Molecular Cloning and Expression of a Hexameric Drosophila Heat Shock Factor Subject to Negative Regulation

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Summary

We report the cloning of the transcriptional activator of heat shock genes, HSF, from Drosophila. The predicted sequence of Drosophila HSF protein is surprisingly divergent from that of yeast HSF, except in regions important for DNA binding and oligomerization. A segment of the DNA binding domain of HSF bears an intriguing similarity to the putative DNA recognition helix of bacterial sigma factors, while the oligomerization domain contains an unusual arrangement of conserved hydrophobic heptad repeats. Drosophila HSF produced in E. coli under nonshock conditions forms a hexamer that binds specifically to DNA with high affinity and activates transcription from a heat shock promoter in vitro. In contrast, when HSF is expressed in Xenopus oocytes, maximal DNA binding affinity is observed only after heat shock induction. These results suggest that Drosophila HSF has an intrinsic affinity for DNA, which is repressed under nonshock conditions in vivo.

Introduction

All organisms respond to elevated environmental temperatures by rapidly activating the expression of a group of proteins referred to as heat shock or stress proteins. Although the functions of heat shock proteins have remained obscure for many years since the discovery of the phenomenon by Ritossa (1962), recent studies suggest a central role for heat shock–induced proteins and their constitutive counterparts in mediating protein–protein interactions, protein folding, and the transport of proteins across membranes (for review see Morimoto et al., 1990). The synthesis of heat shock proteins is subject to both transcriptional and posttranscriptional control in eukaryotic cells (for reviews see Craig, 1985; Lindquist, 1986). Heat shock–inducible transcription is mediated by a positive control element, the heat shock element (HSE), defined as three repeats of a 5-nucleotide [_GAA_] module, arranged in alternating orientation (Pelham, 1982; Amin et al., 1988; Xiao and Lis, 1988). Multiple copies of the HSE are found upstream of all heat shock genes.

A heat shock transcriptional activator, termed heat shock factor (HSF), binds to HSEs and activates transcription of heat shock genes in vitro (Wu, 1984a, 1984b; Parker and Topol, 1984; Topol et al., 1985). Although the sequence of the HSE has been highly conserved in evolution, HSF purified from yeast, Drosophila, and human cells differs in molecular size (150 kd, 110 kd, and 83 kd, respectively; Sorger and Pelham, 1987; Wu et al., 1987; Goldenberg et al., 1988). Yeast and higher eukaryotes also differ in the regulation of HSF activity. In yeast, HSF bound constitutively to the HSE apparently stimulates transcription when phosphorylated under heat shock conditions. In Drosophila and vertebrate cells, HSF is unable to bind to the HSE unless the cells are heat shocked (for a review see Wu et al., 1990). The heat-inducible binding of HSF appears to be a major regulatory step in the pathway to heat shock gene activation in higher eukaryotes.

The induction and reversal of HSF binding activity in vivo does not require new protein synthesis (Zimarino and Wu, 1987; Kingston et al., 1987; Zimarino et al., 1990a). In addition, HSF extracted from nonshocked cell cytosol can be activated in vitro by heat (Larson et al., 1988), low pH (Mosser et al., 1990), and by interaction with antibodies raised to the active form of HSF (Zimarino et al., 1990b). These results suggest that the preexistent, inactive form of HSF can assume the active conformation without an enzymatic modification of protein structure.

In this study, we describe the molecular cloning of Drosophila HSF and present evidence that cloned HSF synthesized in Escherichia coli or translated in vitro in a reticulocyte lysate at non-heat shock temperatures binds to DNA with maximal affinity. In contrast, cloned HSF expressed in Xenopus oocytes binds to DNA with maximal affinity only after heat shock induction, suggesting that HSF is under negative control in higher eukaryotic cells.

Results

Purification and Microsequencing of HSF

We purified Drosophila HSF to about 95% homogeneity by a modification of the procedure described previously (Wu et al., 1987). Two independently purified, 4 μg preparations of the 110 kd polypeptide were digested with trypsin, and the resulting peptides were subjected to reverse phase liquid chromatography. Essentially identical elution profiles were observed for both peptide preparations (data not shown). Individual HSF peptides were subjected to microsequence analysis; six peptides yielded identical amino acid sequences in duplicate.

Isolation of cDNA Clones for Drosophila HSF

Two 20-mer oligonucleotides with 32-fold degeneracy, based on the predicted nucleotide sequences of HSF peptide 27 and peptide 29 (see Experimental Procedures), were used to probe a Drosophila genomic library. We initially identified two genomic DNA clones that contained a common, ~1800 bp SalI–EcoRI fragment. This SalI–EcoRI fragment, which hybridized with both oligonucleotide

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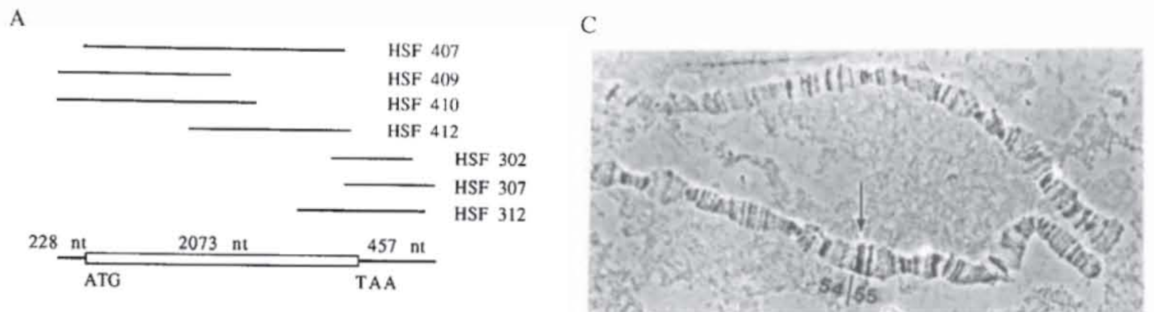

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-138 AACCGAAG.\CTGTGTAAAACAAAAGCGGTACGCCAAGAAAGTGAAGTGTAATAACTAAACTAAACAAGTAAAACGCGAGTTTTTCACTT
                                                                                    peptide 16
   1                                                 M  S  R  S  R  S  S  A  K [A  V  Q  F  K]
 -48 CGCGAAAGGTTTGTGAGCTGCAAAATTGTTAAGGCTGCTGGTCACTTTATGTCCAGGTCGCGTTCATCCGCTAAAGCCGTGCAGTTCAAG
  15 H  E  S  E  E  E  E  E  D  E  E  E  Q  L  P  S  R  R  M  H  S  Y  G  D  A  A  A  I  G  S
  43 CACGAATCGGAGGAAGAGGAAGAAGACGAGGAGGAGCAGCTGCCTTCCAGGAGAATGCACTCTTACGGAGACGCCGCGGCCATCGGAAGC
                                                                                    peptide 23
  45 G  V  P  A  F  L  A  K  L  W  R  L  V  D  D  A  D  T  N  R  L  I  C  W  T  K [D  G  Q  S
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  75 F  V  I  Q  N  Q  A  Q  F  A] K  E  L  L  P  L  N  Y  K  H  N  N  M  A  S  F  I  R  Q  L
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                             peptide 39
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 135 K  R  N  S  P  F  L  L  D  Q  I  K  R  K  I  S  N  N  K  N  G  D  D  K  G  V  L  K  P  E
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                                                                           peptide 29
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                                                     peptide 27
 225 I  V  Q  P  S  R  N  M  S  G  V  K  R  H [V  Q  L  M  I  N  N  T  P  E  I  D  R] A  R  T
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 255 T  S  E  T  E  S  E  S  G  G  G  P  V  I  H  E  L  R  E  E  L  L  D  E  V  M  N  P  S  P
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1033 GCTTCTCCCATGGCCCAAAGTGTGAGTCAATCGCCGGCCCAACATGATGTCTACACAGTCACCGAGGCGCCCGATTCTCATGTCCAGGAG
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1123 GTGCCAAACAGTCCGCCTTATTACGAGGAGCAGAATGTGCTTACCACGCCCATGGTGCGGGAGCAGGAGCAGCAGAAGCGTCAGCAGCTT
 405 K  E  N  N  K  L  R  R  Q  A  G  D  V  I  L  D  A  G  D  I  L  V  D  S  S  S  P  K  A  Q
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1573 GTGCCCACCAACAGTACGCTGCTGGATGCCAATCAGGCCTCGACATCGAAGGCAGCGGCCAAGGCGCAAGCATCTGAGGAAGAGGGCATG
                                        Stu I
 555 A  V  A  K  Y  S  G  A  E  N  G  N  N  R  D  T  N  N  S  Q  L  L  R  M  A  S  V  D  E  L
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 675 R  Q  M  Q  T  Q  S  S  V  L  N  T  P  R  H  E  L  *
2023 CGCCAGATGCAGACGCAAAGTTCGGTTTTAAATACGCCACGTCACGAGTTGTAACTTATATTAAGTAGCAGCAGCTCAGCCGTTAAATTG
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2203 TAATCACATTAATAACCACTATAATCATATGAATGATCTGTATATGCCTTATGCACTATATATATGATATATAGACTAACCAGTTCTTGT
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                                                                 Apa I
2383 AGTTTGCACTAACCGATCGAGAGTACAGTGAAAGCTGATCGCATCGTATACGATTTAAGATCGCACGCTTCAGTTTATACATTTTACACA
2473 CTACTCTTATCATTATTTCAAAATAAATTGTTTAATGTCGCGTTGAGCTGCAAATGAAAAAAAAAAAAAAAAAAAAAAAA
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Figure 1. Cloning and Sequence Analysis of Drosophila HSF

