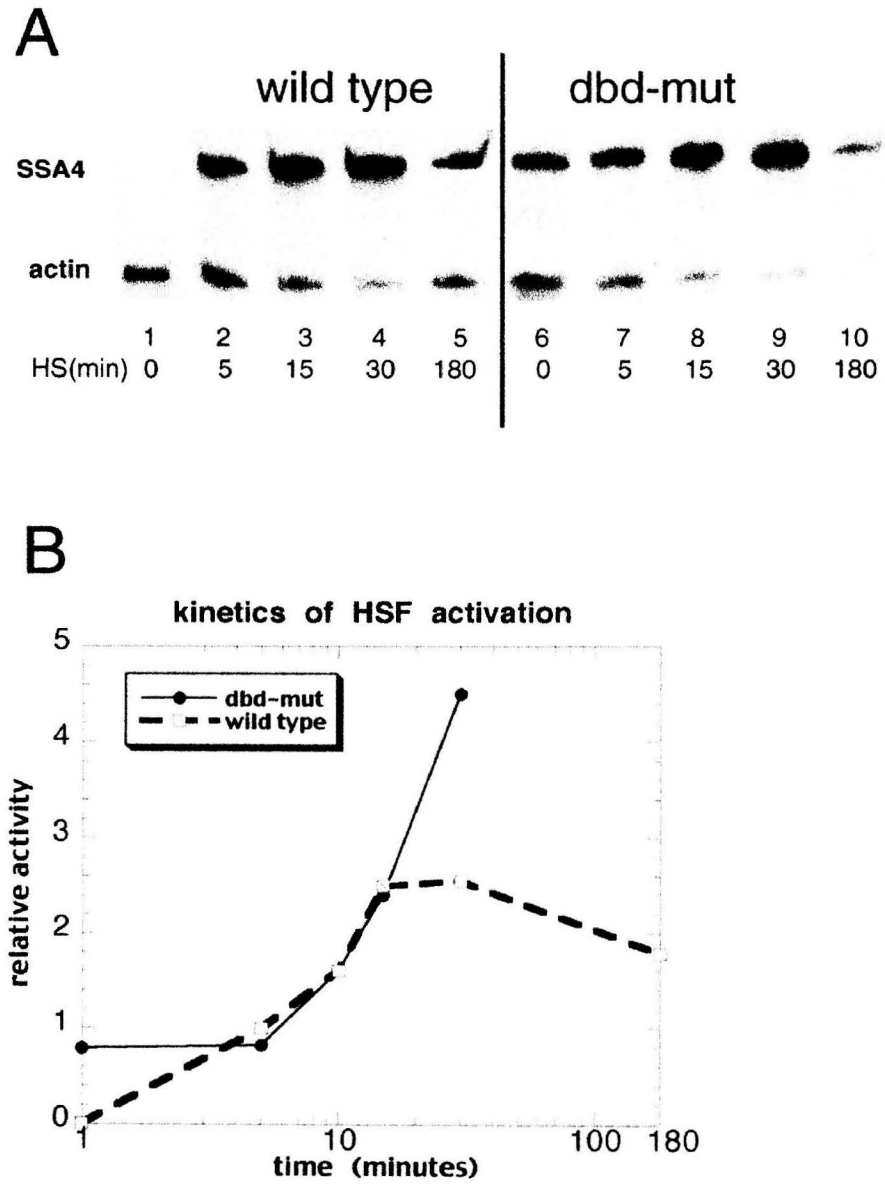
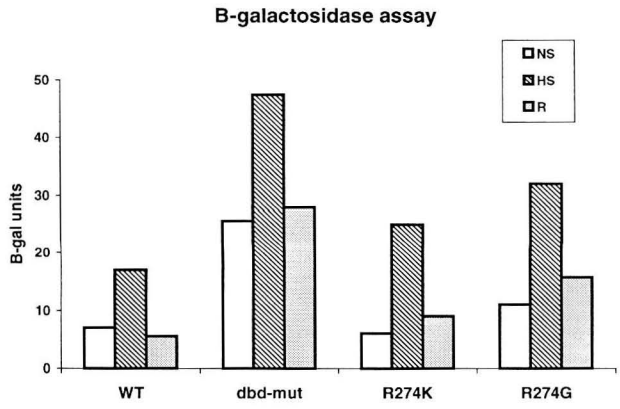

A
wild type
dbd-mut
SSA4
actin
1 2 3 4 5 6 7 8 9 10
HS(min) 0 5 15 30 180 0 5 15 30 180
B
kinetics of HSF activation
dbd-mut
wild type
relative activity
0 1 2 3 4 5
1 10 100 180
time (minutes)

Figure 5

Figure 5. Kinetics of wild type and *dbd-mut* HSF activation. **A,** Primer extension analysis of the activation kinetics of wild type and *dbd-mut* HSFs. Cells are harvest after 0, 5, 15, 30 or 180 minutes of heat shock, and HSP70 mRNA levels were measured by primer extension. Actin mRNA was measure at the same time as internal control. *dbd-mut* HSF (lane 6-10) is activated slower than wild type (lane 1-5). **B,** Kinetics plot of wild type and *dbd-mut* HSFs activation. The units are arbitrary. The ration of HSP70 mRNA transcription to actin mRNA transcription at 5 minutes of heat shock in wild type cells was set to 1 and all the other quantitative data (obtained from phosphoimager plate) were normalized accordingly. X-axis is drawn logarithmically. The transcriptional activities of *dbd-mut* HSF after 30 minutes of heat shock were not shown, because progressive cell death caused inaccurate measurement.



Supplemental Figure

Supplemental Figure. β-galactosidase activity assay of wild type and mutant HSFs. Reporter plasmid $(HSE)_4$/LacZ was transformed into the wild type and mutant strains and β-gal activities of were measured using ONPG as the substrate. The units were calculated according to the formula provided by Clontech.

CHAPTER SIX

DROSOPHILA HEAT SHOCK TRANSCRIPTION FACTOR INTERACTS WITH THE POSITIVE ELONGATION FACTOR P-TEFb TO STIMULATE THE TRANSCRIPTION OF HEAT SHOCK GENES

This chapter is in collaboration with Michael Reid.

ABSTRACT

Promoter-proximal pausing during transcriptional elongation is an important way of regulating many diverse genes. In case of Drosophila Hsp 70 gene, RNA polymerase II is bound to Hsp70 promoters even in the absence of heat shock, and paused after initiating a short transcript approximately 25 nucleotides. Heat shock induces polymerase II hyperphosphorylation and in turn releases the arrested RNA polymerase II into productive elongation. To understand the mechanisms that regulate this process, RNA interference was used to identify the kinase(s) involved in heat shock response. We demonstrate that repressing the expression CDK9, the kinase subunit of positive transcription elongation factor b (P-TEFb), significantly affects the Hsp70 mRNA transcription. Immunoprecipitation showed that P-TEFb is bound to Heat Shock Transcription Factor (HSF) upon heat shock, and the complex can phosphorylate polymerase II C-terminal domain (CTD) in vitro. We also demonstrate that CTD kinase activity associated with HSF is inhibited by either RNAi targeting CDK9, or CDK9 inhibitor DRB. Taken together, our reports suggest that HSF interacts with P-TEFb and releases the promoter-paused polymerase II into efficient elongation.

