HSF access to heat shock elements in vivo depends critically on promoter architecture defined by GAGA factor, TFIID, and RNA polymerase II binding sites

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Chromatin structure can modulate gene expression by limiting transcription factor access to gene promoters. We examined sequence elements of the *Drosophila hsp70* promoter for their ability to facilitate the binding of the transcription factor, heat shock factor (HSF), to chromatin. We assayed HSF binding to various transgenic heat shock promoters in situ by measuring amounts of fluorescence at transgenic loci of polytene chromosomes that were stained with an HSF antibody. We found three promoter sequences that influence the access of HSF to its binding sites: the GAGA element, sequences surrounding the transcription start site, and a region in the leader of *hsp70* where RNA polymerase II arrests during early elongation. The GAGA element has been shown previously to disrupt nucleosome structure. Because the two other critical regions include sequences that are required for stable binding of TFIID in vitro, we examined the in vivo occupancy of the TATA elements in the transgenic promoters. We found that TATA occupancy correlated with HSF binding for some promoters. However, in all cases HSF accessibility correlated with the presence of paused RNA polymerase II. We propose that a complex promoter architecture is established by multiple interdependent factors, including GAGA factor, TFIID, and RNA polymerase II, and that this structure is critical for HSF binding in vivo.

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In living cells, DNA and histones are packaged into nucleosomes that can be further compacted to varying degrees and with other proteins to form chromatin. Examinations of a variety of genes in vivo suggest that gene expression is modulated by chromatin structure, particularly at gene promoters, such that tightly packed, nucleosome-inaccessible chromatin represses transcription, whereas more open chromatin that is accessible to nucleosome digestion is transcriptionally competent (for review, see Elgin 1988; Gross and Garrard 1988; Felsenfeld 1992). Gene induction is sometimes accompanied by an alteration of chromatin structure. For example, nucleosomes are displaced from the promoters of the yeast *pho5* gene upon phosphate deprivation (Almer and Horz 1986; Almer et al. 1986) and the mouse mammary tumor virus long terminal repeat (MMTV LTR) upon hormone

stimulation (Pina et al. 1990). In contrast, promoter architectures can also be preset for transcription. Nucleosome-hypersensitive regions encompass the promoters of *Drosophila* heat shock genes both before and during heat shock (Wu 1980; Keene et al. 1981; Costlow and Lis 1984).

In vitro experiments have shown clearly that the assembly of nucleosomes onto DNA templates effectively represses the transcription of genes in those templates (Knezetic et al. 1986; Lorch et al. 1987). The most significant obstacle imposed by nucleosomes appears to be the initial binding of the basal transcription machinery to gene promoters (Knezetic et al. 1986; Lorch et al. 1987; Losa and Brown 1987; Workman and Roeder 1987). Recently published experiments demonstrate that the K_d of the basal transcription factor TATA-binding protein (TBP) binding to TATA element-containing DNA is reduced >10,000-fold in the presence of prebound nucleosomes (Imbalzano et al. 1994). Binding of TBP before or together with nucleosome assembly allows subsequent

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transcription, whereas postassembly addition of TBP does not (Workman and Roeder 1987; Becker et al. 1991). Furthermore, when the entire basal transcription apparatus is bound to a promoter prior to the assembly of nucleosomes, RNA polymerase II can transcribe through core histones on the body of a gene (Lorch et al. 1987; Losa and Brown 1987; Izban and Luse 1991).

One protein that appears to counter the inhibition of nucleosomes bound to promoters *in vivo* is the *Drosophila* GAGA factor (Biggin and Tjian 1988; Soeller et al. 1993). Deletion of GAGA elements, GA repeats to which GAGA factor binds, results in the loss of nuclease hypersensitivity and transcription activity of *hsp26* (Lu et al. 1992). Purified GAGA protein has also been shown to bind to the GAGA elements of *hsp70* DNA that have been assembled into nucleosomes *in vitro* and concomitantly displace nucleosomes from the DNA (Tsukiyama et al. 1994). In addition, mutation of the GAGA elements of *hsp70* significantly reduces transcription activation *in vivo* (Lee et al. 1992). Heat shock gene transcription is rapidly activated upon heat shock presumably because GAGA factor is bound to promoters prior to induction and maintains a nuclease-hypersensitive, disrupted nucleosome configuration (O'Brien et al. 1995; C. Giardina and J.T. Lis, unpubl.).

The transcription of heat shock genes is activated by the heat shock transcription factor HSF. Upon heat shock, HSF molecules form trimers and bind cooperatively to conserved sequence elements in heat shock promoters, the heat shock elements (HSEs) (for review, see Lis and Wu 1993). HSEs are composed of arrays of five base pair modules, each of which binds an HSF monomer (Perisic et al. 1989). Once bound to these arrays, HSF rapidly stimulates transcription (Parker and Topol 1984; O'Brien and Lis 1993). *In vitro* experiments have shown that HSF binding is strongly inhibited by the assembly of HSEs into nucleosomes (Taylor et al. 1991). *In vivo*, the HSEs of most heat shock genes are cleared of nucleosomes and are readily accessible to HSF. Many factors other than GAGA factor are bound to heat shock promoters prior to heat shock, including transcriptionally paused RNA polymerase II located between 21 and 35 nucleotides downstream of the transcription start site (Gilmour and Lis 1986; Rougvie and Lis 1988; Giardina et al. 1992; Rasmussen and Lis 1995), the TBP-containing complex TFIID (Wu 1984; Giardina et al. 1992), and presumably most of the other basal transcription factors (for review, see Roeder 1991; Zawel and Reinberg 1993). Some or all of these factors might also influence chromatin structure and therefore influence HSE accessibility.

We examined the roles of several *hsp70* promoter regions in generating HSF-accessible HSEs *in vivo*. A variety of transgenic fly lines containing altered *hsp70* promoters were analyzed for their ability to bind HSF. HSF binding was detected by indirect immunofluorescence on the polytene chromosomes of larval salivary glands (Silver and Elgin 1976). Our analysis of transgenic polytene chromosomes stained with an HSF antibody indicate that at least three promoter sequences facilitate the

binding of HSF to chromatin, including binding sites for GAGA factor, TFIID, and RNA polymerase II. These data reveal a complex architecture of multiple, interdependent factors assembled on the *hsp70* promoter, the structure of which is critical to HSF binding *in vivo*.