(A) Schematic representation of seven HSF cDNA clones aligned with reference to the reconstructed full-length HSF cDNA clone. HSF 302, 307, and 312 were isolated from an oligo(dT)-primed cDNA library, and HSF 407, 409, 410, and 412 were isolated from a random-primed cDNA library. The open bar represents the 2073 nucleotide HSF open reading frame.

(B) Nucleotide sequence of the HSF cDNA and predicted amino acid sequence. The entire DNA sequence presented has been sequenced at least twice, from overlapping cDNA clones. Start and stop codons and a polyadenylation signal are highlighted by reverse print. Two single restriction sites (StuI and ApaI) that were used for generation of 3′ deletion mutants are noted. Sequences in the open reading frame that match the sequences of the six HSF tryptic peptides are boxed.

(C) In situ hybridization of digoxigenin-substituted HSF DNA (coding sequences) to Drosophila salivary gland polytene chromosomes. The cytological locus of hybridization (55A) is indicated by the arrow.

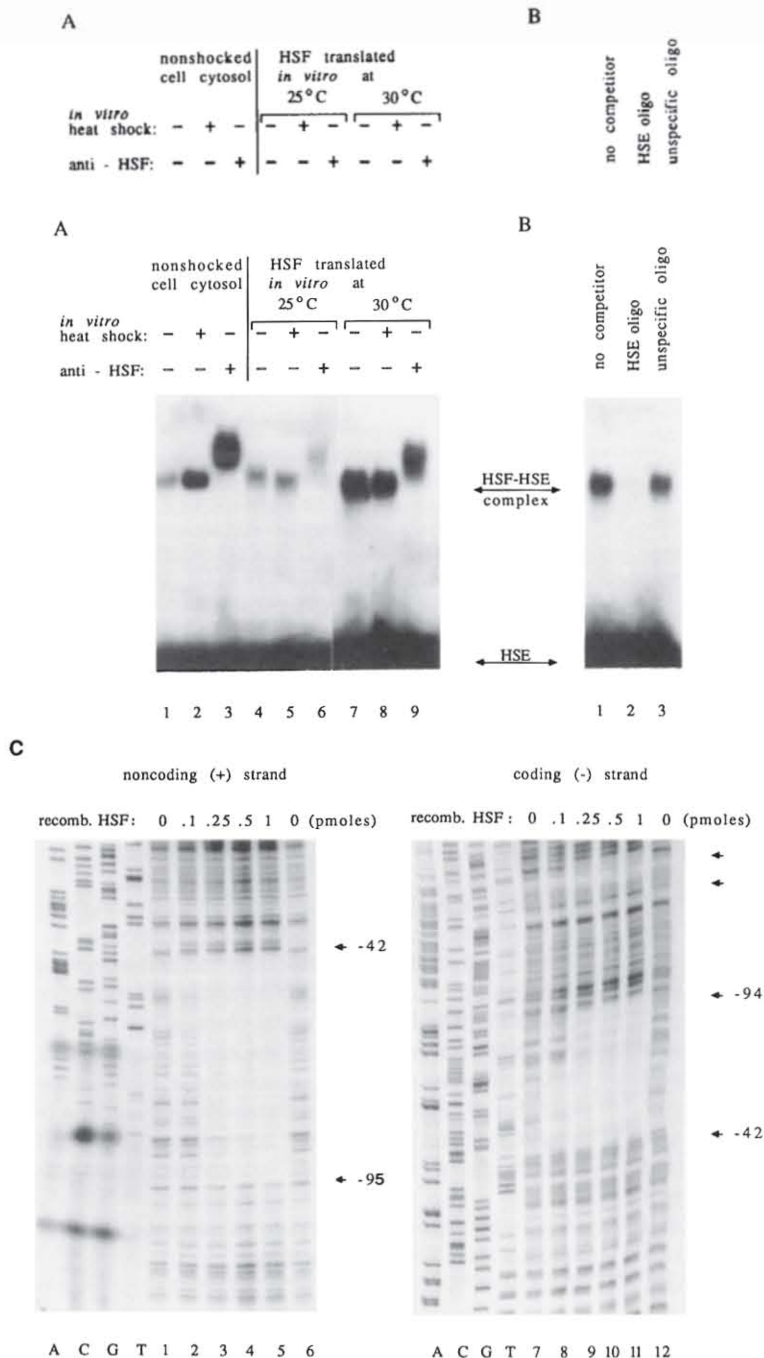

Figure 2. DNA Binding Activity of Recombinant HSF

(A) Gel mobility shift analysis of natural and recombinant HSF. Cytoplasmic extracts from unshocked SL-2 cells (lanes 1–3) and HSF translated in vitro at 25°C or 30°C (lanes 4–9) were subjected to in vitro heat shock (+) for 10 min at 34°C (lanes 2, 5, and 8) or kept at 0°C (−) (lanes 1, 4, and 7). Samples in lanes 3, 6, and 9 were incubated at room temperature with a 1:60 dilution of polyclonal anti-HSF serum prior to gel shift analysis. Identical translations of antisense HSF RNA showed no DNA binding activity.

(B) Gel mobility shift assay of HSF translated in vitro at 30°C in the absence of competitor DNA (lane 1) with a 40-fold excess of unlabeled HSE (lane 2) or a similar excess of synthetic DNA from the *hsp70* gene, positions +40 to +80 (lane 3).

(C) DNAase I protection analysis. Recombinant HSF extracted from E. coli was incubated with 5′ ^{32}P-labeled *hsp70* promoter DNA, digested with DNAase I, and analyzed by electrophoresis on an 8% sequencing gel (left panel; noncoding strand) or 6% (right panel; coding strand) sequencing gel. Amounts of HSF used for each reaction are indicated. The total protein concentration in all samples was normalized by the addition of extracts of bacteria transformed with the expression vector alone. The lanes marked A, C, G, and T are dideoxy sequencing reactions.

(D) Nucleotide sequence of the *hsp70* promoter from position −185 to +10. The sequences in lowercase are from the plasmid vector. Three upstream HSEs and the TATA sequence are boxed. The start site and direction of transcription are indicated. Brackets indicate sequences protected by the recombinant HSF. There is a clear DNAase I footprint on both strands over the two proximal HSEs, and some protection on the coding strand also occurs on the third HSE (position −174 to −186), the noncoding strand of which was not analyzed.

probes, was then used to isolate cDNA clones from a random-primed and an oligo(dT)-primed cDNA library. The 2.8 kb of HSF cDNA sequence reconstructed from six overlapping cDNA clones reveals a single open reading frame of 691 amino acids (2073 nucleotides) (Figure 1A). We were able to locate the sequences of all six HSF tryptic peptides within the 691 amino acid open reading frame, and thus conclude that this reading frame encodes Drosophila HSF (Figure 1B). The molecular mass of Drosophila HSF, calculated from the deduced amino acid sequence, is 77,300 daltons, significantly lower than the apparent mass of 110,000 daltons measured by SDS gel electrophoresis (Wu et al., 1987). Evidently, Drosophila HSF has an anomalous mobility on SDS gels; a similar anomaly was observed with yeast HSF (Sorger and Pelham, 1988; Wiederrecht et al., 1988). For purposes of discussion, we continue to use the molecular size of HSF protein as measured by SDS gel electrophoresis. The Drosophila HSF protein sequence predicts an acidic protein (pI = 4.7). The overall distribution of charged residues along the length of the protein sequence is nonuniform: the N-terminal one-third of HSF (amino acids 1–240) is rel-

atively basic (predicted pI = 10.25), while the C-terminal two-thirds (amino acids 240–691) is relatively acidic (predicted pI = 4.1). In addition, there is an unusual N-terminal cluster of nine acidic residues in a row (amino acids 18–26).