INTRODUCTION:

Eukaryotic mRNA synthesis is catalyzed by multi-subunit RNA polymerase II and proceeds through multiple stages referred to as preinitiation, initiation, elongation and termination (Conaway and Conaway, 1999). Although transcriptional regulation usually occurs at the initiation stage, by a selective binding of RNA polymerase II (pol II), general transcriptional initiation factors (TFIIA, TFIIB, TFIID, TFIIE, TFIIF, and TFIIH) and Mediator complexes to specific promoters (Lee and Young, 2000), recent studies have clearly demonstrated that the transcription of certain genes is regulated at the promoter escape and early elongation stage (Conaway et al., 2000). The expression of viral genes encoded by HIV-1 provides the most striking demonstration of regulation at transcription elongation stage. Before T cell activation, the HIV long terminal repeat (LTR) promoter produces mostly short transcripts; upon T cells activation, HIV-1 encoded Tat suppresses premature arrest by RNA polymerase II and promotes efficient elongation of full length HIV transcripts (Price, 2000; Taube et al., 1999). Similarly, Drosophila Hsp70 gene and Hsp 26 gene (Lis and Wu, 1993; Rougvie and Lis, 1988), human Hsp70 (Brown et al., 1996), c-myc (Krumm et al., 1994; Strobl and Eick, 1992), and c-fos (Plet et al., 1995) are also regulated by transcriptional elongation.

Phosphorylation of the RNA polymerase II large subunit C-terminal domain (CTD) is critical for the transition from the pre-initiation complex to the elongation complex (Conaway and Conaway, 1999). The promoter-paused Pol II is largely hypo-phosphorylated (pol IIa), whereas hyper-phosphorylated pol II (pol IIo) is capable of

highly productive elongation (Lis et al., 2000). The CTD of RNA polymerase II shows a remarkable feature: it consists of tandem repeats of an evolutionarily conserved motif of heptapeptide, $Y^1S^2P^3T^4S^5P^6S^7$ (Bregman et al., 2000). Twenty-six repeats are present in the yeast subunit, 45 in Drosophila, and 52 in the mammalian subunit (Riedl and Egly, 2000). The heptapeptides may be either perfect or imperfect iteration of the consensus sequence, and deletions of the mouse, Drosophila, or yeast CTD turned out to be lethal (Riedl and Egly, 2000). Five out of the seven amino acids in the heptapeptide repeats have the potential to be phosphorylated. Multiple CTD kinases have been identified so far and were shown to phosphorylate distinct positions within the repeats (Bregman et al., 2000). The first CTD kinase characterized was the cyclin-dependent kinase (CDK) $p34^{cdc2}$, however, its physiological significance remains unclear (Sterner et al., 1995). Three other CDKs, CDK7, CDK8, and CDK9 emerged as major candidates involved in CTD phosphorylation in vivo (Riedl and Egly, 2000). CDK7 (yeast KIN28), associated with cyclin H (yeast CCL1), is a subunit of the general transcription factor TFIIH. It is suggested that the CTD kinase activity of CDK7/cyclin H is responsible for converting pol IIa to pol IIo at the promoter clearance stage (Svejstrup et al., 1996). CDK8 (yeast SRB10) and its partner cyclin C (yeast SRB11) were initially isolated as suppressors of truncation mutation in the CTD, and later characterized as a CTD kinase within holoenzymes (Liao et al., 1995; Thompson et al., 1993). CTD phosphorylation by SRB10/SRB11 kinase prevents the assembly of the pre-initiation complex and thereby represses the transcription of specific genes (Hengartner et al., 1998). Positive Elongation Factor b (P-TEFb) is composed of CDK9 and one of several cyclins including T1, T2 and K (Fu et al., 1999; Peng et al., 1998). The kinase activity of P-TEFb is

necessary to overcome the effect of negative elongation factor DSIF, a heterodimer composed of Spt4 and Spt5 (Yamaguchi et al., 2001), and numerous studies have shown that P-TEFb is required to prevent the arrest of elongating pol II (Conaway et al., 2000). Interestingly, CDK9 preferentially phosphorylates CTD that has been already pre-phosphorylated by other kinases such as TFIIH (Marshall et al., 1996), suggesting that while TFIIH probably acts only at the initiation step, P-TEFb remains associated with the elongation polymerase (Ping and Rana, 1999).

Previous studies have shown that RNA polymerase II is bound to Drosophila Hsp70 and Hsp26 promoters in the absence of heat shock, and paused after initiating a short transcript approximately 25 nucleotides (Rougvie and Lis, 1988). Heat shock causes CTD phosphorylation and releases the arrested RNA polymerase II into productive transcription by over 100 fold (Lis and Wu, 1993; Obrien et al., 1994; Rougvie and Lis, 1988). In order to understand the detailed mechanism of this process and the kinase(s) involved in heat-shock induced CTD phosphorylation, we screened several genes that could affect CTD phosphorylation and pol II elongation using RNA Interference (RNAi). We observed that repressing CDK9 expression significantly affects HSP70 mRNA expression, indicating the important role of P-TEFb in heat shock response. We also demonstrated that P-TEFb associates with Drosophila Heat Shock Transcription Factor (HSF) by immunoprecipitation. We propose that upon heat shock, HSF binds to HS promoters and recruits P-TEFb, which in turn, phosphorylates paused pol II and stimulates transcription elongation.

MATERIALS AND METHODS

Plasmid DNA Construction. The V5 epitope of a Drosophila expression vector pAc5.1/V5-His A (Invitrogen) was substituted with FLAG epitope using ApaI and AgeI restriction sites, and the reconstructed plasmid was named as pAc5.3/FLAG-His. Spt4, Spt5, Spt6, Cdk7, Cdk8, Cdk9, Cyclin C, Cyclin H, Cyclin L, Cyclin T, CTK-1 and dodo cDNAs were obtained by PCR using Drosophila Embryonic cDNA library (Clontech, amplified according to the manufacturer's instructions) as the template, and pfu polymerase (Stratragene). In case of Spt6, only the first 3000 bp was used to simplify the cloning process. The cDNAs were then cloned into pAc5.1/FLAG-His in-frame with FLAG epitope and 6-His tag.

dsRNA Synthesis. DNA sequences (approximately 700 bp in length; for genes smaller than 700 bp, full length cDNA sequences were used) to be used as dsRNA synthesis were obtained by PCR using Taq polymerase (Qiagen), Drosophila Embroynic cDNA library (Clontech, amplified according to the manufacturer's instructions) as the template, and primers each contained a T7 promoter sequence on its 5' end (GAA TTA ATA CGA CTC ACT ATA GGG AGA), followed by sequences specific for the targeted genes. The in vitro transcription was carried out in 1X transcription buffer (80 mM HEPES-KOH, pH7.5, 2.5 mM spermidine, 40 mM DTT, 25 mM $MgCl_2$), 4mM of each NTPs, 300 nM purified DNA templates, 1X RNAsecure (Ambion) and 30U of T7 RNA polymerase. All components other than T7 polymerase were added, heated at 60°C for 15 minutes, and then RNA polymerase was added and incubated at 37°C for 6 hours. The dsRNA products were ethanol precipitated and resuspended in RNA storage buffer (Ambion).

The dsRNA were annealed by incubation at 65°C for 10 min followed by slow cooling to room temperature right before use.

Transfection and RNA Interference in Drosophila Cell Culture. Drosophila Schneider 2 cells were transfected using Effectene transfection kit (Qiagen) and the protocol is provided by the manufacturer. 24 hours after transfections, cells were diluted to a final concentration of 2 x 10^6 cells/ml in Drosophila Expression System serum-medium (Invitrogen). 1 ml of cells was plated per well of a six-well cell culture dish. 0 μg (control) or 40 μg (RNAi) of dsRNA was added directly to the media and followed immediately by vigorous agitation. The cells were incubated for 30 min at room temperature followed by addition of 2 ml Schneider's media containing 10% FBS (Gibco). The cells were incubated for an additional 2 days to allow for turnover of the targeted protein. Expression of recombinant proteins and the effectiveness of RNAi were analyzed by western blot using 1:1000 dilution of Anti-FLAG M2 monoclonal antibody (Sigma).