Results

Indirect immunofluorescence with anti-HSF antibody

To understand which factors or combinations of factors are necessary to create a chromatin structure accessible to HSF, we examined transgenic *Drosophila* lines containing genes with alterations in key elements of the *hsp70* promoter (Lee et al. 1992). Polytene chromosomes from the salivary glands of transgenic larvae were analyzed by indirect immunofluorescence with an anti-*Drosophila* HSF antibody (anti-dHSF) generated in our lab. This antibody distributes on polytene chromosomes in a heat-dependent manner such that it is generally localized along each chromosome arm prior to heat shock (Fig. 1A) but specifically distributes to over one hundred distinct sites after heat shock (Fig. 1B). Immunoblot analysis with this antibody shows that it strongly recognizes *Drosophila* HSF (dHSF), which runs at 110 kD, and weakly detects an additional high molecular mass protein (running at ~180 kD) in *Drosophila* Kc cell extracts (Fig. 1C, lane 3). Neither band is present when identically prepared filters are probed with preimmune serum and the same secondary antibody (lane 2). The higher molecular weight band is not detected by an independently generated dHSF antibody (data not shown; Westwood et al. 1991). The immunofluorescence patterns shown in Figure 1, A and B, are virtually identical to patterns displayed by the independently generated dHSF antibody (Westwood et al. 1991). Furthermore, we do not detect any anti-dHSF signal at loci that contain genes with a mutated HSE (described below; see Fig. 3). Therefore, we conclude that our antibody is specifically recognizing HSF on salivary gland chromosomes and that the higher molecular mass band seen by immunoblot analysis does not contribute to the signals reported in this study.

We used our anti-dHSF antibody to determine the HSF accessibility of several variant heat shock promoters in transgenic fly lines. For these experiments, the chromosomal locations of each inserted gene were first mapped by *in situ* hybridization (data not shown). Then the patterns of HSF-dependent fluorescence at the identified loci were compared on chromosomes from transgenic and wild-type larvae. Figure 1D shows an example of indirect immunofluorescence on a transgenic chromosome containing the *hsp70-yp1* hybrid gene, *L1* (described in Fig. 2), which has two HSEs and is located at the 26F locus. The arrow indicates a relatively intense band at 26F that is not present on the wild type chromosome (Fig. 1E).

To quantitatively compare the amounts of HSF that bind to the modified heat shock promoters used in this study, we measured HSF-related fluorescence at trans-

Figure 1. Anti-dHSF antibody is specific and recognizes HSF bound to transgenic heat shock promoters. (A) Staining pattern of affinity-purified anti-dHSF antibody on unshocked salivary gland polytene chromosomes from an *Adh^{fno}, cn; ry⁵⁰²* (ACR) larva. (B) Anti-dHSF staining pattern on heat-shocked salivary gland chromosomes. (C) Immunoblot of *Drosophila* Kc cells probed with affinity-purified anti-dHSF antibody (lane 3) or rat preimmune serum (lane 2). Sizes of markers (lane 1) are indicated at left. HSF migrates with an apparent molecular mass of 110 kD. (D) Anti-dHSF staining pattern on a portion of chromosome 2L from the heat-shocked, transgenic line *L1B*. The site of *L1* insertion, 26F, is indicated with an arrow. *L1* is described in Fig. 2A. (E) Anti-dHSF staining pattern on chromosome 2L from a heat-shocked, wild-type (ACR) larva. The arrow indicates locus 26F.

genic loci. For this quantitative analysis, chromosomes stained with anti-dHSF were scanned with a confocal laser and digitized images were collected. The amounts of fluorescence signal on transgenic loci and a standard endogenous HSF binding site were readily measured from the digitized images. The locus used as a standard in this study is 87A, which contains 2 copies of *hsp70* per haploid genome. To directly compare different promoter variants and to minimize differences in staining from slide to slide, we generated larvae that were heterozygous for two different inserted genes and used their chromosomes for quantitative analysis. For each promoter construct, HSF fluorescence was measured in two independently generated fly lines. These lines exhibit levels of heat shock transcription and paused polymerase on a *yp1* reporter gene that are close to average measurements of several independent lines (Lee et al. 1992) and therefore do not appear to be greatly influenced by their positions in the genome.

To test the fluorescence response to the number of HSF-binding sites, we measured fluorescence signals from chromosomes that were either homozygous or heterozygous for the *L1* gene [described in Fig. 2A]. From >20 measurements, we found that the ratio of homozygous signal to heterozygous signal at the site of insertion is 1.6 ± 0.1 . Whereas this ratio is not the predicted 2.0, the fluorescence signal does appear to respond to HSE

number and thus provides a measure of relative HSF binding.

Both 5' and 3' regions of the *hsp70* promoter influence HSF-chromatin binding

To identify the regions of the heat shock promoter that are critical for HSF binding in vivo, we first examined genes with large portions of the *hsp70* promoter deleted (Lee et al. 1992). The *hsp70* promoter deletions are fused to a *yp1* reporter gene as illustrated in Figure 2A. The *Up2* gene contains *hsp70* sequences from -245 to -39 , which includes its entire complement of HSEs (numbered I, II, III, and IV in a 5' direction from the transcription start site) and GAGA elements. Not surprisingly, this gene stains relatively intensely with the HSF antibody (Fig. 2B). Deletion of the upstream portion of the *Up2* promoter to -89 generates the gene *Up1*. Although this deletion removes only half of the HSEs present in *Up2*, the *Up1* gene shows no detectable fluorescence (Fig. 2, B and C). The lower limit of detection in this immunofluorescence assay is at least six times lower than the signal we measured on *Up2*, indicating that the extra sequences in *Up2* have a more than additive effect on the binding of HSF. This effect is also reflected in levels of heat shock-induced transcription and amounts

Figure 2. The 5' and 3' regions of the *hsp70* promoter are critical for HSF binding in vivo. (A) Compositions of *Up2*, *Up1*, and *L1* genes are diagrammed at left. Portions of *hsp70* (bold line) were fused to the reporter gene, *yp1* (stippled rectangle). The arrow indicates the transcription start site. (T) TATA element, (G) GAGA element, (H) HSE, numbered I–IV above. Constructs are inserted between the *Rosy* gene and the P element (Ry or P in the open rectangle) in the transformation vector Cp20.1, a derivative of Carnegie 20. To the right of the diagrams, mean amounts of anti-dHSF fluorescence on *Up2*, *Up1*, or *L1*, +/– S.E.M. are reported as percent of fluorescence on one *hsp70* gene, which is equivalent to one-quarter of the fluorescence on the 87A heat shock puff. Reported measurements were made from chromosomes heterozygous for *Up2* and either *Up1* or *L1*. Measurements reported as <16 are below the limit of detection for this analysis as determined by measurements of the weakest staining band (see Materials and methods). (B) Anti-dHSF staining pattern on heat-shocked chromosomes heterozygous for *Up2A* and *Up1D*. The 87A locus is also indicated for comparison. (C) A twofold magnification of the chromosome region containing *Up1D*, taken from B, with no alterations in contrast levels. (D) Anti-dHSF staining pattern on chromosomes heterozygous for *Up2A* and *L1B*. (E) A twofold magnification of the region containing *L1B*, taken from D.

of paused RNA polymerase II on *Up1* and *Up2* (Table 1; reported previously in Lee et al. 1992).