DNA gel blot analysis under standard stringency conditions shows that the Drosophila HSF gene is single copy (J. C., unpublished data). The possible presence of homologous genes that have partial sequence similarity to HSF has not yet been addressed. We localized the Drosophila HSF gene by in situ hybridization to a single cytological position at 55A on the Drosophila polytene chromosome (Figure 1C), which is near the maternal effect loci *eay*, *sub*, and *stau* (55A–F; Schüpbach and Wieschaus, 1989).

Recombinant HSF Is an Active, DNA Binding Transcription Factor in the Absence of Heat Shock

Naturally occurring HSF extracted from the cytosol of nonshocked Drosophila cells shows a basal affinity for DNA, which can be significantly increased by a direct heat treatment in vitro or by reaction with polyclonal antibodies raised to the in vivo activated form of HSF (Zimarino et al., 1990b; Figure 2A, lanes 1–3). The slower mobility of the HSF–HSE complex upon anti-HSF treatment is due to the additional binding of antibody. When recombinant HSF was synthesized by in vitro translation in a rabbit reticulocyte lysate at 25°C, or at 30°C, neither heat treatment (34°C) nor reaction with anti-HSF increased HSF affinity for DNA (Figure 2A, lanes 4–9). The low activity of HSF translated at 25°C is due to reduced translational efficiency at this temperature (data not shown). The specific binding of HSF translated in vitro was demonstrated by a DNA competition experiment (Figure 2B). The constitutive DNA binding activity of HSF synthesized in vitro could be due to an activating substance in the reticulocyte lysate. However, we found that reticulocyte lysates do not activate HSF when incubated with cytosol from unshocked Drosophila cells (data not shown).

We overexpressed HSF in E. coli at 18°C using the T7 RNA polymerase–dependent expression system (Studier and Moffatt, 1986). The cloned HSF protein does not contain additional amino acids introduced by fusion with the expression vector. Cloned HSF protein isolated from E. coli showed maximal binding affinity without heat or anti-HSF treatment (data not shown; see also Figure 5B, lanes 3 and 4). HSF expressed at low levels in bacteria also showed maximal affinity without heat or anti-HSF treatment; hence, overexpression per se does not lead to activation (data not shown). Specific binding of HSF produced in E. coli was confirmed in vitro by a DNAase I protection assay, which shows binding to the HSEs upstream of the *hsp70* gene (Figures 2C and 2D). The DNAase I protection pattern is identical to the pattern obtained with natural HSF purified from heat-shocked Drosophila cells (Wu et al., 1987). The data suggest that cloned HSF protein synthesized outside the environment of a higher eukaryotic cell has an intrinsic affinity for DNA.

We tested the ability of HSF produced in E. coli to function as a transcription factor in an in vitro transcription system derived from Drosophila embryos (Soeller et al., 1988; Biggin and Tjian, 1988). Addition of the cloned protein to the transcription extract resulted in a 7-fold increase of transcription from a promoter carrying two HSEs, relative to the transcription from the same promoter lacking HSEs (Figure 3). Hence, recombinant HSF protein is capable of functioning as a transcription factor in a binding site–dependent manner, apparently without further modification by a heat shock–induced enzymatic activity.

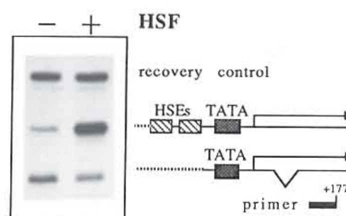

Figure 3. Transcriptional Stimulation by Recombinant HSF In Vitro

Primer extension analysis of RNA synthesized by nonshocked Drosophila embryo transcription extracts supplemented with 0.2 μl of E. coli extract from HSF-expressing cells (+) or with extract from cells transformed with the expression vector only (−). As an internal control for transcription from the template carrying two HSEs, the same template deleted of the HSEs (as well as a 30 bp downstream region) was mixed in the reaction. RNA originating from the template lacking HSEs is thus distinguished by a 30 nucleotide decrease in size. As a further control for RNA recovery, a defined amount of RNA synthesized from a T_7 promoter upstream of the *hsp70* sequences inserted into pBluescript was introduced into each transcription reaction along with the stop solution. Schematic drawings of the two templates are aligned with the primer extension products of the respective transcripts.

Recombinant HSF Expressed in Xenopus Oocytes Shows Heat Shock–Inducible DNA Binding Activity

Naturally occurring HSF in crude extracts of unshocked Drosophila, Xenopus, and vertebrate cells shows a basal

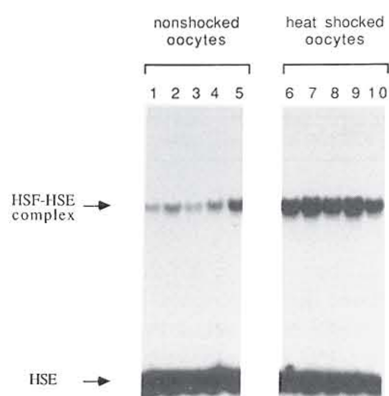

Figure 4. DNA Binding Activity of HSF Expressed in Xenopus Oocytes

Gel mobility shift assay of HSF extracted from individual oocytes. Extracts of each of five nonshocked (18°C) oocytes (lanes 1–5) and five heat-shocked (36°C, 10 min) oocytes (lanes 6–10) were individually analyzed. The positions of the HSF–HSE complex and free HSE are indicated.

affinity for DNA by in vitro assays, which is increased about 10-fold when cells are induced by heat shock (Zimarino et al., 1990a). We tested the activity of recombinant HSF synthesized after microinjection of Xenopus oocytes with HSF RNA transcribed in vitro. The endogenous Xenopus HSF is below the level of detection in these experiments (data not shown). Although there is some fluctuation in the basal DNA binding activity of the recombinant protein in crude extracts of individual unshocked oocytes (Figure 4, lanes 1–5), DNA binding activity is significantly induced (5-fold, on average) after heat shock for 10 min (Figure 4, lanes 6–10). The amount of Drosophila HSF protein synthesized in oocytes subjected to heat shock was equivalent to the synthesis in control oocytes, as determined by [^{35}S]methionine incorporation and SDS gel electrophoresis (data not shown). Thus, in contrast to the full DNA binding capacity of HSF synthesized in E. coli or in a reticulocyte lysate, the intrinsic affinity of HSF for DNA is suppressed in nonshocked Xenopus oocytes. The results suggest that the naturally occurring form of HSF in unshocked Drosophila cells is under negative control, which is relieved upon heat shock.

Oligomeric State of HSF in Solution

The apparent molecular mass of cloned HSF, purified from E. coli extracts, was determined to be about 105 kd by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) (see Figures 5C and 5D). This size is in agreement with the apparent mass (110 kd) of the natural protein purified from Drosophila cells (Wu et al., 1987); the 5 kd difference could be due to gel mobility fluctuations or to posttranslational modification of the natural protein. We measured the native size of cloned HSF by pore exclusion limit analysis (Andersson et al., 1972). In this procedure, proteins are electrophoresed for extended periods (about 24 hr) on nondenaturing polyacrylamide gradient gels; each protein migrates until it reaches the pore exclusion limit, which is dependent, to a first approximation, on the size of the protein. The major species of cloned HSF separated on the native gel migrates with an estimated size of 690 kd (Figure 5A). There are also minor species that migrate above and below the 690 kd species, and very large aggregates near the origin of electrophoresis are also visible. We also measured the native size of HSF bound to the HSE by pore exclusion limit analysis of the protein–DNA complex (Huet and Sentenac, 1987; Hooft van Huijsduijnen et al., 1987). Cloned HSF protein forms a major complex with ^{32}P-labeled HSE that migrates with a size of 690 kd, in addition to minor, unresolved complexes of higher and lower mobility (Figure 5B, lanes 3 and 4). Since the HSE contribution to the overall protein–DNA complex is negligible (assuming one native HSF molecule binds to one or two HSEs), this result suggests that the major DNA binding form of HSF has a molecular size approximating 690 kd. No HSF–HSE complexes could be detected near the origin of electrophoresis, suggesting that the very large HSF complexes observed in Figure 5A are aggregates that lack biological activity. The unusually large size of cloned HSF free in solution and when bound to DNA could be related to high HSF concentrations em-