Primer Extension Analysis. Total RNA of transfected and/or dsRNA treated cells was prepared using Sigma GenElute Mammalian Total RNA Isolation Kit. 5 ng of 5' end-^{32}P-labeled oligonucleotides (described below) were hybridized to 5~8 μg of total RNA in TE pH7.4 containing 20 0mM KCl. Total final volume was adjusted to 12 μl. The oligonucleotides were annealed by heating the reaction mixture at 65°C for 20 minutes, followed by gradual cooling to room temperature. Then 25 μl of reverse transcriptase buffer (20 mM Tris pH 8.7, 10 mM $MgCl_2$, 5 mM DTT, 300 μM four dNTPs, 10 μg/ml

actinomycin D) with 3 to 5 units AMV reverse transcriptase was added to the annealing product, and the reaction mixture was incubated at 42°C for 1 hour. The samples were electrophoresed on denaturing 8M urea-6% polyacrylamide gels. Dried gels were exposed to Molecular Dynamics PhosphoImager plates, which were then scanned on the Molecular Dynamics ImageQuant.

The oligonucleotides used for primer extension analysis are as follows:

HSP 70: 5' ACTTTAACTTGCACTTTACT 3'

Actin: 5' CAGAGCCGTTGTCGACAACC 3'

Establishment of Permanent Cell Line. Drosophila Cdk9 cDNA was cloned into pDS47/V5-His A vector and pAc5.1/V5-His A vector (Invitrogen) in-frame with V5 epitope and 6-His Tag, or pAc5.3/FLAG-His in-frame with FLAG and 6-His Tag. Drosophila cyclin T was cloned into pAc5.1/V5-His A in-frame with V5 and 6-His tag. The recombinant plasmids were mixed with pCoHYGRO (Invitrogen) with 3:1 ratio, and transformed into Schneider 2 cells using the Effectene transfection kit (Qiagen). Stable transformants were selected by adding hygromycin-B to 300 μg three days after transfection.

Co-immunoprecipitation. Schneider 2 cells stably expressing recombinant CDK9-V5 under the control of the actin promoter, or co-expressing CDK9-FLAG and cyclin T-V5 were heat shocked at 37°C for 25 minutes and collected by centrifugation. Cell pellets were resuspended in A buffer (10mM HEPES, pH7.5, 15mM KCl, 0.1mM EDTA, 5mM $MgCl_2$) containing 1mM PMSF and 0.2% NP-40. After votexing for 30 seceonds, cells

were centrifuged at 10,000g and the supernatant was isolated as the cytosolic fraction. The pellet was then resuspended in A buffer containing 0.5M NaCl and 1mM PMSF, incubated at 4°C for 20 minutes, centrifuged at 14,000g and the supernatant was isolated as the nuclear extract. Anti-HSF coupled Protein G Sepharose (Pharmacia), or anti-V5 antibody coupled PGS, or anti-FLAG coupled PGS, or Ni-NTA resin (Qiagen) were then added to nuclear extract and incubated at 4°C for 1 hour. The beads were then washed with D (25mm Hepes, pH7.4, 100mm KCl, 1mm EDTA and 0.2% Triton X-100) buffer 3 times, and analyzed by 8% SDS-PAGE. Western blotting was performed using 1:5000 dilution of anti-V5, 1:2000 dilution of anti-FLAG M2 Ab (Sigma) and anti-HSF monoclonal antibody and Alkaline Phosphatase based detection system (Pierce).

In-vitro Kinase Assay with DRB. Schneider 2 cells stably transformed with DS47-Cdk9 were heat shocked at 37°C for 25 minutes and nuclear extract was prepared. Anti-HSF antibody coupled Protein G Sepharose was used to precipitate HSF and HSF-associated proteins. After three washes with D buffer, 5µl of the beads were used in a 25µl kinase reaction contained 20mM HEPES, pH7.5, 10mM $MgCl_2$, 0.1mg/ml bovine serum albumin, 10µM ATP, 50µCi of [γ-^{32}P]ATP, 200 ng of purified recombinant GST-CTD, and various concentrations of DRB as indicated. The reactions were incubated at room temperature for 30 minutes. After spinning out Protein G Sepharose, GST-affinity resin (Stratagene) was added to the supernatant to precipitate GST-CTD. After final washes, precipitated GST-CTD was revolved by 8% SDS-PAGE and exposed to X-ray autoradiography. Reaction products were quantified by PhosphoImager and ImageQuant program.

RESULTS

Heat Shock induces global dephosphorylation of the largest subunit of RNA polymerase II

Since the phosphorylation of the RNA polymerase II largest subunit C-terminal domain (CTD) is critical for the transition from the pre-initiation complex to the elongation complex, monoclonal antibody H14, which detects phosphorylated serine^{5} within the heptapeptide repeats of CTD, was used to determine the phosphorylation state of pol II upon heat shock. As shown in figure 1A-T, the overall levels of phosphorylated pol II detected by H14 steadily decreased during the 10-minute period of heat shock. Before heat shock, phosphorylated pol II is universally distributed within the nucleus (1A); after 4 minutes of heat shock, only a few spots, presumably at heat shock loci, could be detected by immunofluorescence (1K, M, O, Q, S). Considering that phosphorylated pol II represents the actively elongating complex, the data suggest that upon heat shock, the transcription of most cellular genes is suspended, and pol II is primarily engaged in the transcription of the induced heat shock genes. This observation could explain the previous finding that HS response takes precedence over other developmental events and alters the normal programs of gene activity (Edwards, 1998). Western blotting using H14 antibody showed similar result, with the total amount of Ser^{5}-phosphorylated pol II significantly decreasing upon heat shock, while control protein, Karyopherin α3 showed very little change (Figure 1U).

Repressing CDK9 expression by double-stranded RNA interference causes reduced transcription of HSP70 mRNA

While the majority of the serine[5] residues in the CTD of pol II becomes dephosphorylated upon heat shock, promoter-paused pol II at heat shock loci is phosphorylated and released into elongation competent polymerase (Obrien et al., 1994). Evidently, the binding of HSF to HS promoter recruits specific kinase(s) that phosphorylates pol II and promotes efficient elongation. In order to identify the CTD kinase(s) that is recruited by HSF and other factors that affect the transcription of HSP70 mRNA, double-stranded RNA Interference (RNAi) was used to repress the expression of potential CTD-kinase candidates and some non-kinase elongation factors, the effect of knocking out a specific gene was analyzed by measuring the HSP70 mRNA level by primer extension. Besides the three major CTD kinase complexes, CDK7/Cyclin H, CDk8/Cyclin C, and CDk9/Cyclin T, the following proteins were also studied using RNAi in order to understand their roles in heat shock response: Spt4, Spt5, Spt6, cyclin L, CTK-1 and dodo. Spt4 and Spt5 are the components of DSIF, a heterodimer that is capable of both stimulating and repressing pol II elongation (Wada et al., 1998), and it has been reported that Spt5 is present at uninduced heat shock gene promoters (Andrulis et al., 2000). Spt6 is found to be recruited to heat shock loci within 2 minutes of a heat shock, and associated with actively transcribing Hsp genes (Andrulis et al., 2000). Mutations of cyclin L affect the transcription of Hsp mRNA in *C. elegans* (personal communications with Dr. Sternberg). CTK-1 is the Drosophila homologue of yeast CTD kinase CTK1, which when mutated, results in an altered pattern of CTD phosphorylation (Jona et al., 2001; Patturajan et al., 1999). dodo is the Drosophila homologue of yeast

Ess1, a peptidyl-prolyl cis/trans isomerase that has been shown to bind selectively to phosphorylated CTD (Morris et al., 1999).

Drosophila S2 cells were treated with double-stranded RNA corresponding to the above described genes, and the effects of gene knockout were analyzed by primer extension of Hsp70 and actin (as an internal control) mRNA. As shown in Figure 2A, knocking out the expression of HSF (hsf, lane 13) abolished the transcription of Hsp70 mRNA completely, without greatly affecting the transcription of actin mRNA demonstrating the functional specificity of HSF. Knocking out Spt4 (lane 1) and dodo (lane 12) had very little effect on the transcription of both genes, and knocking out CDK7 (lane 4), CDK8 (lane 5), cyclin L (lane 9), and CTK-1 (lane 11) did not seem to affect the transcription of HSP 70 gene significantly either. The level of Hsp70 mRNA was greatly reduced in Spt5 (lane 2) and Spt6 (lane 3) knockouts, indicating that these two proteins are important for Hsp gene expression. However, the expression of actin mRNA was greatly reduced as well, suggesting that Spt5 and Spt6 are likely general elongation factors involved in the transcription of many genes. Knocking out either subunit of P-TEFb, CDK9 (lane 6) and Cyclin T (lane 10) reduces the Hsp 70 mRNA significantly, furthermore, in CDK9 knockouts, Hsp70 mRNA expression was reduced by 78%, while the actin mRNA was only down by 65%, strongly suggesting the involvement of P-TEFb in heat shock response. The quantitative data of relative Hsp70 and actin mRNA levels, obtained by phospho-imaging, is shown in Figure 2B.