Because HSF can bind DNA cooperatively, it is possible that the additional HSEs in *Up2* produce an HSF binding site with much higher affinity for HSF than that present in *Up1*. However, in vitro binding experiments

with the same portions of the *hsp70* promoter found in *Up2* and *Up1* have shown only a two-fold difference in HSF binding (Table 2). For these experiments, we used a mixed probe mobility-shift assay in which a limiting amount of HSF was added to a mixture of end-labeled DNA fragments derived from the *hsp70* promoter. The bound and free DNAs of the binding reaction were separated on a non-denaturing gel, isolated, and analyzed on

Table 1. Levels of paused RNA polymerase II and heat shock-induced transcription

Gene	Heat shock transcription (% <i>hsp70</i>)	Paused polymerase (% <i>hsp70</i>)
<i>Up2</i>	23	78
<i>Up1</i>	<0.2	<12
<i>L1</i>	23	67
<i>dmHSE-L1</i>	1	41
<i>mmGAGA-L1</i>	4	16
<i>L1+23</i>	6	19
<i>L1-12</i>	1	<12

Heat shock transcription levels for *hsp70-yp1* fusion genes and *hsp70* were determined by Northern analysis. Amounts of paused RNA polymerase II were determined by nuclear run-on assays. Both transcription and paused polymerase levels are reported as percent of signal from one copy of *hsp70* and were originally reported in Lee et al. (1992). The listed genes are described in Figs. 2–4.

Table 2. Relative Binding of HSF to *hsp70* promoter fragments in vitro

HSF binding sites	<i>hsp70</i> fragment	K_{relative}
HSE I–IV	–495 → –15	1.0
HSE I + II	–89 → +84	0.5
HSE I	–68 → +84	0.4
HSE II	–89 → –68	0.08
HSE I + II _{mmGA}	–89 → +62	0.25

Relative binding constants (K_{relative}) were determined for HSF binding in vitro to various fragments of the *hsp70* promoter, including the entire *hsp70* promoter (HSE I–IV), HSEs I and II together, HSE I alone, HSE II alone, and the *hsp70* portion of *mmGAGA-L1* (HSE I + II_{mmGA}), a gene that contains five point mutations in its GAGA element. Relative binding constants for a given fragment are reported as relative to the HSE I–IV fragment. Calculation of K_{relative} is described in Materials and methods.

a denaturing gel. Relative binding constants were calculated from measurements of amounts of each DNA fragment in the bound and free DNA pools (Liu-Johnson et al. 1986). Using this technique we determined that the relative binding constant for HSF binding to a fragment containing the *hsp70* portion of *Up2* versus the *hsp70* portion of *Up1* is approximately twofold whereas the difference measured in vivo by immunofluorescence is at least sixfold. This discrepancy suggests that the additional GAGA elements in *Up2* may play a significant role in facilitating HSF–chromatin interactions, as we will further demonstrate below.

Surprisingly, we find that sequences other than HSEs and GAGA elements can contribute to HSF's access to its binding sites in chromatin. This is demonstrated by HSF fluorescence on the gene *L1*. *L1* contains the same HSEs and GAGA element as the *Up1* gene (Fig. 2A). However, unlike *Up1*, *L1* also contains sequences from –39 to +62, including the transcription start site and a part of the leader of *hsp70*. Data reported previously has shown that *L1* is transcribed during heat shock at levels similar to *Up2* (summarized in Table 1). Similarly, *L1* shows relatively strong fluorescence with anti-dHSF, almost as much as *Up2*, indicating that the –39 to +62 region of *hsp70* also contributes significantly to HSF binding (Fig. 2D,E). This region does not contain any HSEs or GAGA elements, suggesting that several different regions and elements of the heat shock promoter can functionally compensate for each other to permit HSF to bind HSEs in vivo.

It is interesting to note that the measurements of HSF fluorescence on *Up2* and *L1* are greater than or equal to, respectively, the calculated amount on one copy of *hsp70* (Fig. 2A). *Up2* contains the same number of HSEs as *hsp70* but appears to have more HSF bound. Likewise, *L1* has half as many HSEs as *hsp70* but shows equal an amount of HSF fluorescence. The measurements of *hsp70* were obtained from the 87A locus, which contains 2 copies of the gene per chromosome homolog, whereas the *Up2* and *L1* genes were present in single copy on heterozygous chromosomes. Because we have seen non-linearity in fluorescence measurements when the number of genes at a locus is doubled (as in the comparison of *L1B* homozygotes and heterozygotes), the measurements of HSF on the 87A copies of *hsp70* are certainly under-represented with respect to single copies of *Up2* and *L1*. Furthermore, we might also be measuring differences in HSF association that result from specific positioning in chromosomes. For example, measurements of the individual lines of *Up2* differ by a factor of two (Fig. 2A). These measurements suggest that all sites of insertion are not the same, perhaps attributable to predefined amounts of chromatin packing or to the presence of other regulatory elements or enhancers that might facilitate or repress binding at transgenic promoters.

GAGA elements and HSEs are necessary for HSF binding

To pinpoint elements more clearly in the upstream portion of the *hsp70* promoter that contribute to HSF acces-

sibility, we examined another series of transgenic lines with specifically directed mutations in the chimeric heat shock promoters (Fig. 3A). All were derived from the *L1* construct that contains a minimal but functional heat shock promoter (Table 1; Lee et al. 1992). In the first *L1* mutation, HSE I was destroyed by two point mutations at highly conserved bases in the HSF binding motif that are critical to HSF binding in vitro (Fernandes et al. 1994). These mutations reduce heat shock-induced transcription >20-fold, although the formation of a paused polymerase is affected only minimally (Table 1). We do not detect any fluorescence over the loci into which this gene, *dmHSE-L1*, has inserted (Fig. 3A–C). As mentioned above, this result confirms that the fluorescence pattern we observe is HSE dependent. It also implies that HSE II in the context of *L1* is not sufficient on its own to bind HSF. HSE II contains only two 5 base pair units that closely match the HSF-binding consensus, whereas HSE I contains four. In vitro experiments measuring the relative binding of HSF to different binding sites have shown that HSE II has five times less affinity for HSF than HSE I (Table 2). This data agrees with previously published results from in vitro footprinting that showed that HSF binding to HSE II depends on cooperative interactions with HSF bound to the stronger HSE I (Topol et al. 1985).

We also examined the role of GAGA factor in HSF binding with a promoter containing multiple point mutations in the GAGA element of *L1* (*mmGAGA-L1*) (Fig. 3A). The mutations in this gene not only reduce paused polymerase and heat shock transcription levels more than fivefold (Table 1) but reduce HSF binding to nondetectable levels as well (Fig. 3D and E). However, these mutations alter the sequence of the overlapping HSE II. When measured in vitro, these mutations reduce HSF binding only by a factor of 2 (Table 2). In vivo the fluorescence signal is reduced at least sixfold suggesting that a functional GAGA element contributes significantly to HSF binding when the DNA is packaged into chromatin. We have also examined by indirect immunofluorescence an *hsp26* promoter lacking its two GAGA elements (Lu et al. 1993). Unlike *hsp70*, the HSEs and GAGA elements of *hsp26* do not overlap. Even though this gene has a full complement of intact HSEs, it has greatly reduced, but not abolished, HSF binding in vivo (data not shown). Because GAGA elements are necessary for the formation of nuclease-hypersensitive chromatin surrounding heat shock promoters (Lu et al. 1992), our results suggest that this open chromatin structure plays an important role in generating accessible HSEs in vivo.

