DNA sequence requirements for generating paused polymerase at the start of hsp70

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RNA polymerase II is transcriptionally engaged but paused ~25 nucleotides from the start site of the hsp70 gene of Drosophila melanogaster in uninduced (non-heat-shocked) flies. Here, we identify regions of the hsp70 promoter that are required for formation of this paused polymerase. Various hsp70 promoter sequences are substituted for promoter sequences of a yolk protein gene, ypl, which, in males, is normally not expressed and has no paused polymerase. Run-on assays with nuclei of male transgenic flies are used to measure the level of paused polymerase on the hybrid genes. Sequences that reside upstream of the hsp70 TATA element, when fused upstream of the ypl TATA element, specify the formation of a paused polymerase on the 5' end of this hybrid gene. Within this region are multiple copies of the GAGA element, which is known to bind a constitutively expressed factor. This element appears to play a role in generating the pause. Also, in the absence of much of this upstream region, hsp70 sequences in the vicinity of the transcriptional start and pause site participate in specifying the pause. Deletions of the pause site reduce the level of paused polymerase but do not lead to constitutive transcription. However, a connection between transcription and pausing is seen. The level of paused polymerase on the various hybrid hsp70–ypl promoters correlates with the promoter's potential to direct heat-induced transcription.

[Key Words: RNA polymerase pausing, transcriptional regulation, heat shock genes, GAGA factor, promoter, Drosophila]

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Heat shock treatments of Drosophila cells trigger a rapid and dramatic increase in the transcription of the hsp70 gene. This upsurge in transcription is detectable within several tens of seconds and the shift to the fully induced level of transcription is complete within 3 min [Belyaeva and Zhimulev 1975; T. O'Brien, E. Wong, and J.T. Lis, unpubl.]. During this induction, the density of transcribing RNA polymerase II on the hsp70 gene increases ~200-fold, as estimated from measurements of pulse-labeled transcripts [Lis et al. 1981b], amounts of cross-linking of RNA polymerase to the hsp70 gene in vivo [Gilmour and Lis 1985], and quantification of transcripts produced by nuclear run-on assays [O'Brien and Lis 1991]. The fully induced hsp70 gene is packed with approximately one to two polymerases per 100 bp [Corces et al. 1981].

The hsp70 promoter in uninduced cells appears poised for a rapid and intense change in transcription rate. The promoter is punctuated by nuclease hypersensitive sites located at known regulatory elements and also at the transcription start and leader regions [Wu 1980; Costlow and Lis 1984]. RNA polymerase II is already present on the 5' end of the uninduced hsp70 gene as detected both in intact cells by UV cross-linking [Gilmour and Lis 1986] and in nuclei by nuclear run-on assays [Rougvie and Lis 1988]. Nuclear run-on assays demonstrate further that in uninduced Drosophila cells, this RNA polymerase II complex synthesizes an ~25-nucleotide nascent RNA and then pauses or arrests. This polymerase complex can escape the pause and transcribe into the body of the gene if nuclei are treated with either 0.6% Sarkosyl or high salt [Rougvie and Lis 1988]. In cells, the rate of escape from the pause is enhanced by heat shock. The recruitment of new polymerases to the hsp70 promoter appears to be inherently fast and not rate limiting over the entire range of hsp70 gene expression [O'Brien and Lis 1991; C. Giardina and J. Lis, unpubl.]. Thus, a major control of hsp70 expression appears to be at the level of early elongation, that is, the rate of escape of the paused polymerase into the body of the hsp70 gene.

A full understanding of the mechanism of elongation control of the hsp70 gene requires identification of the DNA sequences and proteins responsible for generating the paused RNA polymerase II complex. Known se-
Results