To ensure that RNAi effectively repressed the expression of the targeted genes, the degradation of FLAG epitope-tagged recombinant proteins were examined by western blotting. First, FLAG-tagged recombinant proteins were transfected into S2 cells. 24

hours later, half of the transfected cells were treated with dsRNA. Both control and RNAi treated cells were harvest 48 hours later, and probed with anti-FLAG antibody. As shown in Figure 2C, recombinant proteins could be detected in untreated cells (odd numbered lanes, shown by arrows), but were not detectable in RNAi treated cells (even numbered lanes), demonstrating that RNAi effectively knocked out the expression of the targeted genes.

CDK9 and HSF associate with each other upon heat shock

Results obtained from RNAi led us to analyze whether HSF interacts with P-TEFb upon heat shock. First, Schneider 2 cell line constitutively expressing 6-His and V5-epitope tagged recombinant CDK9 was established. Nuclear extract of heat-shocked cells were then immunoprecipitated with anti-HSF monoclonal antibody or with Ni-NTA, and the samples were probed with anti-HSF and anti-V5 antibody. As shown in Figure 3A, recombinant CDK9 was detected in the precipitated samples using PGS-coupled anti-HSF antibody (3A, lane 3), and HSF was also detected in the precipitated fractions using Ni-NTA (3A, lane 5), indicating that HSF and CDK9 interact with each other during heat shock.

Similar results were obtained when recombinant CDK9 and Cyclin T were both introduced into S2 cells. In this case, stable cell line constitutively expressing FLAG-epitope tagged CDK9 and V5-epitope tagged Cyclin T was established, and nuclear extract of heat-shocked cells were immunoprecipitated with anti-HSF antibody, anti-FLAG antibody and anti-V5 antibody. The results of western blotting were shown in Figure 3B. Lane 1 to lane 6 were probed with anti-V5 and anti-FLAG antibodies, and

lane 7 to lane 12 were the same samples probed with anti-HSF and anti-FLAG antibodies. Clearly, both recombinant CDK9 and Cyclin T can be detected in the precipitated fractions when PGS-coupled anti-HSF antibody was used (HSF, lane 2, shown by arrows), and vise versa, HSF can also be detected in the precipitated fractions when either PGS-coupled anti-FLAG antibody (CDK9, lane 10) or PGS-coupled anti-V5 antibody (CycT, lane 12) was used. We conclude that P-TEFb interacts with HSF upon heat shock.

Repressing CDK9 expression by RNA Interference significantly reduces CTD kinase activity associated with HSF

In order to demonstrate that HSF recruits P-TEFb in response to heat shock and P-TEFb in turn phosphorylates pol II CTD and releases the paused polymerase, in vitro kinase assay was used to study the kinase activity associated with HSF. First, extracts from non-shock or heat-shocked cells were immunoprecipitated with PGS-anti-HSF antibody, and then, the precipitates (HSF and associated factors) were mixed with recombinant GST-CTD and γ^{32}P-ATP. As shown in figure 4A, only the samples precipitated from heat-shocked nuclear extract exhibited significant CTD kinase activity (HS nu, lane 4), while precipitates from non-shock extracts or heat-shock cytoplasmic extract showed very little CTD kinase activity (4A, lane 1-3). Two major products were observed, one around 80 KDa and the other around 130 KDa (4A, lane 4, shown by arrows). The molecular weight of recombinant GST-CTD is about 80 KDa, so the 80 KDa band detected by autoradiography is probably GST-CTD phsophorylated only at a few sites, not significant enough to cause a shift on SDS gel, whereas the 130 KDa band

is likely the hyperphosphorylated form migrating much slower. The third band seen on the gel, around 50 KDa, is probably a partial degradation product.

When CDK9 expression was repressed by RNAi, the kinase activity associated with HSF was nearly reduced to background level (Figure 4A, lane 5-8), indicating that CDK9 is responsible for the CTD kinase associated with HSF (compare lane 4 and 8). It is possible that a second kinase (or more) also co-immunoprecipitates with HSF, since a very weak 130 KDa band was observed, however, CDK9 is obviously the major CTD kinase recruited by HSF.

To ensure that CDK9 expression was effectively repressed by RNAi, a specific S2 cell line was used in the above described kinase assay. C-terminal V5-epitope tagged CDK9 under Drosophila DS47 promoter (for moderate level of constitutive expression) was stably expressed in this cell line, so the expression of CDK9 could be monitored by western blotting using anti-V5 antibody. Figure 4B shows the western blotting results of the extracts used in the previous in vitro kinase assay. Without RNAi treatment, recombinant CDK9 was a nuclear protein expressed at low levels (DS47-CDK9, lane 2 and 4), and RNAi effectively eliminated the expression of recombinant CDK9 (lane 6 and 8). Since the double-stranded trigger RNA was targeting the first 700 bp of coding region of CDK9, we assume the expression endogenous CDK9 was repressed as well by RNAi.

Heat Shock Factor-associated kinase is sensitive to DRB inhibition

The nucleotide analog 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) is a potent inhibitor of mRNA synthesis by dramatically increasing the frequency of pol II

arrests after initiation (Conaway and Conaway, 1999). Since it is demonstrated that P-TEFb is extremely sensitive to DRB inhibition (Marshall et al., 1996), the inhibitory effect of DRB on the kinase(s) recruited by HSF was examined by in vitro kinase assay. The nuclear extract of heat-shocked cells were immunoprecipitated with PGS-coupled anti-HSF antibody and the precipitates were mixed with recombinant GST-CTD and γ^{32}P-ATP as described before. When various concentration of DRB was added into the reaction mix, it is clear that the kinase associated with HSF is highly sensitive to DRB (Figure 5A, lane 2-8, and 5B). At 10 μM of DRB, the CTD kinase activity was reduced nearly by 50%. Quantitative data obtained from phospho-imaging was plotted in figure 5B. The DRB-sensitivity profile seems to indicate that there might be at least two kinases associated with HSF, the first one, accounting for 75% to 80% of the total kinase activity, was inhibited by DRB at less 10 μM. A much higher concentration of DRB, around 100 μM, was required to inhibit the activities of the second kinase (figure 3B). Since the IC_{50} of DRB was estimated as 2.5 μM for P-TEFb (Kim and Sharp, 2001), this result is consistent with previous RNAi analysis, and strongly suggests that P-TEFb is associated with HSF upon heat shock, and is likely the major kinase that is recruited by HSF to phophorylate pol II in vivo.

DISCUSSION

Drosophila Hsp70 gene is rapidly and vigorously activated by heat shock. An instantaneous heat shock triggers a 200-fold increase in the level of Hsp70 transcription in 3 minutes (Lis, 1998). Two mechanisms were suggested to explain this rapid

activation. First, the open chromatin structure depleted of histones at the heat shock promoter could provide HSF and general transcription machinery rapid access to specific sequences (Lis and Wu, 1993). Second, even before heat shock, TBP and pol II are already bound to heat shock promoters and primer for transcription (Lis, 1998). Approximately one pol II is transcriptionally engaged, but paused, on each hsp70 gene (Rougvie and Lis, 1988). In vivo UV-crosslinking showed that the paused pol II is largely hypophosphorylated (IIa form), and the transition into elongationally competent form (IIo form) requires CTD phosphorylation (Obrien et al., 1994). In this study, we provide evidence that during heat shock, HSF recruits elongation factor P-TEFb to phosphorylate and release the promoter-paused pol II and stimulate transcriptional elongation. First, we found by RNAi screening that among the four CTD kinase candidates examined, only CDK9/Cyclin T knockouts significantly reduced the transcription of Hsp70 mRNA. Second, we demonstrated that HSF, CDK9 and Cyclin T associate with each other in nuclear extracts of heat shocked cells. Third, the kinase associated with HSF is capable of CTD phosphorylation, and the CTD kinase activity can be largely eliminated by either repressing CDK9 expression or inhibiting the CDK9 activity by DRB.