The paused polymerase and transcription start site regions also influence HSF accessibility

Comparison of the *Up1* and *L1* genes described above (Fig. 2) indicates that the –39 to +62 region of the *hsp70* promoter also contributes to HSF binding. Using indirect immunofluorescence, we examined more carefully the importance of two portions of this downstream region for their effects on HSF binding. Sequences were deleted

Figure 3. The GAGA element and promoter-proximal HSE are required for HSF binding. (A) Schematic diagrams of *L1*, *dmHSE-L1*, and *mmGAGA-L1* are shown at left and drawn as in Fig. 2A. Crosshatching indicates mutated promoter elements. *dmHSE-L1* contains two-point mutations in HSE I, whereas the GAGA element of *mmGAGA-L1* contains five-point mutations. To the right of the diagrams is a summary of anti-dHSF fluorescence measurements on loci containing *L1*, *mmGAGA-L1*, and *dmHSE-L1* inserts in heat-shocked chromosomes that were heterozygous for *L1* and one of the other two genes. Measurements are reported as described in Fig. 2A. (B) Anti-dHSF staining pattern of heat-shocked chromosomes heterozygous for *L1B* and *dmHSE-L1 4*. The 87A locus is also indicated. (C) A twofold magnification of the chromosome region containing the *dmHSE-L1 4* insert shown in B. (D) Anti-dHSF staining pattern on chromosomes heterozygous for *mmGAGA-L1 1* and *L1B* with locus 87A also indicated. (E) A twofold magnification of the chromosome region containing the *mmGAGA-L1 1* insert taken from D.

from the 3' end of the *hsp70* portion of *L1* to either +23 or -12 to generate *L1+23* and *L1-12*, respectively (Fig. 4A). The *L1+23* gene lacks *hsp70* sequences from +23 to +62, which is the region where the paused polymerase complex is found (Giardina et al. 1992; Rasmussen and Lis 1995). *L1+23* shows a threefold reduction in HSF-related fluorescence (Fig. 4B), similar to the fourfold reduction in pausing RNA polymerase II previously reported (Table 1). In contrast, *L1-12* contains a 3' deletion to -12 and so is additionally lacking the *hsp70* transcription start site. We observe no HSF binding at the sites of insertion in two independently generated *L1-12* lines (Fig. 4D,E). However, an HSF band at a third site was detected over background (data not shown). We believe that this anomalous signal is attributable to a position effect in which the inserted gene has activated a previously masked HSE in the surrounding genomic sequences. This explanation is supported by the facts that this third site is not transcriptionally active, as determined by Northern blotting, and that no protection is observed on the HSEs of this inserted gene when analyzed by in vivo DNase I footprinting (data not shown).

Whereas the indirect immunofluorescence assay affords high sensitivity and the ability to quantify relative amounts of factor binding, the level of resolution is low, tens of kilobases at best. To confirm our immunofluorescence observations, we reexamined a subset of the chimeric genes at high resolution by in vivo DNase I footprinting (Fig. 5). Protection from DNase I cleavage at

both HSEs of *L1* is observed when HSF is added to *L1* DNA in vitro (asterisks, Fig. 5A) and in nuclei from heat-shocked flies (Fig. 5B, cf. lanes 3 and 4). In contrast, the HSEs of *L1-12* are not protected either prior to or during heat shock (Fig. 5C, lanes 3,4). The lack of footprint on *L1-12* is not because of poor nuclei preparation, as the endogenous *hsp70* promoter in DNA from the same *L1-12* nuclei shows a heat shock-dependent footprint (Fig. 5D).

The patterns of nuclease digestion of *hsp70*, *L1*, and *L1-12* prior to heat shock are not identical, particularly in the region of HSE I. These minor differences are reproducible and reflect a successive decrease in overall sensitivity to DNase I with successive deletions of the *hsp70* promoter. Why this decrease in sensitivity is most apparent in HSE I remains unclear. An intermediate level of nuclease sensitivity and heat shock-dependent protection is observed over both HSEs of the *L1+23* gene (data not shown). When primers of the opposite strand (transcribed strand) are used in the LMPCR reactions (data not shown), the levels of HSE protection on *L1*, *L1+23*, and *hsp70* are similar to those shown in Figure 5. These footprinting results are reproducible and are consistent with the indirect immunofluorescence data. Our observations with the *L1+23* and *L1-12* genes suggest that factors on the pause region and on or near the transcription start site, namely the transcriptionally engaged RNA polymerase II and remaining preinitiation complex proteins, are critical for HSF to access the promoter.

Figure 4. The pause region and transcription start site of *hsp70* influence HSF binding. (A) Schematic diagrams of *L1*, *L1+23*, and *L1-12* transgenes are shown at left. The *hsp70* portion of *L1* was deleted from the 3' end to either +23 or -12 to create *L1+23* and *L1-12*, respectively. The rest of the diagrams are as in Fig. 2A. To the right of diagrams is a summary of anti-dHSF fluorescence measurements on *L1*, *L1+23*, and *L1-12*. All measurements were taken from chromosomes of heat-shocked larvae that were heterozygous for *L1* and either *L1+23* or *L1-12*. Measurements are reported as described in Fig. 2A. (B) Anti-dHSF staining pattern on heat-shocked chromosomes heterozygous for *L1B* and *L1+23 D-7*. The 87A heat shock locus is also indicated. (C) A twofold magnification of the region containing the *L1+23 D-7* insert, taken from B. (D) Anti-dHSF staining pattern on chromosomes heterozygous for *L1-12 D* and *L1B* with the 87A locus also indicated. (E) A twofold magnification of the *L1-12 D* insert taken from D.

The transcription start site and paused polymerase regions of *hsp70* enhance TFIID binding in vivo

The dependence of HSF binding on the paused polymerase region and transcription start site sequences might be explained by studies that have demonstrated that a purified *Drosophila* TFIID complex contacts sequences of the *hsp70* promoter downstream of the TATA element to approximately +35 and that specific contacts within this region (from -2 to +2 and at +17, +18, +19, +28, and +31) are necessary for the TFIID complex to stably associate with the promoter (Emanuel and Gilmour 1993; Purnell et al. 1994; Verrijzer et al. 1995). This data led us to hypothesize that the effects of the 3' deletions on HSF binding might in fact be linked to a reduction in TFIID on the *L1+23* and *L1-12* promoters. To test this hypothesis, we examined the occupancy of the TATA elements in the *L1*, *L1+23*, and *L1-12* genes by in vivo footprinting. Because treatment of nuclei with DNase I did not produce enough nicks in TATA element DNA to indicate a footprint (Fig. 5), we instead used KMnO_4 as a DNA modifying agent (Rubin and Schmid 1980; Sasse-Dwight and Gralla 1989). KMnO_4 modifies thymines in single-stranded and strained double-stranded DNA. Previous work has shown that two of the thymines in the *hsp70* TATA element of purified DNA are hypersensitive to KMnO_4 but are protected from modification in treated fly nuclei or cultured cells (Giardina et al. 1992; C. Giardina and J.T. Lis, unpubl.). We

attribute this in vivo protection to TBP binding since an identical pattern of protection was produced when purified TBP was bound to DNA in vitro (Giardina et al. 1992). In Figure 6A, the indicated hypersensitive sites in the TATA element of *L1* (lane 2, denoted by asterisks) are protected in nuclei (cf. lanes 2 and 4). These sites are less protected in *L1+23* nuclei (lanes 6,8) and appear completely unprotected in *L1-12* nuclei (lanes 10,12). The results shown in Figure 6A indicate that alterations of some of the key nucleotides in the *hsp70* start site and leader sequences reduce TBP binding, and presumably TFIID binding, in vivo. Decreased amounts of TFIID would in turn result in a loss of RNA polymerase II recruited to the promoter. Furthermore, the loss of one or more of these components appears to reduce levels of HSF binding as described above (Figs. 4 and 5).