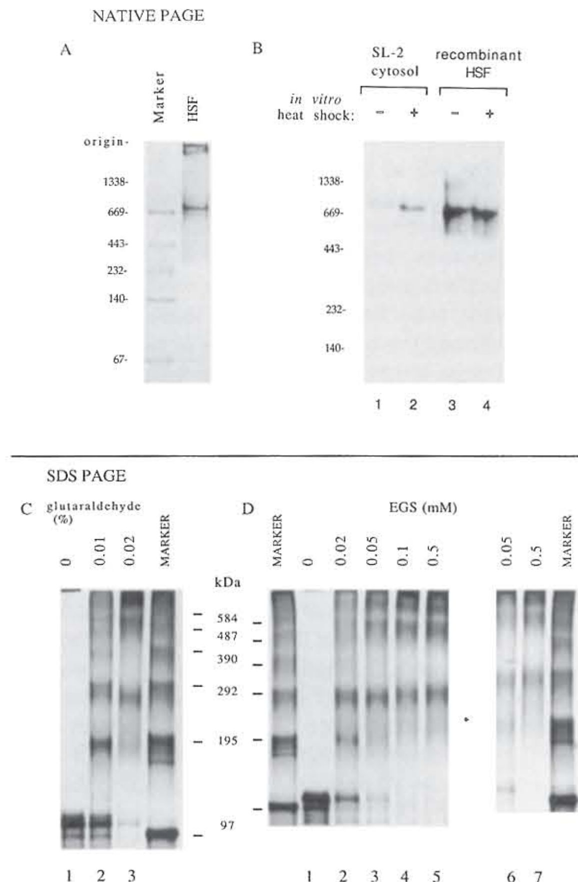

Figure 5. Estimation of the Native Size of HSF

(A) Pore exclusion limit analysis of HSF. Purified, cloned HSF (5 μg/12 μl sample volume) was electrophoresed on a nondenaturing 4%–20% polyacrylamide gel until the limit of migration was reached. The gel was stained with Coomassie blue. The marker lane shows molecular size markers: thyroglobulin tetramer (1338 kd), thyroglobulin dimer (669 kd), apoferritin (440 kd), catalase (232 kd), lactate dehydrogenase (140 kd), and bovine serum albumin (67 kd).

(B) Pore exclusion limit analysis of the HSF–^{32}P-HSE complex. Three microliters of Drosophila SL-2 cell cytosol (lanes 1 and 2) and 0.5 μl of an extract from E. coli expressing HSF (lanes 3 and 4) were heat shocked (+) in vitro at 34°C or incubated at 0°C (−) for 10 min. The samples were incubated for 10 min with ^{32}P-labeled HSE under standard gel shift conditions and electrophoresed on a nondenaturing, 3%–12% polyacrylamide gradient gel until the limit of migration. The gel was stained with Coomassie blue, dried, and subjected to autoradiography. The positions of marker proteins are indicated.

(C) Glutaraldehyde cross-linking of cloned HSF. Purified HSF (2 μg/10 μl) was treated for 5 min at room temperature with glutaraldehyde as indicated. After quenching, about 1 μg of cross-linked HSF was separated on a 4%–6% SDS–polyacrylamide gel and silver stained. The minor polypeptides below the 105 kd HSF protein probably represent degradation products. The marker lane contains cross-linked phosphorylase b (Sigma); cross-linked thyroglobulin was also used as a marker (not shown). A similar resolution of HSF oligomers was obtained by SDS gel electrophoresis in a phosphate buffer (Weber and Osborn, 1969).

(D) EGS cross-linking of cloned HSF. Lanes 1–5, purified HSF (2 μg/10 μl) was treated for 10 min at room temperature with EGS as indicated; lanes 6 and 7, similar EGS treatment of HSF diluted to 2 μg/ml. The cross-linked products were precipitated with 15% TCA, washed twice with ice-cold acetone, and dissolved in Laemmli sample buffer. Cross-linked products were analyzed by SDS gel electrophoresis as above. Introduction of ovalbumin into the cross-linking reaction revealed no interaction between HSF and the monomeric ovalbumin protein.

ployed in the analysis of the cloned protein. However, we find that natural HSF from the cytosol of SL-2 cells also migrates with an apparent size of 690 kd when bound to DNA (Figure 5B, lanes 1 and 2). We propose that the state of HSF active for binding to an HSE is composed of a hexamer of the 105 kd or 110 kd subunit. Although large oligomers of HSF other than hexamers could conceivably account for the observed gel mobilities, we favor a hexameric association because of evidence that the native HSF molecule is composed of three (or multiples of three) DNA binding subunits (Perisic et al., 1989).

We confirmed the oligomeric nature of cloned HSF by chemical cross-linking. Cloned HSF protein cross-linked with limiting amounts of glutaraldehyde (Landschulz et al., 1989), and when analyzed on an SDS gel displayed a ladder of cross-linked products whose apparent sizes are approximate multiples (up to six) of the 105 kd HSF monomer (Figure 5C, lane 2). HSF oligomers were sized relative to cross-linked phosphorylase b markers (97 kd monomer). Increasing the glutaraldehyde concentration enhanced the abundance of HSF trimer and hexamer, in addition to larger species at the limiting mobility of the gel. Similar results were obtained with the bifunctional reagent EGS (Abdella et al., 1979) (Figure 5D, lanes 1–5). More importantly, a 100-fold dilution of cloned HSF protein (to 2 μg/ml) gave essentially the same abundance of HSF oligomers (Figure 5D, lanes 6 and 7), suggesting that the oligomerization of HSF in trimers and hexamers is not due to an artificially high concentration of the cloned protein.

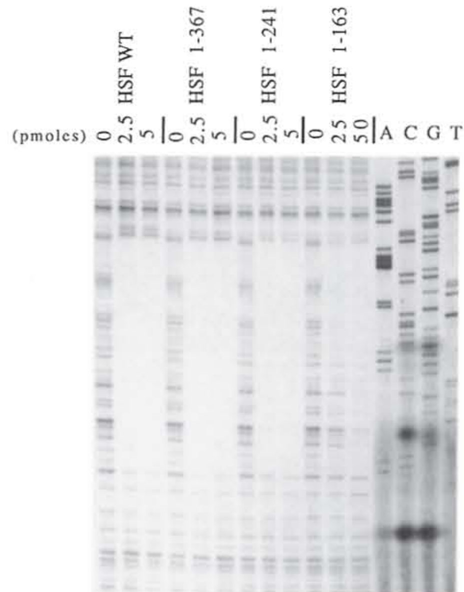

Figure 6. Deletion Analysis of HSF

DNAase I protection analysis of HSF mutants. A labeled fragment from the *hsp70* promoter was incubated with the indicated amounts of wild-type or mutant HSF proteins, expressed in E. coli with the T_7 system. Footprinting reactions were performed essentially as described in Figure 2C.

Regions Important for Specific and High Affinity Binding to DNA

Deletion analysis of HSF reveals an N-terminal region important for specific binding to DNA. C-terminal truncations of HSF protein, up to residue 163 (HSF 1–163), are fully capable of binding to DNA, but HSF 1–163 shows a distinctly lower affinity for the *hsp70* promoter compared with the affinity of full-length HSF (Figure 6). From the HSF protein concentrations required to achieve roughly 50% binding to DNA, we estimate that HSF 1–163 binds with about 50-fold lower affinity relative to the binding of full-length HSF. The binding of HSF 1–241 and HSF 1–367 differ from full-length HSF by no more than 2-fold. Hence, we conclude that HSF 1–163 is sufficient for binding specifically to HSEs, while an adjacent region, from residues 164 to 241, increases the affinity by 25- to 50-fold.