P-TEFb was identified as a kinase/cyclin heterodimer that was critical for overcoming an early block to transcriptional elongation (Marshall et al., 1996). Studies on HIV-1 transcription showed that HIV-encoded Tat protein interacts with Cyclin T1 to recruit the P-TEFb complex to promote productive elongation (Price, 2000). Strong transcriptional pause sites are also found in several mammalian genes, such as Ig-κ and c-myc (Barboric et al., 2001). In all these cases, the rates of pol II initiation exceed the

rates of elongation, resulting in pol II stalled at promoter-proximal region. Transcription factor NF-κB is shown to activate Ig-κ gene by recruiting P-TEFb and stimulating elongation via P-TEFb mediated CTD phosphorylation (Barboric et al., 2001). In this study, we show that HSF uses the same machinery to stimulate the transcriptional elongation of Hsp genes. Notably, we demonstrated for the first time that CTD kinase complex is associated with HSF upon heat shock (figure 4A). The fact that HSF-associated CTD kinase is highly sensitive to either low concentration of DRB, or RNAi specifically targeted to CDK9 provides convincing evidence that P-TEFb is the major kinase that interacts with HSF and phosphorylates pol II.

Our co-immunoprecipitation results revealed that only a small fraction of P-TEFb is associated with HSF (figure 3B), indicating that HSF and P-TEFb may only interact with each other near promoter region but dissociate shortly after pol II enters into productive elongation. In fact, after heat shock, P-TEFb was found at the 3' ends of heat shock genes (Andrulis et al., 2000). Therefore, while HSF remains bound to promoter region, it is likely that P-TEFb soon dissociates with HSF and travels along with phosphorylated pol II.

The rapid dephosphorylation of the majority of pol II we observed can also be explained by the redistribution of P-TEFb. Immunofluorescence analysis of Drosophila polytene chromosomes revealed that P-TEFb is normally located at more than 200 loci, but upon heat shock, it rapidly redistributes to heat shock loci (Lis et al., 2000). While our studies suggest that HSF is responsible for the recruitment of P-TEFb to heat shock loci, how heat shock induces P-TEFb to dissociate from other genes remains unknown.

It seems that a second CTD kinase other than P-TEFb also associates with HSF, whose activity is inhibited at much higher concentration of DRB. Although CDK7/Cyclin H is a major CTD kinase that has been shown involved in the very early stages of elongation, it is unlikely that CDK7 plays a role in heat shock response. Repressing CDK7 with RNAi did not significantly affect the Hsp70 transcription. Furthermore, studies from other labs revealed that heat shock puffs are devoid of CDK7 (Bensaude et al., 1999). A more likely candidate is MAP kinase, since it was reported that stress activates ERK1/2 and induces extensive CTD phosphorylation (Venetianer et al., 1995).

In summary, several lines of evidence show that HSF interact with P-TEFb to phosphorylate CTD and stimulate elongation. Since activation domains of HSF were mapped to both N-terminus and C-terminus (Wu, 1995), it would be interesting to analyze which domains are required for its interaction with P-TEFb, and whether HSF interact with CDK9 directly or through Cyclin T. Mutations and deletions that abolish this interaction will provide further insights into the regulation of heat shock response.

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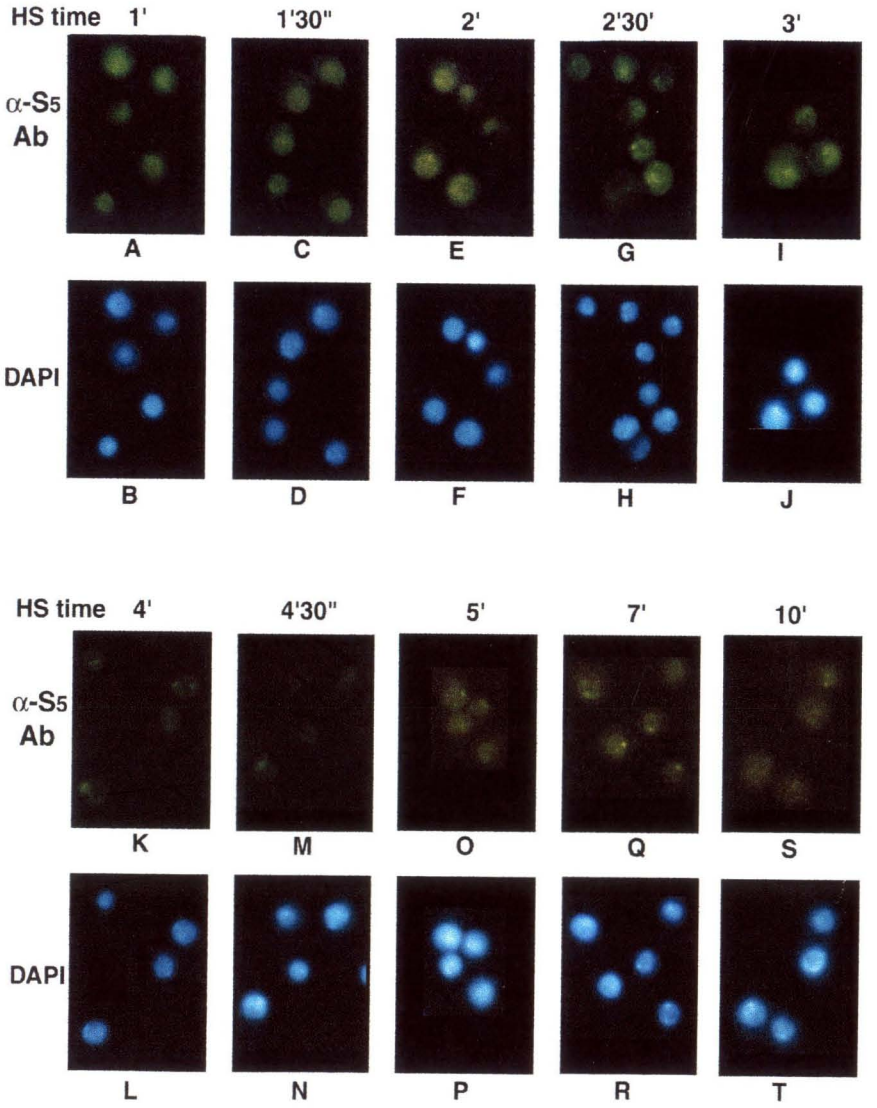
HS time 1'
1'30"
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Figure 1A-1T