GAGA elements are essential for the binding of TBP to *hsp70* in vivo

Because the binding of TFIID appears to be crucial for subsequent HSF binding, we investigated whether the GAGA element influences TBP-promoter association. Figure 6B shows a KMnO_4 footprinting experiment on the *mmGAGA-L1* gene. The reactive thymines of the TATA element in naked DNA are indicated by asterisks. These nucleotides are not protected in DNA from KMnO_4 -treated *mmGAGA-L1* nuclei (cf. lanes 2 and 4).

Figure 5. HSE occupancy of *L1*, *L1-12*, and *hsp70* genes in intact nuclei. (A) HSF binds to the HSEs of *L1* in vitro. The *L1* promoter DNA fragment in the presence (lane 2) or absence (lane 1) of HSF, subsequently digested with DNase I is shown. Brackets and asterisks indicate nucleotides in HSEs that are protected from DNase I by the addition of HSF. The *hsp70* portion of *L1* is diagrammed (far left) as in Fig. 2A. (B) The HSEs of *L1* are occupied in vivo, as seen in DNase I digestion patterns of *L1* DNA from heat-shocked and non-heat-shocked *L1A* nuclei. DNAs were amplified with primers matching the Ry marker and polylinker sequences in the transformation vector to specifically view the *L1* gene. (C) No footprint is observed on *L1-12* HSEs during heat shock. DNAs from nuclease-treated, heat-shocked and non-heat-shocked *L1-12E* nuclei were amplified with Ry primers to view the *L1-12* inserted gene. (D) The HSEs of *hsp70* are oc-

cupied in *L1-12E* flies. The same DNA samples shown in C were amplified with *hsp70*-specific primers. For B–D: (Lanes 1–3) Nuclei prepared from non-heat-shocked adult flies; (lanes 4–6) nuclei from heat-shocked flies. (Lanes 1,6) naked DNA digested with 3.2 Worthington units of DNase I; (lanes 2,5) untreated nuclei; (lanes 3,4) nuclei treated with 9.6 units of DNase I; (Lane 7) G-ladder of *L1-12* DNA.

These data indicate that at least one GAGA element is required for TBP to access the TATA element of *hsp70*. We infer that TBP is unable to bind to the TATA element in the absence of GAGA factor because the promoter-containing chromatin has not been sufficiently opened.

The binding of TBP may be necessary but not sufficient for HSF access

We examined the *Up2* and *Up1* genes by KMnO_4 footprinting to further test our observation that GAGA elements influence TBP binding in vivo. The additional sequence elements in *Up2* that are not contained in *Up1*, namely extra GAGA elements and HSEs, appear to compensate for the loss of the transcription start and paused polymerase regions in terms of HSF binding (Fig. 2). Data reported previously show that *Up1* is not transcriptionally active during heat shock, whereas *Up2* is strongly transcribed (Table 1). Interestingly, we find that the TATA sequences of both *Up1* and *Up2* genes appear to be protected from KMnO_4 modification (Fig. 6C, lanes 2 and 4 and lanes 6 and 8, respectively). One reactive thymine in the TATA region of *Up1* appears to be protected at an intermediate level, similar to that seen for the *L1+23* gene (Fig. 6A, lanes 6,8). However, unlike *L1+23*, *Up1* does not detectably bind HSF, does not contain detectable paused RNA polymerase II, nor is it efficiently expressed during heat shock. Taken together with these observations, our footprinting data suggest that perhaps a TBP-containing complex is bound to *Up1* but is probably unable to recruit RNA polymerase II to the promoter. Furthermore, because *Up1* does not appear

to bind HSF (Fig. 2), we infer that the paused polymerase might play a key role in generating accessible HSEs.

In contrast to *Up1*, the TATA element of the *Up2* promoter appears more completely protected from KMnO_4 modification. The *Up2* protection pattern is qualitatively different from that of *Up1*, as a different thymine within the TATA element is protected and the protection extends beyond the TATA element toward the start of the gene (denoted by asterisks, Fig. 6C). Because *Up1* and *Up2* contain the same TATA element and leader sequences of *yp1* and because *Up2* has a paused polymerase and is efficiently expressed during heat shock (Lee et al. 1991), our footprinting data imply that the additional GAGA elements in *Up2* serve to direct a transcriptionally competent TBP complex to the promoter that then facilitates the recruitment of RNA polymerase II, thus generating the more extensive footprint shown in Figure 6C. This complex *Up2* promoter architecture is accessible to HSF, unlike the *Up1* promoter (Fig. 2).

Discussion

Through examinations of transgenic constructs with mutations and deletions in several regions of the *hsp70* promoter by indirect immunofluorescence, we identified promoter elements that play a role in allowing HSF to access HSEs in vivo. We found that the GAGA element, a region containing the paused polymerase, and a region containing the transcription start site all influence HSF binding to varying degrees. We also demonstrated that the loss of the paused polymerase and transcription start

Figure 6. TBP binding in vivo depends on downstream promoter sequences and an intact GAGA element. (A) KMnO₄ footprinting of *L1*, *L1-12*, and *L1+23* nuclei. These LM PCR products were generated with primers matching the coding strand of the *Ry* marker gene and indicate modified T's in the opposite strand. Asterisks denote the KMnO₄ hypersensitive T's in the TATA element at -28 and -32 that are protected by the binding of TBP. (Lanes 1–4) DNA from *L1B* nuclei; (lanes 5–8) DNA from *ND L1+23*; (lanes 9–12) DNA from *L1-12E*. (Lanes 1,5,9) Naked DNA treated with DMS and piperidine to indicate positions of G's in the transcribed strand; (lanes 2,6,10) naked DNA treated with KMnO₄ to show positions of reactive T's in the transcribed strand; (lanes 3,7,11) untreated nuclei; (lanes 4,8,12) KMnO₄-treated nuclei. (B) KMnO₄ footprinting of the *mmGAGA-L1* gene. DNA was isolated from KMnO₄-treated nuclei of *mmGAGA-L1 2-5b* flies and used in LM PCR reactions with *Ry* primers. Asterisks indicate hyperreactive T's of the TATA element. Lanes 1–4 are arranged as in A. (C) Occupancy of *Up2* and *Up1* TATA elements. KMnO₄ footprinting with *Up2A* flies was performed as described in Materials and methods followed by gel electrophoresis purification of *Up2* restriction fragments away from *hsp70* DNA. The purified *Up2* fragments were then used in LM PCR reactions with coding strand primers matching the *hsp70* portion of the *Up2* gene. DNAs from *Up1D* flies were not gel purified and were amplified with *Ry* primers. Lanes 1–4 and 5–8 are arranged as in A. Asterisks indicate the protected T's in or near the TATA element.

regions reduces TBP binding on the *hsp70* TATA element. These data suggest that the TBP-containing complex, TFIID, might play a significant role in establishing HSF-accessible chromatin. However, we also observed occupancy of the TATA element of a gene that does not bind HSF (*Up1*), suggesting that the presence of TBP is necessary but not sufficient for establishing accessible HSEs. This same *Up1* gene also does not generate paused RNA polymerase II. This implies further that the presence of paused polymerase is key to forming an open and HSF-activatable heat shock promoter.