Conserved Sequences between Drosophila and Yeast HSF

We compared the primary amino acid sequence of Drosophila HSF with the published sequence of yeast HSF (Wiederrecht et al., 1988; Sorger and Pelham, 1988). It is striking that despite the high degree of homology among heat shock proteins between species as diverse as E. coli and Drosophila (about 50% identity, for *hsp70*; Bardwell and Craig, 1984), the sequences of Drosophila and yeast HSF have diverged over a large portion of the proteins. A dot matrix plot of sequence similarities revealed two major and two minor regions of local conservation (Figure 7A). Among the four regions, region A is most conserved between Drosophila and yeast HSF. Out of 66 amino acids, 33 are identical (50% identity; 73% similarity, allowing for conserved substitutions) (Figure 7B).

Conserved region B shows 44% identity and 67% similarity in 33 amino acids. Region B is contained within a larger region of yeast HSF that is required for trimerization of the yeast factor (Sorger and Nelson, 1989). Regions C and D show 27% identity, 41% similarity, and 28% identity, 51% similarity, respectively. These regions are not involved with DNA recognition, since they can be deleted without affecting the DNA binding function. Regions C and D are notably represented by polar amino acids, and among the 23 identical residues combined for both regions, 10 are serines or threonines, potential candidates for phosphorylation. Four of the identical residues are acidic.

Discussion

We have cloned the transcriptional activator of heat shock genes, HSF, from Drosophila. A large portion of the predicted amino acid sequence of Drosophila HSF is unexpectedly divergent from the sequence of yeast HSF. Four local regions show significant homology, including domains important for DNA binding and oligomerization. Drosophila HSF produced in E. coli at non-heat shock temperatures forms a hexamer that binds specifically to DNA with high affinity and activates transcription from a heat shock promoter in vitro. When HSF is expressed in Xenopus oocytes, maximal DNA binding affinity is observed only after heat shock induction, suggesting that

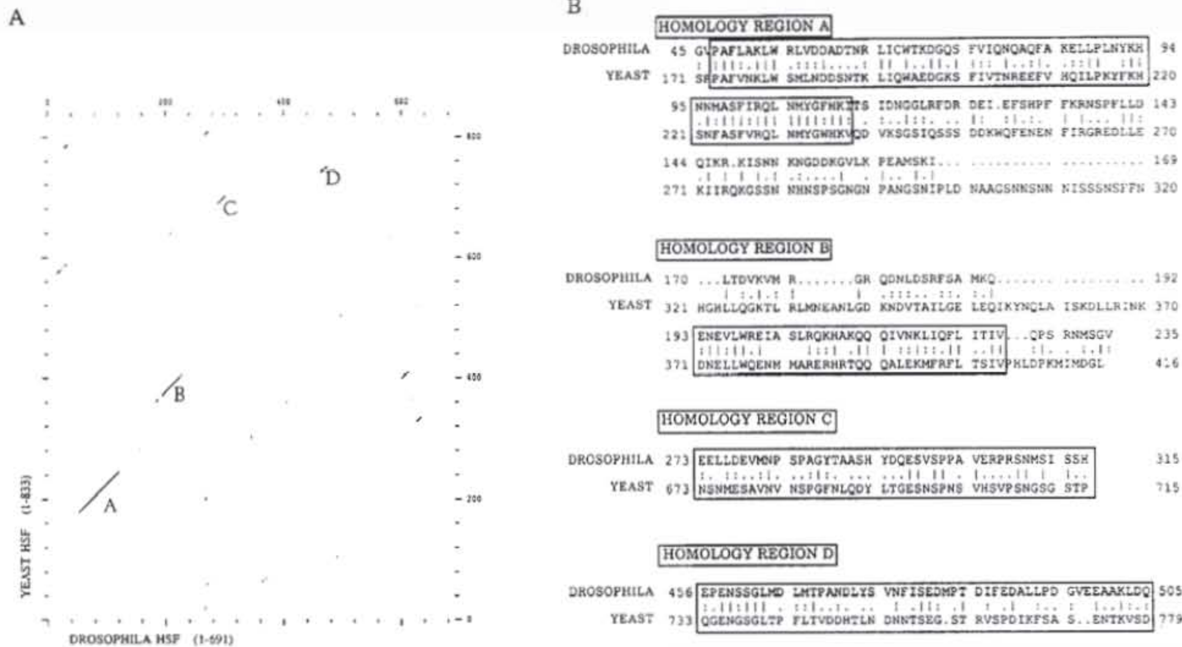

Figure 7. Sequence Comparison of Drosophila and Yeast HSF

(A) Dot matrix plot of conserved amino acids between Drosophila HSF (horizontal) and yeast HSF (vertical), using the UWGCG sequence analysis programs Compare (window/stringency 30/17) and Dotplot.

(B) Amino acid alignment of conserved regions A–D, using the UWGCG sequence analysis program BestFit, with default parameters. Vertical lines indicate amino acid identities. ":" indicates similar amino acids, according to Dayhoff, as normalized by Gribskov and Burgess (1986). Conserved regions A–D are boxed. There are sequence similarities that extend beyond the somewhat arbitrary boundaries imposed on each conserved region.

the binding of HSF to DNA is under negative control in higher eukaryotic cells.

Does HSF Form a Trimer or Hexamer?

The native size of HSF as estimated by pore exclusion limit electrophoresis suggests that a significant fraction of cloned HSF protein forms a hexamer free in solution. A similar size estimation of cloned or natural HSF protein bound to the HSE suggests that an HSF hexamer binds to DNA with high affinity. Chemical cross-linking of a dilute HSF solution shows that cloned HSF protein is composed of trimers, hexamers, and very large complexes beyond the limit of gel analysis. Taken together, these results indicate that HSF probably exists in equilibrium as trimers, hexamers, and even larger oligomers free in solution, but the oligomeric state that binds to an HSE (three alternating [__GAA__] modules) is primarily hexameric. The question thus arises whether one or two subunits of a hexamer participate in the recognition of each [__GAA__] module. If one HSF subunit interacts with one [__GAA__] module, three subunits of the hexamer remain free, in principle, to bind to another HSE. It will be important to determine the stoichiometry of HSF binding to HSE.

Previous reports have shown that the oligomerization state of HSF in Drosophila and yeast is primarily trimeric (Perisic et al., 1989; Sorger and Nelson, 1989). Evidence for trimerization of Drosophila HSF was based on the observation of a 350 kd cross-linked HSF product visualized by protein blot (Western) analysis. It is possible that the full oligomerization potential of HSF was not in evidence in those preparations of natural HSF, or that the cloned protein associates more readily as a hexamer. The conclusion that yeast HSF is a trimer was based on the associative properties of recombinant yeast HSF proteins that had roughly half of the protein sequence deleted. It may also be important to determine the subunit composition of the full-length yeast HSF protein in solution.

Sigma Homology in the DNA Binding Domain

Among the four regions conserved between Drosophila and yeast HSF, the 66 amino acid region A is most conserved (50% identity). This region is included within the DNA binding domains of both Drosophila and yeast HSF (this paper; Wiederrecht et al., 1988) and may therefore organize a structural domain for specific DNA recognition. In E. coli, heat shock genes are positively regulated by a special sigma subunit of RNA polymerase, σ^{32} (Gross et al., 1990). We compared the DNA binding domains of Drosophila HSF and yeast HSF with the σ^{32} protein sequence and found a short conserved region, which is also represented in the major E. coli sigma subunit, σ^{70} (Figure 8A). Intriguingly, this region includes the putative helix-turn-helix DNA binding motif common to sigma factors. While residues comprising the turn between the two helices are apparently not conserved in HSF, there are two conserved pentapeptides located in the putative DNA rec-

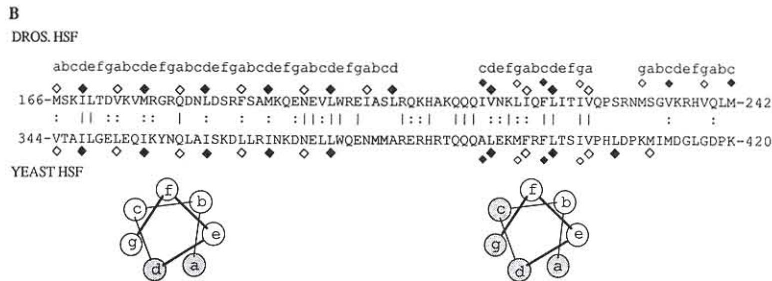

Figure 8. Conserved Structural Motifs

(A) Alignment of protein sequences conserved between Drosophila HSF, yeast HSF, σ^{32}, and σ^{70}. Similar residues are stippled. The putative helix-turn-helix motif of σ^{32} (26 residues) is located between L-253 and L-278; the three residues comprising the turn are boxed. The Drosophila HSF sequence shows 27% identity/46% similarity to the σ^{32} sequence in the block of 26 amino acids.