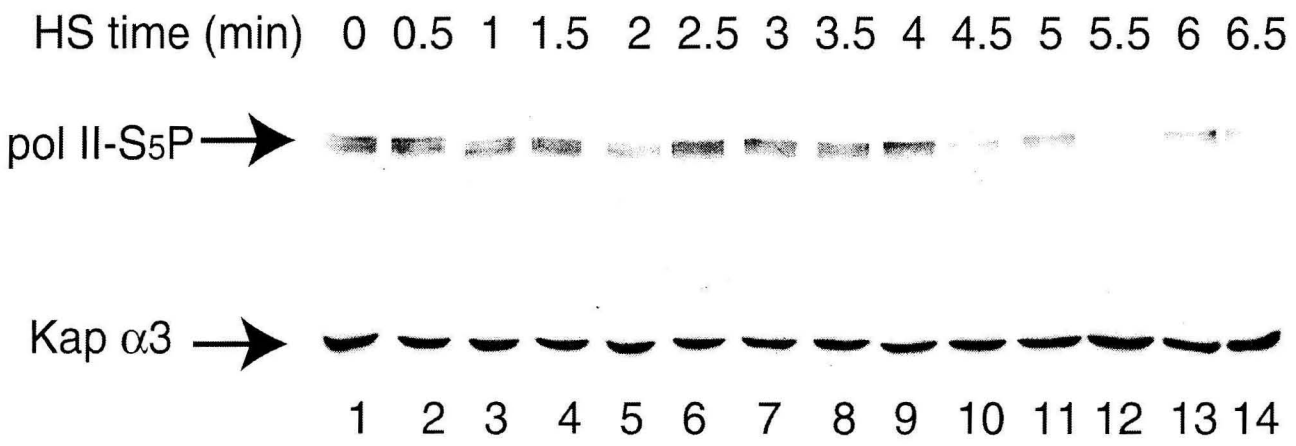

HS time (min) 0 0.5 1 1.5 2 2.5 3 3.5 4 4.5 5 5.5 6 6.5
pol II-S5P
Kap α3
1 2 3 4 5 6 7 8 9 10 11 12 13 14

Figure 1U

FIGURES AND FIGURE LEGENDS.

Figure 1. RNA polymerase II is largely dephosphorylated upon heat shock.

1A-1T. Immunofluorescence staining of Serine 5-phosphorylated RNA polymerase II during 10 minutes time course of heat shock. Drosophila S2 cells heat-shocked at 37°C at the indicated times were fixed immediately after heat shock and stained with H14 monoclonal antibody (from Covance, recognizes phosphorylated ser-5 of the CTD YSPTSPS repeats). The fluorescence intensity is decreasing gradually during the fist 3 min of heat shock (compare panel A, C, E, G, I). From 4min onward, phosphorylated CTD can only be detected on a few loci (presumably HS genes) (Panel K, M, O, Q, S). DAPI staining (panel B, D, F, H, J, L, N, P, R, T) was performed at the same time to shows the nuclei.

1U. Western blotting analysis of RNA pol II serine 5 phosphorylation during the time course of heat shock. Drosophila S2 cells were heat shocked for the indicated time and lysed directly in SDS loading buffer immediately after heat shock. Samples were probed with Covance H14 antibody (1:5000 dilution). Upper arrow shows the Serine 5 phosphorylated pol II. To ensure that identical amount of protein were loaded in each well, anti-Kap α3 antibody as used at the same time an internal control (lower arrow).

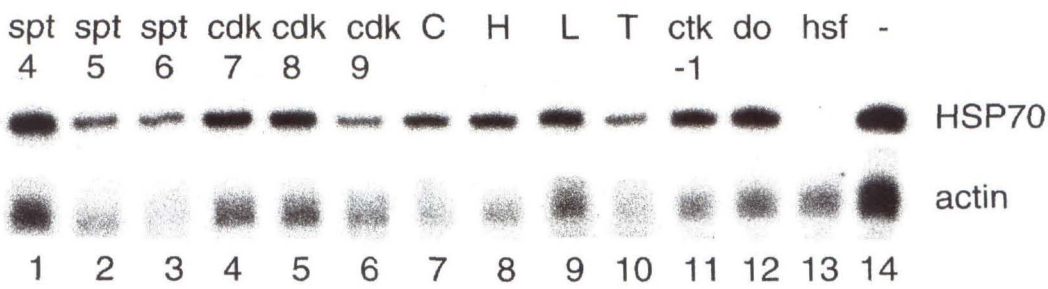


Figure 2A

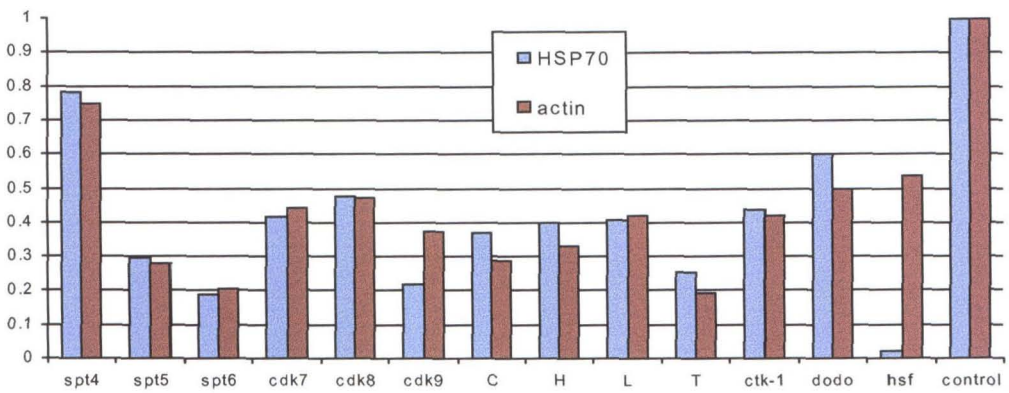


Figure 2B

Figure 2. Repression of CDK9 expression by RNA Interference significantly reduces the expression of Hsp70 mRNA.

2A. Primer extension analysis of HSP70 and actin mRNA after various targeted genes were repressed by RNAi. Drosophila S2 cells were transiently transfected with C-terminal FLAG epitope tagged Spt4, Spt5, Spt6, Cdk7, Cdk8, Cdk9, Cyclin C, cyclin H, Cyclin L, Cyclin T, CTK-1, dodo. 24 hours after transfection, cells were treated with 30 μg of dsRNA corresponding to the first 700 bp of the coding regions (or the entire coding region for genes smaller than 700 bp). 48 hours after dsRNA treatment, cells were harvested, and the expression of HSP70 mRNA was examined by primer extension. Actin mRNA was examined at the same time as an internal control. The specific gene targeted by RNAi was indicated on top of the image. hsf, cells treated with dsRNA corresponding to the first 700 bp coding region of HSF. -, cells treated with no dsRNA.

2B. Relative expression levels of Hsp70 and actin mRNA in RNAi treated cells. Quantitative data of primer extension (obtained from PhosphoImager plate) in 2A was plotted. mRNA expression levels of control cells were set as 1, and all the others were normalized accordingly.