Our data were generated with the use of chimeric genes that have been reintroduced into the *Drosophila* genome at random. It is possible that some of their characteristics might be influenced both by their position in the genome and their somewhat artificial sequence composition. By examining at least two transgenic lines for each gene, we have been able to identify the effects of genomic positioning. The effects of changing native heat shock promoter sequence, however, are more difficult to control. For example, deletions of the upstream or downstream regions of the promoter might serve to bring a

nucleosome positioned on either the flanking *ry* or *yp1* genes closer to the HSEs and TATA element. We have taken two approaches to minimize such effects. First, we examined additional genes with only minor changes to some of the predicted critical promoter elements (such as the five point mutations in *mmGAGA-L1*) and find that their behaviors agree with the more significantly altered genes (*Up1* and *Up2*). Second, when constructing genes with deletions in the downstream regions of the heat shock promoter, we were careful to maintain spacing between the *hsp70* and *yp1* sequences. For example, in *L1-12* the *hsp70* promoter sequence ends at -12 and is fused to the 5' end of *yp1* starting at -13.

The conclusions we have drawn from our study of these chimeric genes are summarized in Figure 7. We propose that compact chromatin (Fig. 7A) is wedged open by the binding of GAGA factor at conserved sites within a heat shock promoter (Fig. 7B). Once the chromatin has been sufficiently opened, a TFIID complex can bind and is stabilized by contacts with nucleotides in the start and leader of the gene (Fig. 7C). The formation of a stable TFIID-promoter complex is followed by the assembly of

Figure 7. A model depicting the formation of an HSF-accessible heat shock promoter structure. Prior to heat shock, nucleosomes are displaced from compact chromatin (A) by GAGA factor when it binds to GAGA elements in a heat shock promoter (B). This opening of chromatin enables other factors to bind the promoter including TFIID, RNA polymerase II, and other general transcription factors required to form a transcription complex. RNA polymerase II then synthesizes a short RNA and repositions itself in the pause region (C). When all factors are in place, a structure is formed in which the HSEs are accessible to HSF. Upon heat shock (D), HSF molecules form trimers and readily bind prepared HSEs.

a complete transcription complex. In the case of the uninduced *Drosophila* heat shock genes, RNA polymerase II not only binds to the start site, but begins to transcribe a short RNA and waits at the pause site. This binding and repositioning of the polymerase completes a structure on the promoter in which the HSEs are fully exposed, so that, upon heat shock, HSF can easily bind to the HSEs and rapidly activate transcription (Fig. 7D).

The results reported here suggest that the displacement of nucleosomes by GAGA factor is paramount to building a potentiated heat shock promoter. We have demonstrated that a GAGA element is necessary for TBP to bind to the TATA element of *hsp70* in vivo, the first step in transcription complex assembly. Presumably GAGA factor must first bind to DNA and displace nu-

cleosomes that cover the TATA element. Previous work has demonstrated that TBP alone has a very low affinity for TATA elements that are assembled into nucleosomes (Imbalzano et al. 1994). In addition to binding at the TATA element, our investigation of the *L1+23* and *L1-12* genes revealed that strong TBP binding to the *hsp70* TATA element in vivo depends not only on GAGA binding sites, but on the presence of specific sequences at the start site and in the pause polymerase region as well. Stable binding of purified TFIID to the *hsp70* promoter in vitro depends on the presence of specific nucleotides in these downstream sequences (Purnell et al. 1994). Stable binding of a TFIID complex appears to be critical for generating a paused RNA polymerase II, as deletion of the transcription start and pausing regions results in the concomitant loss of TFIID and paused polymerase (see above; Lee et al. 1992).

We have uncovered a case where TBP appears to be bound to a gene that does not have paused polymerase, and, in fact, is not detectably transcribed during heat shock. This gene, *Up1*, is composed of the *yp1* TATA element, transcription start site, and sequences downstream. We infer from our data that perhaps a variant form of TFIID has bound to *Up1*, one which is defined by *yp1* TATA and downstream sequences and cannot support the formation of a complete transcription apparatus. Alternative TBP-containing complexes, such as SNAP_c, have been previously documented (Sadowski et al. 1993). Furthermore, it has been shown that the selection of a particular TBP complex that will bind to a promoter depends both on promoter sequence and other transcription factors associated with that promoter (Das et al. 1995; Verrijzer et al. 1995). Thus it is possible that the addition of extra GAGA elements to the *yp1* TATA element and downstream sequences, as found in *Up2*, serves to direct the binding of a form of TFIID that subsequently allows the assembly of general transcription factors and a paused RNA polymerase II. However, the footprints on the TATA elements of *Up1* and *Up2* (Fig. 6C) must be interpreted with caution, as they are derived from the *yp1* gene. The interaction of TBP with the *yp1* TATA element has not been characterized as thoroughly as this interaction with the *hsp70* TATA (Giardina et al. 1992). Nonetheless, the strong protection that we observe on the *Up2* TATA is expected given the previously measured levels of expression and of paused polymerase (Table 1).

The paused polymerase could affect the formation of accessible HSEs in a number of ways. Its presence might result in further opening up chromatin structure at the promoter. This explanation is supported by recently published experiments with a transgenic fly line carrying a mutated TATA element in the *hsp26* promoter. These experiments showed that a functional TATA element, which directs the assembly of the entire transcription complex, contributes modestly to the promoter's hypersensitivity to nuclease digestion (Lu et al. 1994). Probably the large complexes of both TFIID and RNA polymerase II proteins cause further rearrangement of nucleosomes and other chromatin-bound factors. Likewise,

specific contacts between the polymerase and HSF might also serve to guide HSF onto its binding sites. However, since purified HSF has a high affinity for HSEs and does not require ancillary factors to bind HSEs in vitro (Parker and Topol 1984; Clos et al. 1990), it is likely that the most important function of the complex structure formed on the promoter prior to heat shock is to open chromatin and expose the DNA sequences that contain heat shock elements.

Materials and methods

Fly lines

Generation of *Up1*, *Up2*, *L1*, *dmHSE-L1*, *mmGAGA-L1*, *L1+23*, and *L1-12* fly lines was reported previously (Lee et al. 1992). All stocks were maintained at 24°C. Indicated samples were heat treated for 30 min at 36.5°C.