Strategy for mapping sequences that set up paused polymerase

The paused RNA polymerase II at the start of the uninduced hsp70 gene can be detected in nuclear run-on assays (Rougvie and Lis 1988). Although this engaged polymerase fails to transcribe in a standard run-on assay buffer, it does transcribe efficiently if either 0.6% Sarkosyl or 0.8 M KCl is included in the reaction. Both conditions block transcriptional initiation but allow transcriptionally engaged RNA polymerases to elongate (Hawley and Roeder 1985; Cai and Luse 1987). We reasoned that in vitro-mutated hsp70 promoters could likewise be assayed for paused polymerase in the context of normal chromatin after the stable integration of these promoters into the Drosophila genome. We used P-element-mediated germ-line transformation (Rubin and Spradling 1982) to generate transgenic flies that contain single chromosomal inserts of these modified promoters. To distinguish the RNA products of the introduced promoter from those arising as a result of transcription of the five endogenous copies of the hsp70 gene, we marked the modified hsp70 promoters by fusion to the Drosophila yolk protein gene yp1. The endogenous yp1 gene is transcribed only in females (Garabedian et al. 1986); therefore, run-on assays were performed only with male nuclei. The nontransformant control shows an undetectable level of yp1 transcripts if run-on assays are done either in the absence or presence of Sarkosyl (data not shown). This demonstrates that the endogenous yp1 gene of males contains little or no transcribing or paused polymerase and thus provides a low background for detecting transcription from the introduced hsp70-yp1 genes.

The general strategy for identifying the DNA sequences that set up paused polymerase was to construct various hybrid genes by fusing hsp70 to the yp1 sequences at positions within their respective promoter regions. Each construct was used to generate transgenic lines. Nuclei were isolated from adult males of each line and allowed to transcribe in the presence of radioactive NTPs. Sarkosyl was present (except in the controls) during the run-on transcription assays permitting the paused RNA polymerase, if present, to transcribe. The resulting radiolabeled, run-on transcripts were analyzed by hybridization to gel-separated restriction fragments of yp1 and hsp70 genes that had been blotted onto nylon membranes. The amount of radiolabeled RNA that hybridized to each fragment was quantified using a Betagen Betascope. The number of paused RNA polymerase II complexes on the fusion constructs was calculated by comparing the hybridization signal of the yp1 gene to that of the hsp70 gene standard. We have estimated that the uninduced hsp70 genes have one RNA polymerase per gene for each of the five genomic copies (Gilmour and Lis 1986; O’Brien and Lis 1991). To minimize any effects of genomic position, we examined two or more independent transformants of each construct.

Paused polymerase on a single gene can be detected in nuclei isolated from adult flies

Run-on assays with nuclei from transgenic males are shown in Figure 1. Run-on transcription in the absence of Sarkosyl produced abundant histone H1 RNAs but very little RNA homologous to hsp70. In contrast, when nuclei were treated with Sarkosyl and allowed to transcribe during a short (5 min) run-on reaction, synthesis of RNAs homologous to the 5′ end of the hsp70 gene was greatly stimulated. This result is like that seen with nuclei isolated from cell culture (Rougvie and Lis 1988) and demonstrates that the hsp70 genes in tissues of adult flies, like those in cell culture, contain a paused polymerase.

The run-on assays with adult nuclei can also detect RNA polymerase on an hsp70-yp1 hybrid. The nuclei used to generate the initial test results shown in Figure 1 were isolated from a transgenic line that contains a single-copy insert of the hsp70-yp1 hybrid gene called UP1x5. This hybrid gene contains five tandem copies of an hsp70 upstream sequence, nucleotides −89 to −38, fused to position −38 of the yp1 gene promoter (see Materials and methods, Fig. 2). Like the hsp70 gene, normal run-on reactions in the absence of Sarkosyl produce no detectable yp1 transcripts from the hybrid gene, indicating that polymerase is not efficiently transcribing this gene in the absence of heat shock. This is also supported by analysis of RNAs by Northern blot hybridization (Kraus et al. 1988). However, in the presence of Sarkosyl, transcription is seen on the 5′ but not the 3′ fragment, indicating that a transcriptionally engaged polymerase is present on the 5′ end of the gene. This transcription of yp1 is from the hybrid gene and not the endogenous yp1 gene as determined by the finding that nuclei from adult males of the ACR control [nontransformed line that is the parent of all the transformed lines described in this
and histone genes are included on each blot, and $hspH_ypl$, $hspO$, sequences from -89 to -38 fused hsp70 of the upstream region between -89 and -38 to the $hsp70-ypl$ gene at -38 region in the transcriptional start region. A dimer [UPlx2] generates paused polymerase on the hybrid gene at a level 55% that of an $hsp70$ gene, or, as mentioned in the initial test above, a pentamer [UPlx5] of this region fused to yp1 sequence generates an even higher level (Fig. 2). Thus, $hsp70$ sequences that reside entirely upstream of the TATA box can specify the formation of paused polymerase when fused upstream of the yp1 gene.