(B) Comparison of the heptad repeats of hydrophobic amino acids found in Drosophila and yeast HSF sequences. The two sequences are aligned without gaps using conserved region B as defined by the Bestfit sequence analysis program as the starting frame of alignment. The repeats are made up of hydrophobic residues at positions a (open diamonds) and d (filled diamonds), in the nomenclature for coiled coils $(a\ b\ c\ d\ e\ f\ g)_n$. The small diamonds represent a third array of hydrophobic residues out of register by 1 residue from the second array. Heptad repeats of the yeast HSF sequence are taken from Sorger and Nelson (1989). Backbone illustrations of hypothethical α-helices are shown with the positions of hydrophobic residues stippled.

ognition helix (region 4.2) of the sigma factors (Gribskov and Burgess, 1986; Helmann and Chamberlin, 1988). We suggest that the homology to the putative recognition helix of sigma factors may define an α-helical element of the HSF DNA binding domain that is important for DNA interaction.

Overlapping Heptad Repeats of Hydrophobic Residues

Two lines of evidence implicate sequences within and surrounding conserved region B in the self-association of Drosophila HSF. First, C-terminal deletions that remove 78 residues between amino acids 163 and 241 reduce the affinity for DNA, but not the specificity, by as much as 50-fold. Second, region B of yeast HSF has been shown directly to mediate trimerization of a truncated yeast HSF protein (Sorger and Nelson, 1989). These workers first noted an array of heptad repeats of hydrophobic residues in the yeast HSF oligomerization domain and proposed a triple-stranded coiled-coil model for the yeast HSF trimer. A second, heptad array of hydrophobic residues located 18 amino acids C-terminal to the first array was suggested to contribute to the stability of the trimeric interface.

We found the first and second array of hydrophobic amino acid repeats in Drosophila HSF (Figure 8B, large diamonds). In addition, we discovered a third array of hydrophobic residues, positioned 1 residue out of register with the second array (Figure 8B, small diamonds). When the second and third array of heptad repeats are viewed in a backbone model of an α-helix, it becomes evident that the helix has hydrophobic residues juxtaposed at four positions on one helical face (Figure 8B). Such a helix would have the potential to associate simultaneously with two neighboring helices of the same type by hydrophobic interactions characteristic of leucine zipper coiled coils (Landschulz et al., 1988; O'Shea et al., 1989). Although it is unclear at present how the three arrays of hydrophobic heptad repeats might direct hexamerization of HSF, the remarkable degree of conservation suggests that they all have functional roles. It is possible that the HSF hexamer is formed by a combination of dimeric and trimeric coiled-coil interactions.

The conserved amino acids in the oligomerization domain are not limited to hydrophobic residues. Identical residues include polar amino acids (three glutamines in a row [QQQ]), hydrophobic (W, F, I, L), basic (R, K), and acidic (E) amino acids. Although hydrophobic interactions are the major stabilizing force between coiled coils, additional specificity may be conferred by charged or polar interactions, mediated by residues outside the heptad repeat (Cohen and Parry, 1990). The conserved residues may also be involved with interactions of the HSF subunit with other proteins (see below).

Transcriptional Activity and Phosphorylation

The conserved serine and threonine residues in regions C and D of Drosophila and yeast HSF suggests that these residues could be sites for heat shock–induced phosphorylation, leading to a transcriptionally active HSF protein (Sorger and Pelham, 1988). However, we find that Drosophila HSF produced in E. coli at low temperature can stimulate transcription from a heat shock promoter in vitro. This result appears to be inconsistent with phosphorylation as a requirement for transcriptional competence and suggests that cloned HSF protein is able to fold to a transcriptionally active conformation in bacteria. A requirement for phosphorylation may become evident when transcriptional activity is measured by assays that are closer to the conditions within eukaryotic cells. An alternative possibility is that HSF synthesized in E. coli at 18°C is phosphorylated like the natural protein in heat-shocked Drosophila cells because of increased kinase and/or lowered phosphatase activity in bacterial cells. It will be important to determine the extent of phosphorylation of HSF isolated from heat-shocked Drosophila cells, particularly at the conserved serines and threonines.

A Model for Heat Shock Regulation in Higher Eukaryotes

The naturally occurring form of HSF in Drosophila cells binds to DNA with high affinity only under stress conditions. Cloned HSF synthesized in E. coli or in a rabbit reticulocyte lysate shows maximal affinity for DNA without a heat shock; this affinity is suppressed when HSF is synthesized in Xenopus oocytes. Our results suggest that HSF protein has an intrinsic tendency to fold to the active conformation, which is suppressed in higher eukaryotic cells. The suppression in vivo could be due to a redirection of the folding of HSF to a conformation that is unable to oligomerize or to an association of HSF with a specific inhibitory substance.

We speculate that heat shock proteins may participate in the suppression of HSF activity. There are precedents for the function of heat shock proteins as molecular detergents or chaperones in protein–protein interactions (for reviews see Pelham, 1990; Rothman, 1989). For example, *hsp90* forms a complex with steroid hormone receptors; binding of hormone causes dissociation of *hsp90* and formation of an active receptor complex (Joab et al., 1984; Catelli et al., 1985; Sanchez et al., 1985, 1987; Pratt et al., 1988; Denis et al., 1988; Picard et al., 1988). The BiP/GRP78 protein, a member of the *hsp70* family, complexes with incompletely folded or assembled molecules such as immunoglobulin heavy chains that lack a light chain (Haas and Wabl, 1983; Bole et al., 1986) or monomer subunits of the influenza hemagglutinin prior to assembly of the hemagglutinin trimer (Gething et al., 1986; Copeland et al., 1988). There have been many suggestions that heat shock proteins negatively autoregulate their synthesis (DiDomenico et al., 1982; Bonner, 1982; Craig, 1990; Morimoto et al., 1990; Beckmann et al., 1990; Gross et al., 1990; Georgopoulos et al., 1990).

From the earliest studies of the heat shock response, the bewildering multiplicity of stress inducers have presented a challenge to the search for a common stress signal transduction pathway. Besides heat, inducers of the stress response include drugs affecting energy metabolism, oxidizing agents, sulfhydryl reagents, chelating agents, heavy metals, ionophores, amino acid analogs, etc. (Ashburner and Bonner, 1979; Nover et al., 1984). We and others have shown that the inactive state of HSF is easily altered in vitro by physical and chemical changes in the environment. If the inactive form of HSF protein is maintained in a metastable state by diverse molecular forces, for example, by an essential combination of hydrophobic, charged, and polar interactions, then the disruption of a subset of these forces by any one inducer of the stress response could be sufficient to trigger a change of state. In this view, a common biochemical pathway for transduction of the heat shock signal eluded searchers for almost three decades precisely because such a common pathway may not exist. Instead, a solution to the enigma of stress signal transduction may be found in the molecular architecture of HSF protein itself and in the interactions with its negative regulators.

Experimental Procedures

Purification of HSF and Digestion with Trypsin

HSF was purified as described in Wu et al. (1987) with one modification. Nuclear extract from heat-shocked Schneider line 2 cells was purified by chromatography on heparin–Sepharose CL-6B (Pharmacia) and two affinity chromatography steps on HSC oligo-Sepharose. The HSF fraction from the second affinity column was then fractionated by reverse phase HPLC on an Aquapore RP 300 (C_8) column (2.1 × 30 mm, Applied Biosystems). HSF was eluted by a 10 ml gradient of 0% to 70% acetonitrile in 0.1% TFA, and 100 μl fractions between 35% and 50% acetonitrile were collected in siliconized microcentrifuge tubes. To identify fractions containing HSF, 5% of each fraction was dried in vacuo in a centrifugal concentrator (Speedvac, Savant), redissolved in sample buffer, subjected to SDS gel electrophoresis, and stained with silver nitrate. The peak of HSF was eluted in one fraction at 44% acetonitrile. In an independent purification, we prepared HSF to the Mono S step (Wu et al., 1987), followed by chromatography on a ~30 μl ProRPC C_1/C_8 column (Pharmacia), under conditions similar to those described for the Aquapore column.