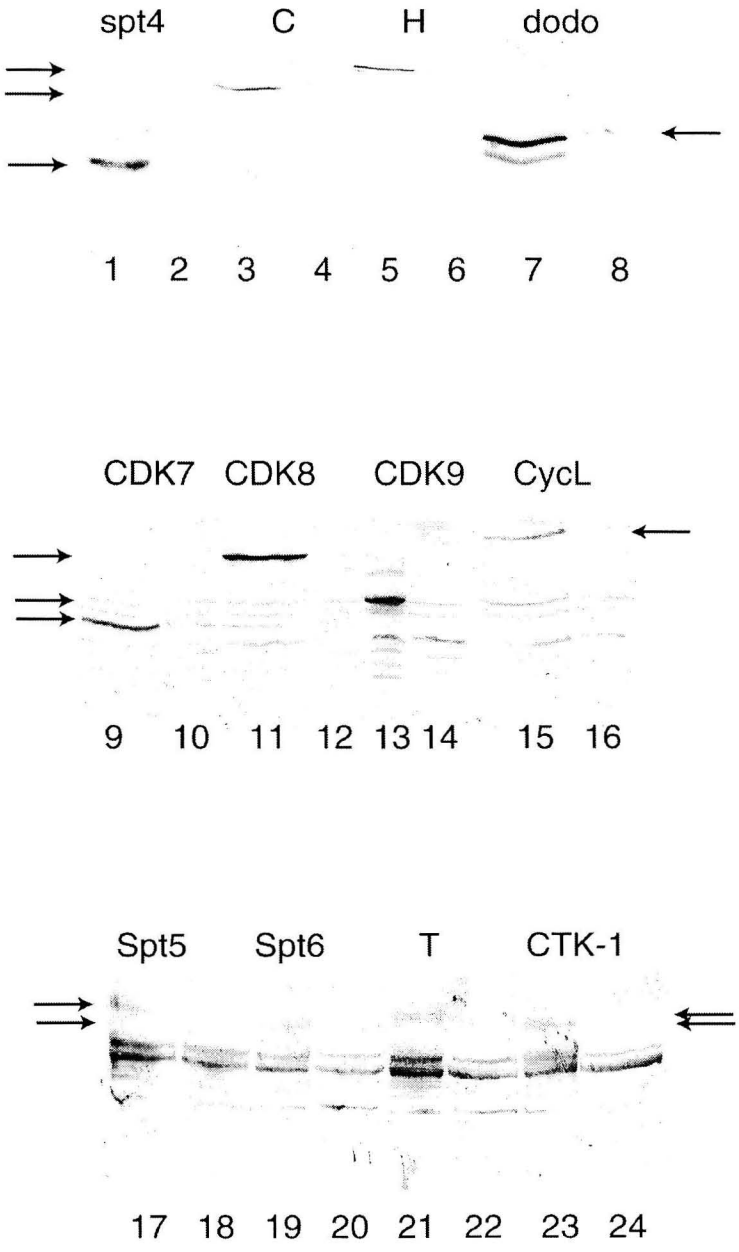

spt4
C
H
dodo
1 2 3 4 5 6 7 8
CDK7 CDK8 CDK9 CycL
9 10 11 12 13 14 15 16
Spt5 Spt6 T CTK-1
17 18 19 20 21 22 23 24

Figure 2C

2C. RNAi treatment effectively repressed the expression of the targeted gene. S2 cells were transiently transfected with C-terminal FLAG epitope-tagged Spt4, Cyclin C, Cyclin H, dodo, CDK7, CDK8, CDK9, Cyclin, Spt5, Spt6, Cyclin T, CTK-1. 24 hours after transfection, cells were treated with 0 μg (lane 1, 3, 5, 7, 9, 11,13,15, 17, 19, 21, 23) or 30 μg of dsRNA corresponding to the first 700 bp of the coding regions (or the entire coding sequence if the gene is smaller than 700 bp) (lane 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24). Western blotting analysis using anti-FLAG M2 antibody showed that the transfected genes were successfully expressed in control cells (indicated by arrows), but their expression was repressed in RNAi treated cells.

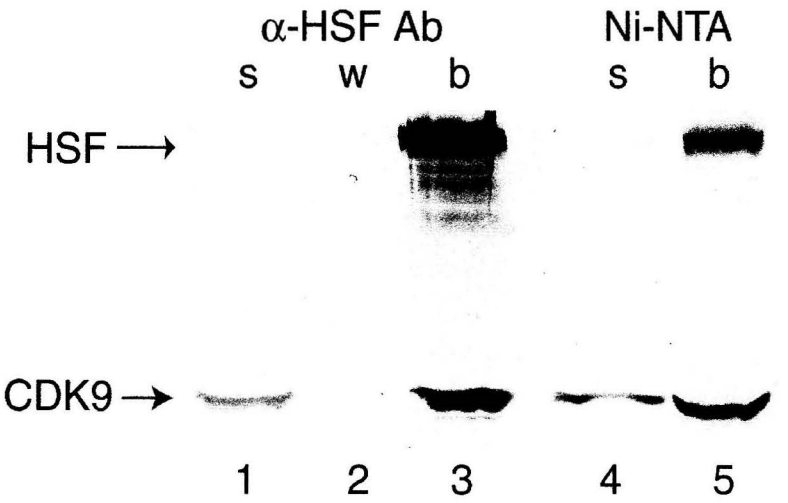
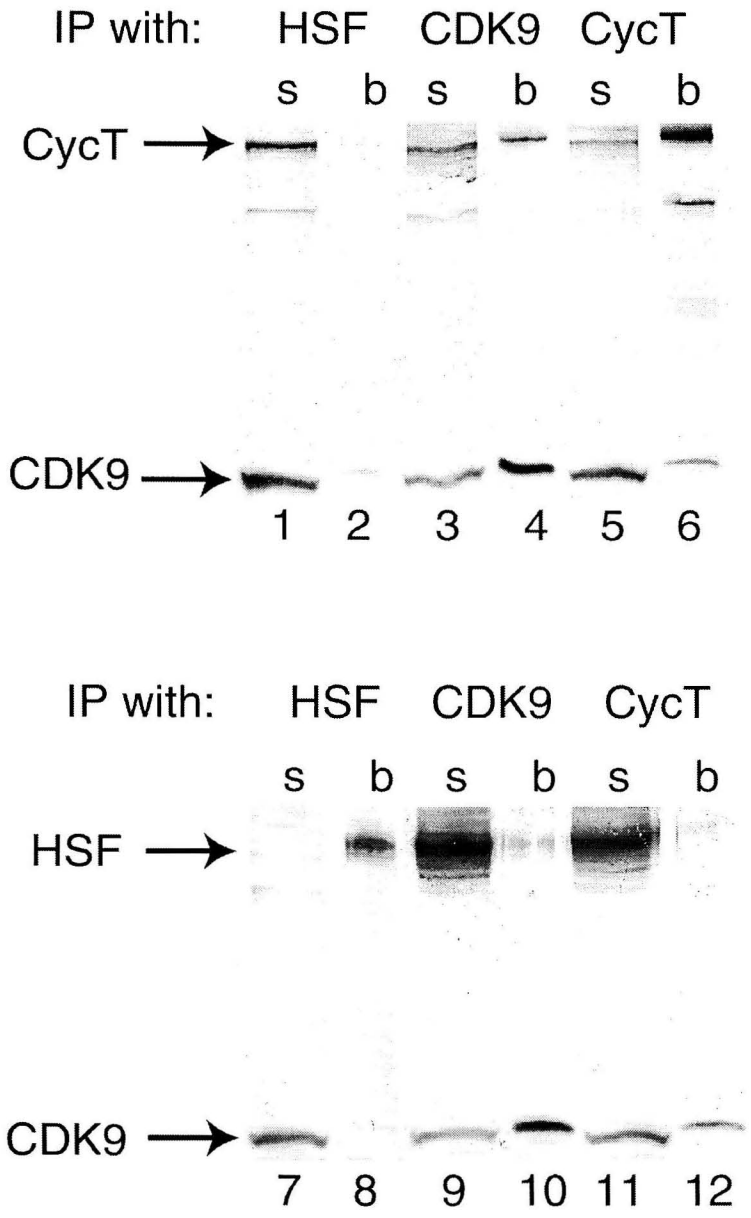


Figure 3A

Figure 3A. CDK9 and HSF associate with other upon heat shock. S2 cells stably transformed with V5 and 6His tagged-CDK9 under actin promoter were heat shocked at 37°C for 25 minutes, nuclear extract was then prepared. anti-HSF antibody coupled-Protein G Sepharose or Ni-NTA beads were added to the nuclear extract. The precipitates were washed and analyzed by western blotting using anti-HSF and anti-V5 antibody. Lane 1-3: immunoprecipitation with anti-HSF antibody. Lane 4 and 5, precipitation with Ni-NTA. **s**, fractions collected as the supernatant of the immunoprecipitation. **w**, fractions collected in first wash after IP. **b**, proteins bound to the beads after three washes.


IP with:
HSF
CDK9
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IP with:
HSF
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HSF
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12

Figure 3B

Figure 3B. P-TEFb and HSF associate with other upon heat shock. S2 cells stably transformed with V5-tagged-Cyclin T and FLAG-tagged-CDK9 (both under actin promoter) were heat shocked at 37°C for 25 minutes, nuclear extract was then prepared. Protein G Sepharose-coupled monoclonal anti-HSF, anti-FLAG (CDK9), and anti-V5 (Cyclin T) antibodies were added to the nuclear extract. The precipitates were washed and analyzed by western blotting. Lane 1-6: immunoprecipitation samples probed with anti-HSF and anti-FLAG antibody. Lane 7-12, same samples probed with anti-V5 and anti-FLAG antibody (lane 7-12). s, fractions collected as the supernatant of the immunoprecipitation. b, proteins bound to the beads after three washes.