Generation of anti-dHSF antibody

Drosophila HSF was overproduced as a glutathione *S*-transferase (GST) fusion protein (GST-dHSF) in *Escherichia coli*. Purification was performed essentially as described (Smith and Johnson 1988) except for the following modifications: Cells from a 50-ml overnight culture were induced with 1 mM IPTG for 3–5 hr, pelleted, and resuspended in 10 ml of buffer A (20 mM Tris at pH 7.4, 0.2 mM EDTA, 1.0 mM DTT, 0.5 mM PMSF, 1.0 M NaCl) for sonication; extract was adsorbed to glutathione-agarose beads and washed twice with 20 ml buffer A, twice with 20 ml buffer B (20 mM Tris at pH 7.4, 0.2 mM EDTA, 0.1 mM NaCl); the fusion protein was eluted in three 1-ml washes with buffer C (buffer B containing 5 mM glutathione).

Anti-dHSF antibody was generated in rats against GST-dHSF and purified over a GST-dHSF-Sepharose affinity column according to manufacturer's directions (CNBr-activated Sepharose 4B, Pharmacia), resulting in a fourfold dilution of HSF-recognizing antibodies as compared to crude serum. Western blots of *Drosophila* Kc cells were probed with a 1:2500 dilution of affinity-purified antibody or a 1:10000 dilution of preimmune sera. Kc cells were collected, resuspended in SDS-loading dye, boiled, and proteins were separated by SDS-PAGE (10% acrylamide). Proteins were transferred to nitrocellulose and detected by chemiluminescence according to manufacturer's directions (ECL, Amersham) except that the filter was blocked and probed in 1% gelatin, 10% calf serum, in TBS (10 mM Tris at pH 7.5, 150 mM NaCl). A 1:10000 dilution of peroxidase-conjugated anti-rat goat IgG was used for secondary antibody probing.

Indirect immunofluorescence

Salivary glands from third-instar larvae were dissected, fixed, squashed, and stained as described (Champlin et al. 1991), with the following modifications: The final fixation solution consisted of 50% acetic acid, 3.7% formaldehyde. Slides of fixed, squashed glands were stained with a 1:100 dilution of rat anti-dHSF in TBS, 1% fetal bovine serum (FBS) for 1 hr in a moist chamber, washed, stained with a 1:200 dilution of rhodamine-conjugated rabbit anti-rat IgG (Sigma) in TBS, 1% FBS, for 1 hr, washed again, and then stained further with 1 µg/ml of Hoechst 33258 (Sigma) in TBS for 10 min. Slides were examined under a 63× Zeiss Planapo objective attached to a Zeiss Universal fluorescence microscope.

Imaging and quantitation of fluorescence

Digitized images were acquired with a Zeiss Axiovert microscope coupled to a Bio-Rad MD600 laser scanner. Single-section images were generated with a wide PMT aperture setting (1.8) and three accumulated scans. All quantitation of images was accomplished with NIH *Image* 1.52b2. Unless otherwise noted, quantitated chromosomes were heterozygous for two transgenic loci to directly compare the genes on the same spreads, thereby reducing variability that might arise from slide to slide. Fluorescence measurements of transgenic loci were standardized to measurements of 87A, which contains four copies of *hsp70* per diploid genome (Ish-Horowicz et al. 1979). At least 23 background measurements were obtained in a similar fashion from the same loci of nontransformed, heat-shocked chromosomes (*Adh^{tm6}*, *cn*; *ry⁵⁰²* parental strain). The means of the background measurements relative to one copy of *hsp70* were subtracted from the means of signals from corresponding transgenic loci, also relative to one *hsp70* gene. These calculated values are reported in Figures 2–4 as mean fluorescence. To determine the lower limit of measurable fluorescence, we measured signal from the weakest observable endogenous band on chromosome arm 3R (at locus 88F) on 25 different samples and calculated the mean HSF fluorescence signal at that locus relative to one copy of *hsp70* as described above. This value is 16 ± 1.6 . Any measurements of mean fluorescence at transgenic loci that were lower than this measured limit of detection are reported as <16.

In situ hybridization

Larval salivary gland squashes for in situ hybridization were prepared as described in Lis et al. (1978), except larvae were not heat shocked. The plasmid HiDev-pXTd3, a derivative of *L1* (called *L2*) cloned into the Carnegie 20 transformation vector (Lee et al. 1992), was used as a probe. HiDev-pXTd3 was labeled by random priming in the presence of biotin-16-dUTP (Enzo Diagnostics) and absence of dTTP. Denatured slides were incubated with ~10 ng of denatured probe in 10 µl of hybridization buffer (50% formamide, 2× SSC, 10% sodium dextran sulfate, 0.5 mg/ml of denatured salmon sperm DNA) overnight at 37°C. Slides were washed twice in 0.05% Triton X-100, PBS, once in 0.1× SSC for 10 min at 55°C, and again in 0.05% Triton, PBS, for 5 min at room temperature. Biotinylated probe was detected according to directions for the Enzo System Detection Kit (Enzo Diagnostics), incubating slides first in the specified dilution of streptavidin-peroxidase followed by incubation in diaminobenzidine solution. Chromosomes were stained with 2% Giemsa in 10 mM sodium phosphate. Slides were mounted in Permount (Sigma) and examined by phase-contrast microscopy with a Zeiss 25× Plan-Neofluar objective.

In vitro analysis of relative binding constants

Analysis of relative binding constants was carried out as reported previously (Xiao et al. 1991). Briefly, a limiting molar amount of purified, baculovirus-expressed *Drosophila* HSF (Fernandes et al. 1994) was mixed with approximately equimolar amounts of pooled DNA fragments and allowed to bind for 16 hr at 24°C. DNA fragments were labeled at the 5' end on one strand using Klenow polymerase and purified by electrophoresis on low-melt agarose gels as described (Sambrook et al. 1989). To generate a fragment containing the entire *hsp70* promoter, an *AccI*-*SnaBI* fragment from plasmid aDm2.39 (Hackett 1985) was end-labeled with [α -³²P]dCTP; for the *hsp70* HSE I and HSE II, an *EcoRI*-*PstI* fragment from plasmid pXT1 (Xiao and Lis

1986) was end-labeled with [α - 32 P]dATP; for the *hsp70* HSE I only, a *Bss*HII–*Pst*I fragment from plasmid pXT1 was end-labeled with [α - 32 P]dCTP; for the *hsp70* HSE II only, a *Bss*HII–*Pvu*II fragment from plasmid pXT1 was end-labeled with [α - 32 P]dCTP; for the regulatory region of *mmGAGA-L1*, an *Eco*RI–*Pvu*II fragment from the *mmGAGA-L1* plasmid (Lee et al. 1992) was end-labeled with [α - 32 P]dATP. Binding reactions were run on a nondenaturing agarose gel to separate bound and free DNAs. Bands of bound and free DNA were cut from the gel and DNAs extracted. Bound and free DNA pools were then analyzed on a denaturing sequencing gel. Relative binding constants were calculated from amounts of each fragment in the bound and free DNA pools as measured on the denaturing gel. For fragments 1 and 2, the relative binding constant (K_{relative}) = $K_1/K_2 = (B_1/F_1)/(B_2/F_2)$ (Liu-Johnson et al. 1986).