The remaining 95% of each of the two purified HSF preparations (each about 4 μg, or 40 pmol) were digested with trypsin essentially according to Stone et al. (1989), in two separate reactions. The HSF fraction was dried in vacuo and redissolved in 8 M urea/0.1 M ammonium bicarbonate (pH 8.1) at a concentration of 0.5 μg/μl HSF. After addition of one-tenth volume of 45 mM dithiothreitol, the sample was incubated for 15 min at 50°C and cooled to room temperature. The sample was then incubated for 15 min at room temperature with iodoacetamide (0.1 vol of 0.1 M solution, Sigma), followed by dilution with 3 vol of 0.1 M ammonium bicarbonate. Trypsin (Sequencing Grade, Boehringer Mannheim) was added at a weight ratio of 1:30 trypsin:HSF, and the sample was incubated for 24 hr at 37°C. The tryptic digest was diluted with an equal volume of 10% acetonitrile, 0.1% TFA and loaded on a Vydac C_{18} reverse phase HPLC column (2.1 × 150 mm, The Nest Group) on an Applied Biosystems Model 130A separations system. Peptides were eluted with a gradient of 0% to 50% acetonitrile in 0.1% TFA, and individual peaks were collected onto glass fiber filters. The filters were dried in vacuo and subjected to amino acid sequence analysis on an Applied Biosystems 477A Protein Sequencer coupled to a 120A analyzer. The sequences of six peptides are: peptide 16 (AVQFK), peptide 23 (DGQ[S/M]FVIQNQAQFA), peptide 27 (XVQLMINNTPEIDR), peptide 29 (FSAMKQENEVL), peptide 32 (FASNFDVPTNSXLLDANQA), peptide 39 (ITSIDNGG).

E. coli Strains and Plasmids

For routine cloning and plasmid amplification we used the strains Xl-1 Blue (Stratagene) or DH-5α (BRL). Lambda gt11, EMBL 3 phage, and their derivatives were propagated in strains Y1090 or LE392, respectively. The strain BL21(DE3) (Studier and Moffatt, 1986) served as host for bacterial expression of HSF. Subcloning of genomic DNA and cDNA inserts and reconstruction of the full-length HSF cDNA were performed with pBluescript II KS(+) (Stratagene). pHSFpoly(A) contains HSF cDNA (positions −15 to +2540, combined from pHSF407 and pHSF312, see Figure 2A) inserted in the EcoRI site of pJC1. pJC1 was constructed by fusing a $(dA)_{100}$ sequence derived from the plasmid pSP65AT (Baum et al., 1988) between the SmaI and BamHI sites of pBluescript II KS(+). This plasmid allows the transcription of HSF RNA containing a poly(A) tail, under the control of the T_3 RNA polymerase promoter for in vitro translation and microinjection studies. The bacterial expression vector pJC10 was constructed by ligation of the ScaI–BglII (blunted) fragment from pET 3C (Rosenberg et al., 1987), which contains the T7 φ10 promoter, translation signals, and transcription terminator, plus the 5′ half of the ampR region, with the ScaI–PvuII fragment from pBluescript II KS(+), containing the 3′ half of the ampR region and the col E1 origin of replication. pJC10 is smaller than pET3C and is a high copy number plasmid allowing high yields in analytical plasmid preparations. pHSFWT was constructed by creation of an NdeI site at the start codon of the HSF cDNA and ligation of an NdeI–BamHI HSF fragment to pJC10 (linearized with NdeI and BamHI). The NdeI–BamHI fragment contains 2532 nucleotides of HSF

sequences from the initiating AUG codon, plus 16 nucleotides at the 3′ end from pBluescript II KS(+). Nested deletion mutants were generated by ExoIII/S1 digestion of pHSFWT cleaved at the StuI and ApaI sites (see Figure 1B) following the manufacturer's protocol (Pharmacia).

Library Screening

The Drosophila genomic library in EMBL 3 and the oligo(dT)-primed cDNA library were gifts from John Tamkun, Jim Kennison, and Matthew Scott. The random-primed cDNA library was a gift of Bernd Hovemann. The genomic library was screened by hybridization with two oligonucleotides: oligo 27, 5′-TT(G/A)ATCAT(G/C)AG(C/T)TG(G/C)-AC(C/T)TT; and oligo 29, 5′-AC(C/T)TC(G/A)TT(C/T)TC(C/T)TG(C/T)-TTCAT. Oligos 27 and 29, representing the coding strand, were derived from peptides 27 and 29. V, L, and I codons of oligo 27 were chosen in accordance with the codon bias of Drosophila. Hybridization was performed at 37°C in 6× SSC, and the final wash was done at 48°C in 3.2 M tetramethylammonium chloride (Wood et al., 1985; Devlin et al., 1988). Plaque hybridization of the cDNA libraries in lambda gt11 was carried out as follows: hybridization and washes at 65°C in 6× SSC and 0.5× SSC, respectively, using an ~1800 bp SalI–EcoRI fragment from genomic clone EMBL 3-104. Twelve cDNA clones were isolated, seven of which were sequenced after subcloning into pBluescript II KS(+).

Preparation of HSF RNA and Translation In Vitro

pHSFpoly(A) (20 μg/ml) was cleaved with XbaI and incubated for 60 min at 37°C in a 50 μl volume containing 40 mM Tris–HCl (pH 8.0), 8 mM $MgCl_2$, 5 mM dithiothreitol, 4 mM spermidine, 400 μM each of ATP, CTP, UTP, and $m^7G(5')ppp(5')Gm$, 40 μM GTP, 50 μg/ml bovine serum albumin, 1000 U/ml RNAase inhibitor (Boehringer Mannheim), and 40 U/ml T_3 RNA polymerase (Boehringer Mannheim). RNA was extracted with phenol-chloroform, precipitated with ethanol, and redissolved in HPLC grade water (Fisher Scientific).

Rabbit reticulocyte lysate (Promega) was treated with Staphylococcus aureus nuclease (Boehringer Mannheim) as described in Maniatis et al. (1982). One microgram of in vitro transcribed HSF RNA was translated for 2 hr at either 25°C or 30°C in a 25 μl volume containing 50% translation lysate, 20 μM of each amino acid, 1000 U/ml RNAase inhibitor, and 0.2 mCi/ml [^{35}S]methionine (1000 Ci/mmol, DuPont-NEN). Small aliquots of the reaction were subjected to SDS gel electrophoresis and fluorography to verify the translational efficiency and accuracy. The remainder was frozen in liquid nitrogen and stored at −80°C.

Expression and Purification of Cloned HSF in E. coli

BL21(DE3) cells transformed with pHSFWT or its derivatives were grown at 37°C to an OD_{600} of 0.6 in M9TB/amp medium (10 g of Bacto-Tryptone [Difco], 5 g of NaCl, 1 g of NH_4Cl, 3 g of KH_2PO_4, 6 g of Na_2HPO_4, 4 g of glucose, 1 mM $MgSO_4$, and 50 mg/liter ampicillin). IPTG was added to 0.4 mM, and the cultures were transferred to 18°C. After 40 to 60 min incubation, 40 mg of rifampicin was added to suppress transcription by bacterial RNA polymerase, and incubation was continued at 18°C overnight, with shaking. Alternatively, cells were harvested 1 hr after IPTG induction. Bacteria were pelleted by centrifugation (6000 × g, 10 min, room temperature) and resuspended in 1/100 volume of buffer CB + 400 mM KCl (buffer CB: 20 mM HEPES [pH 7.6], 1.5 mM $MgCl_2$, 0.1 mM dithiothreitol, 2 mM leupeptin, 10% [v/v] glycerol). After disruption by sonication at 100 mW for 2 min (B. Braun), the lysate was incubated for 30 min on ice. The bacterial debris was removed by centrifugation (6000 × g, 10 min, 4°C), and the supernatant was diluted 2-fold with buffer CB and centrifuged at 100,000 × g at 4°C for 1 hr. The supernatant containing crude HSF was frozen in liquid nitrogen and stored at −80°C.

To purify cloned HSF, 40 ml of the crude supernatant was diluted with buffer CB to a KCl concentration of 100 mM and chromatographed on a 20 ml heparin–Sepharose CL-6B column. HSF was eluted with a linear NaCl gradient (100–500 mM) in buffer CB. HSF activity was monitored by gel mobility shift assays, and active fractions were diluted to 100 mM NaCl with buffer CB. HSF was further chromatographed on a 1 ml Mono Q column (Pharmacia) and eluted with a linear NaCl gradient (100–500 mM) in buffer CB. Active fractions contained the 105 kd HSF protein purified to 90% homogeneity, as determined by SDS gel electrophoresis and silver staining. The total protein concentration was 3.5 mg/ml, as determined by a dye binding assay (Bio-Rad).