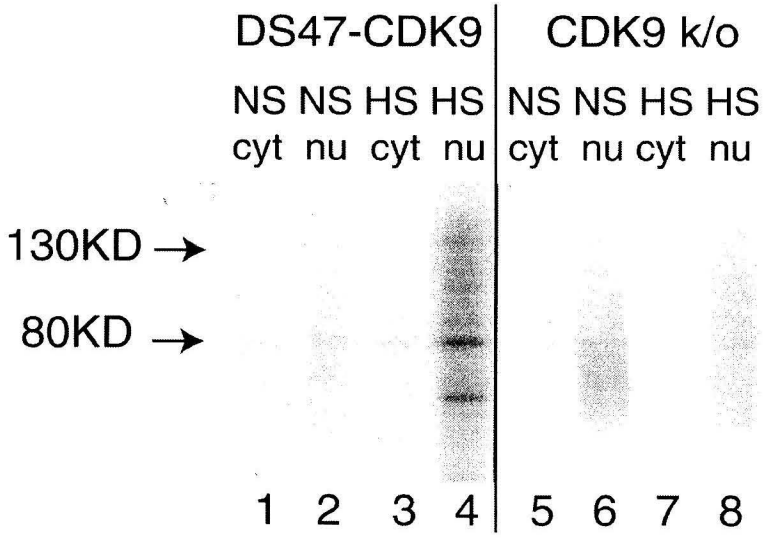

Figure 4A

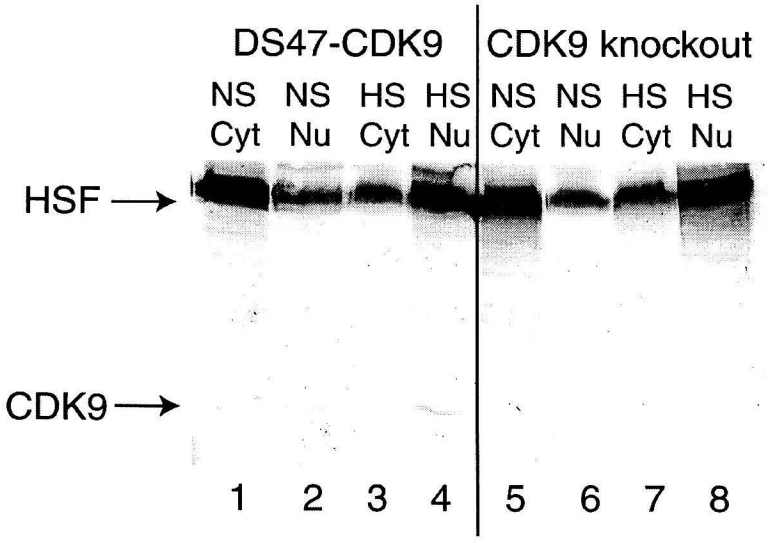

Figure 4B

Figure 4. Repressing CDK9 expression by RNAi significantly reduces the CTD kinase activity associated with HSF.

4A. In vitro kinase assay. S2 cells were stably transformed with V5-tagged-CDK9 under DS47 promoter for low-level, constitutive expression. Cell extracts from control or RNAi treated cells were immunoprecipitated using Protein G Sepharose-coupled anti-HSF antibody. The precipitated HSF and HSF-associated proteins were then mixed with recombinant GST-CTD, kinase buffer and γ^{32}P-ATP. Phosphorylation of GST-CTD was analyzed by 8% SDS gel and autoradiography. The 80KDa band corresponds to the size of recombinant GST-CTD. The 130 KDa is likely hyperphosphorylated form of GST-CTD. NS: nonshock, HS: heat shock, Cyt: cytoplasmic extract, Nu: nuclear extract.

4B. RNAi effectively represses the expression of CDK9. Cell extracts from control or RNAi treated DS47-CDK9 cell line were probed with α-HSF and α-V5 monoclonal antibodies. The expression of recombinant CDK9 was effectively repressed by RNAi (lower arrow). NS: nonshock, HS: heat shock, Cyt: cytoplasmic extract, Nu: nuclear extract

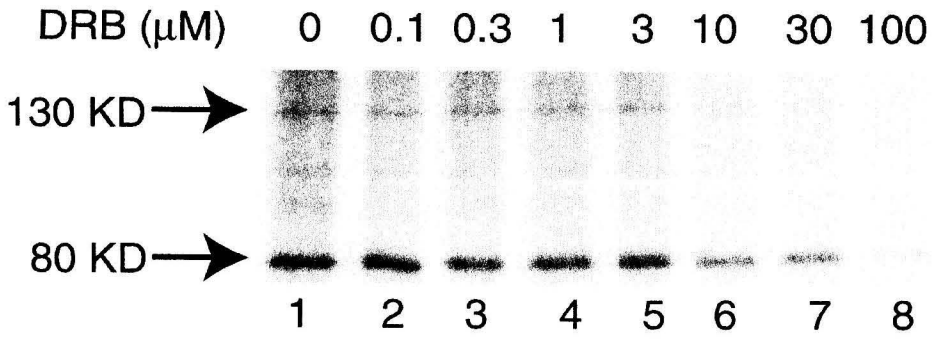

Figure 5A

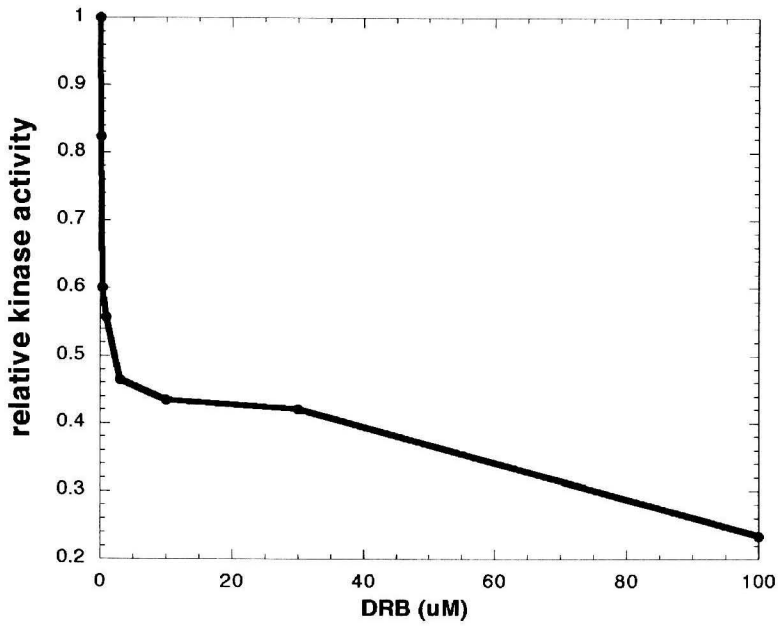

Figure 5B

Figure 5. DRB sensitivity of the CTD kinase(s) associated with HSF.

5A. Nuclear extract of heat-shocked (37°C, 25 minutes) DS47-CDK9 cells was immunoprecipitated with Protein G Sepharose-coupled anti-HSF antibody. The precipitated HSF and HSF-associated proteins were then mixed with recombinant GST-CTD, kinase buffer, γ^{32}P-ATP and the indicated concentration of DRB. The kinase(s) co-precipitates with HSF is highly sensitive to DRB. The lower 80KDa band corresponds to the size of recombinant GST-CTD. The 130 KDa is likely hyper-phosphorylated form of GST-CTD.

5B. Relative CTD kinase activities at different concentration of DRB. Quantitative data of the kinase assay were obtained from PhosphoImaging Plate. The intensity of GST-CTD at 0 mM of DRB was set as 1, and the others were normalized accordingly.