DNase I footprinting in fly nuclei

Nuclei were prepared from 0.6–0.8 gram of heat-shocked or non-heat-shocked adult flies as described (Lee et al. 1992) with the following modifications. Fly homogenization buffer (buffer A) consisted of 0.3 M sucrose, 60 mM KCl, 15 mM NaCl, 15 mM Tris (pH 7.4), 1 mM EDTA, 0.1 mM EGTA, 0.5 mM DTT, 0.1 mM PMSF, and 0.3% Triton X-100. After homogenization and filtration through 35 μ m nylon mesh, the homogenate was mixed with an equal volume of cold buffer A' (same as buffer A, but with 1.75 M sucrose and no Triton X-100) and spun through a sucrose gradient.

The final nuclear pellet was resuspended in DNase I buffer (60 mM KCl, 15 mM NaCl, 15 mM Tris-Cl at pH 7.9, 0.25 M sucrose, 3 mM MgCl₂, 0.05 mM DTT) to 500 μ l, divided into 100- μ l aliquots, and digested with 0 or 9.6 units DNase I (Worthington Biochemical) for 30 sec at 4°C. EDTA was added to 10 mM to stop digestion, nuclei were pelleted, reaction buffer removed, and pellet resuspended in sarkosyl lysis buffer (50 mM Tris-Cl at pH 7.9, 100 mM EDTA, 0.5% Sarkosyl). DNA was purified by proteinase K digestion (50 μ g/sample) at 37°C for 2 hr followed by multiple (usually five) phenol extractions and RNase A digestion. A portion (2 μ g) of the recovered DNA was digested with *Sal*I. Naked DNA was generated by digesting 2 μ g of DNA from untreated nuclei (resuspended in 100 μ l DNase I buffer after restriction) with 3.2 units DNase I on ice for 30 sec. Reactions were stopped as before, mixed with 300 μ l of Sarkosyl lysis buffer, digested with 50 μ g of proteinase K for 1 hr at 37°C, and extracted twice with phenol–chloroform.

All samples were then amplified and labeled by ligation-mediated PCR (LM PCR) as described (Mueller and Wold 1989), with the following modifications. *Taq* polymerase and buffer were purchased from GIBCO–BRL, Life Technologies. For amplification of transgenic DNA, we used primers Ry1T (5'-GT-TGAGCAAGTTTTCGGATGAATTG), which is complementary to the transcribed strand of *Rosy* from 83 to 58 nucleotides upstream of the *Hind*III site that is fused to inserts in the Cp20.1 transformation vector (Simm et al. 1985), and Ry3T (5'-GCT-CAATCAAAGAAGCTTGGCTGCAGGTCGAGG), which is complementary to the last 15 nucleotides of Ry and 19 nucleotides of polylinker sequence in Cp20.1. Ry1T was annealed at 56°C prior to the Sequenase reaction and during the first 18 rounds of amplification in an automatic thermocycler with the following program: cycle 1, 95°C for 3.5 min; 56°C for 2 min; 72°C for 3 min; cycles 2–18, 95°C for 1 min; annealing and elongation steps as in cycle 1. End-labeled Ry3T was subsequently added to PCR reactions that were then cycled six times as before, except that Ry3T was annealed at 70°C instead of 56°C. Two different primers (c70-161, 5'-TCTCTTTTTT-TGGGTCTCTCCC, and c70-141, 5'-CTCCCTCTCTGCAC-

TAATGCTCTCTCACTC) were used for footprinting analysis of *hsp70*. These primers are complementary to the transcribed strand of the 3 copies of *hsp70* in 87C starting at –161 and –141, respectively, and were annealed at 56°C and 70°C, respectively, under the same LM PCR conditions used with Ry1T and Ry3T. DNA used in *hsp70* footprinting was restriction cut with *Alu*I instead of *Sal*I. PCR products were purified and analyzed on 6% polyacrylamide/7 M urea sequencing gels. G-ladders were generated by modifying *L1–12* genomic DNA with DMS, cleaving with piperidine (Sambrook et al. 1989), and sub-jecting fragments to LM PCR as described above.

DNase I footprinting in vitro

An *L1* DNA fragment was generated by PCR amplification of *L1B* genomic DNA with the primers Ry1T and Yp1D2 (5'-ACG-GAGTTGTCCATACGGCCATTG). Binding reactions with 100 ng of PCR product and 1 mg of an HSF–maltose binding protein fusion proceeded as described (O'Brien et al. 1995) and were subsequently treated with 0 or 0.6 units DNase I for 30 sec at room temperature followed by the addition of an equal volume of stop buffer (25 mM EDTA, 1% SDS). DNAs were purified by treating with 20 μ g of proteinase K for 60 min at 60°C followed by phenol extractions. Recovered DNAs were used for primer extensions, following the same steps in the Sequenase reaction of the LM PCR protocol (Mueller and Wold 1989) and using kinase-labeled Ry3T as primer that was annealed at 62°C. Products were then analyzed on a 6% polyacrylamide, 7 M urea sequencing gel.

KMnO₄ footprinting in fly nuclei and extracts

For *L1*, *L1+23*, *L1–12*, and *mmGAGA-L1*, nuclei preparation, DNA purification and LM PCR with Ry1T and Ry3T primers were the same as in DNase I footprinting described above. KMnO₄ and piperidine treatment of nuclei and DNA proceeded essentially as in Giardina et al. (1992).

For *Up1* and *Up2*, flies were collected and homogenized in a Sorvall Omni-mixer at maximum speed on ice with buffer A (used in nuclei preparation for DNase I footprinting). Extracts were filtered successively through 100- and 35- μ m nylon mesh and then treated with KMnO₄ (final concentration 25 mM) or H₂O for 30 sec on ice. Reactions were stopped with an equal volume of stop solution (50 mM EDTA, 1% SDS, 0.4 M β -mercaptoethanol). DNAs were purified as described above in DNase I footprinting in fly nuclei. Half of the H₂O-treated DNA samples were modified with KMnO₄ for “naked DNA” experiments. All *Up1* DNA samples were then cleaved with piperidine and used in LM PCR reactions with primers Ry1T and Ry3T. Purified *Up2* DNAs were first digested with *Eco*RI and *Sty*I and separated on a 1.2% agarose gel. Fragments ~350 bp long were purified from the gel (Quiax II DNA purification kit, Quiagen) to separate *Up2* containing DNA from endogenous *hsp70* sequences. Half of the mock H₂O-treated DNA fragments were then modified with KMnO₄ for naked DNA samples. *UP2* DNAs were reacted subsequently with piperidine and used in LM PCR reactions with specific primers a70-161(TCTCTAT-TCGTTTTGTGACTCTCCC) and c70-141 (see above).

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HSF access to heat shock elements in vivo depends critically on promoter architecture defined by GAGA factor, TFIID, and RNA polymerase II binding sites.

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