Gel Mobility Shift Assay

DNA binding was monitored by the gel mobility shift assay as described previously (Zimarino and Wu, 1987), using a double-stranded, synthetic HSE carrying three [_GAA_] repeats in alternating orientation (Zimarino et al., 1990a). The DNA was labeled with ^{32}P by primer extension as described previously (Wu et al., 1987). For the experiments shown in Figure 2A, 2 μl samples of protein were mixed with 10 fmol of ^{32}P-labeled HSE, 2.5 μg of poly(dI-dC)·poly(dI-dC), 5 μg of yeast tRNA, 0.5 μg of sonicated E. coli DNA, and 0.5 μg of $poly(dN)_5$ in 10 μl of 10 mM HEPES (pH 7.9), 1.5 mM $MgCl_2$, 0.05 mM EDTA, 120 mM NaCl, and 6% glycerol. Samples were incubated on ice for 10 min and electrophoresed on a 1.2% agarose, 0.5× TBE gel. The gel was blotted and dried onto DE 51 paper and autoradiographed.

DNAase I Footprinting

DNA fragments labeled with ^{32}P at one 5′ end were synthesized by the polymerase chain reaction using a combination of one 5′ labeled oligonucleotide primer and one unlabeled primer. An XhoI–AccI fragment (positions −185 to +295) from the *hsp70* gene promoter (locus 87A) cloned into pBluescript I SK(+) served as template for the polymerase chain reaction. The oligonucleotide primers used were: *hsp70* lower strand positions +149 to +177, T_7 sequencing primer (Stratagene), *hsp70* upper strand positions −140 to −120, and *hsp70* lower strand positions +10 to +29. The labeled DNA fragment (50 fmol) was incubated at room temperature with cloned HSF extracted from E. coli under the same conditions as described for the gel mobility shift assays. After 10 min, DNAase I (Pharmacia) was added (300 U/ml), and the incubation was continued for another 2 min. The reaction was stopped by the addition of EDTA and SDS to 10 mM and 1%, respectively, and the DNA was extracted with phenol–chloroform and precipitated with ethanol. Primers that were 5′ end-labeled for the polymerase chain reaction were also used for dideoxy sequencing reactions as a reference.

In Vitro Transcription

Two supercoiled plasmid templates were used for in vitro transcription. p(−50)HSE carries a modified *hsp70* promoter in a pBluescript vector (Stratagene). The modified *hsp70* promoter consists of *hsp70* (locus 87A) sequences from −90 to +296, in which two upstream HSEs were remodeled according to Xiao and Lis (1988), keeping the natural spacing between the HSEs and the *hsp70* TATA box. The p(−50) minigene is similar to p(−50)HSE, except for a deletion of a 30 bp AluI fragment between +41 and +71 and substitution of sequences from −50 to −90 (containing the HSEs) with a synthetic polylinker. Details of these plasmid constructions will be presented elsewhere (P. B. B. et al., unpublished data).

Transcription extracts were prepared from 0–12 hr D. melanogaster (Oregon R, P2) embryos (Soeller et al., 1988; Biggin and Tjian, 1988). Care was taken not to inadvertently heat shock the embryos. Protein from the ammonium sulfate precipitation step was dialyzed to a conductivity equivalent to HEMG:100 mM KCl and stored in aliquots at −80°C (HEMG, Soeller et al., 1988). Transcription with crude embryo extracts was performed according to Heberlein et al. (1985), modified as follows for RNA recovery: after addition of 100 μl of stop mix (minus SDS) and 100 μl of phenol to the transcription reactions, the samples were mixed in an Eppendorf shaker for 2 min. One hundred microliters of chloroform:isoamyl alcohol 24:1 was added, and the mixing was repeated. The aqueous phase was transferred to a fresh tube, reextracted with organic solvent, and nucleic acids were precipitated with ammonium acetate. After thorough washing with 80% ethanol, the pellet was dried in vacuo and dissolved in 9 μl of 250 mM KCl, 2 mM Tris–HCl (pH 7.9), 0.2 mM EDTA; 1 μl of ^{32}P-labeled primer (*hsp70* positions +149 to +177) was added, and the primer was annealed by incubation at 75°C for 5 min, and at 42°C for 20 min. After addition of 25 μl of 50 mM Tris–HCl (pH 8.3), 10 mM $MgCl_2$, 5 mM dithiothreitol, 1 mM EDTA, 1 mM each dNTP, the primer was extended with 7 U of AMV reverse transcriptase (Promega) at 42°C for 45 min.

Translation of HSF RNA by Microinjection in Xenopus Oocytes

Xenopus laevis females were obtained from Nasco or Xenopus 1.

Pieces of ovary were surgically removed and the connective tissue digested with 0.2% collagenase (Sigma type II) in OR-2 medium (Wallace et al., 1973). Stage VI oocytes were incubated for about 12 hr in OR-2 with 1 mM oxaloacetate as exogenous energy source (Eppig and Steckman, 1976) before microinjection. All procedures were performed at 16°C–18°C, except where indicated.

HSF RNA was adjusted to a concentration of approximately 0.4 ng/nl in injection buffer (90 mM KCl, 15 mM HEPES [pH 7.5]). Approximately 25 nl (10 ng) of RNA was injected into each oocyte using a micropipet attached to an adjustable 10 µl Drummond pipettor as described (Westwood, 1988). After 10 hr, groups of injected oocytes were transferred to 1.5 ml microfuge tubes containing approximately 50 µl of OR-2 medium and heat shocked at 36°C for 10 min. Nonshocked oocytes were left at 18°C. The medium was removed and the oocytes rinsed quickly with 100 µl of 0°C homogenization buffer (50 mM KCl, 10 mM HEPES [pH 7.9], 0.5 mM PMSF, 0.5 mM dithiothreitol). Individual oocytes were transferred to fresh tubes and homogenized by repeated pipetting with a micropipettor (10 µl of buffer per oocyte). The lysate was centrifuged for 5 min at 12,000 × g at 4°C, and the supernatant was transferred to a fresh tube, avoiding the top lipid layer. Extracts were either frozen in liquid nitrogen or assayed immediately by the gel mobility shift technique (5 µl of extract in a 10 µl final volume).

Pore Exclusion Limit Electrophoresis

Two microliters (5 µg) of cloned HSF purified to the Mono Q step and high molecular weight marker proteins (Pharmacia #17-0445-01) were electrophoresed on a 4%–20% polyacrylamide (3%–15% glycerol) gradient gel in 0.5× TBE buffer. Electrophoresis was continued for 24 hr at 4°C (20 V/cm), and the gel was stained with Coomassie blue R-250 (Pierce). The long duration of electrophoresis was necessary for proteins to have migrated to their exclusion limit (Andersson et al., 1972).

Size estimation of the HSF–HSE complex was performed by electrophoresis of a mixture of HSF and ^{32}P-labeled HSE (under standard gel shift assay conditions) on a 3%–12% polyacrylamide gradient gel in 0.5× TBE buffer, as above. The gel was stained with Coomassie blue, destained, equilibrated in water, dried, and autoradiographed.

Chemical Cross-Linking

Cloned HSF (2 µg) (Mono Q fraction) was incubated with glutaraldehyde or EGS (Pierce) at room temperature for 10 min in 10 µl of 175 mM NaCl, 15 mM Tris–HCl (pH 8.0), 0.1 mM EDTA, and 1.5 mM $MgCl_2$. Reactions were quenched with 30 mM lysine and 1 vol of 2× Laemmli sample buffer. Samples were heated to 95°C for 5 min; aliquots were separated on an SDS 4%–6% polyacrylamide gel without a stacking gel and silver stained.

In Situ Hybridization

Preparation of chromosomal squashes for in situ hybridization followed standard procedures (Ashburner, 1989). The DNA probe was substituted with digoxigenin-dUTP by a random priming reaction, and hybrids were detected according to instructions supplied with the Genius kit (Boehringer Mannheim).

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GenBank Accession Number

The accession number for the sequence reported in this paper is M38668.

Note Added in Proof

A human HSF cDNA has been recently cloned in this laboratory (S. Rabindran and G. Giorgi, personal communication). The human and Drosophila HSF cDNA sequences are conserved in regions A and B, in addition to a new region (Drosophila HSF Glu-583 to Asp-610) that includes a fourth, conserved, heptad repeat of hydrophobic residues.