The levels of paused polymerase on this series of $hsp70$-yp1 hybrid genes in uninduced flies appear to correlate with their levels of expression in heat shock-induced flies (Fig. 2). The heat-induced expression of the hybrid gene UPl, which contains one copy of the -89 to -38 region of $hsp70$ and has no detectable paused polymerase, is barely detectable in either assays of steady-state RNA (Kraus et al. 1988) or in nuclear run-on assays performed on transgenic lines that have been heat shocked (Fig. 2C). In contrast, UPl2, an $hsp70$-yp1 construct that contains additional $hsp70$ upstream sequences to -256 and has paused polymerase, is expressed during heat shock at a >100-fold higher level than UPl, a level that is 23% that of a native $hsp70$ gene (Fig. 2). In addition, the presence of multiple copies of only the -89 to -38 $hsp70$ region in the $hsp70$-yp1 hybrids also produces both paused polymerase and high levels of heat-induced transcription [cf. UPl1 with UPlx5 and UPlx2 (Fig. 2A,B)]. Thus, the pausing of RNA polymerase on these genes appears to be related to their ability to be induced by heat shock.

hsp70 sequences in the transcriptional start region can complement upstream sequences in setting up paused polymerase

The sequences in and around the pause and transcriptional start site also contribute to the RNA polymerase pausing. The short region of upstream $hsp70$ sequences from -89 to -38 in the UPl construct cannot by itself, in a single copy, generate detectable paused polymerase. However, it can set up paused polymerase when present with additional upstream sequences (above) or with additional sequences from the transcriptional start region. The L1 hybrid gene contains $hsp70$ sequences from -89 to +62, which include the upstream sequence of -89 to -38, the TATA box, the start site, and the leader region where polymerase pauses. This hybrid programs the formation of an intermediate level (67% of an $hsp70$ gene) of the paused polymerase (Fig. 3). A slightly lower level (44%) is seen when an additional 32 bp are deleted (L1 + 30, -89 to +30), whereas deletion of an additional 7 bp of the $hsp70$ leader (L1 + 23) results in a distinctly lower level of paused polymerase (19%).

Figure 1. The initial test for mapping sequences that set up paused polymerase. The run-on assay results shown were obtained using nuclei isolated from adult male flies transgenic for the UPlx5 hybrid gene. This gene contains five copies in tandem of the upstream $hsp70$ sequences from -89 to -38 fused to position -38 of the yp1 gene (see Fig. 2 and Materials and methods). Run-on transcription assays were performed for 5 min with nuclei transcribing in the absence or the presence of 0.6% Sarkosyl, which blocks initiation completely but allows elongation of transcriptionally engaged RNA polymerase (Rougvie and Lis 1988). Both 5' and 3' DNA fragments of the yp1, $hsp70$, and histone $H1$ genes are included on each blot, and their positions are as indicated in the key. In the case of $hsp70$, a fragment from the middle [m] of the gene is also present. Restriction maps of the fragments shown in the key are drawn below the hybridization results. The thick bars represent transcribed sequences.

hsp70 upstream sequences can set up paused polymerase on the yp1 gene and render it heat inducible

In the region between -89 and the $hsp70$ TATA box are two HSEs, which bind HSF [Parker and Topol 1984; Wu 1984], and a GAGA element, which binds a general, constitutively active transcription factor [Gilmour et al. 1989]. The fusion of a single copy of this $hsp70$ upstream region between -89 and -38 to the yp1 gene at -38 (the UPl construct in Fig. 2A) gives a hybrid gene that has no detectable paused polymerase in uninduced transgenic flies, <12% (the limit of detection) of that found on a single $hsp70$ gene (Fig. 2A,B). In contrast, an $hsp70$-yp1 construct that contains additional $hsp70$ upstream sequences to -256, UPl2, has an easily detectable level of paused polymerase that is 78% that of a native $hsp70$ gene. The paused polymerase is also generated in constructs that contain multiple tandem copies of the -89 to -38 region: a dimer [UPlx2] generates paused polymerase on the hybrid gene at a level 55% that of an $hsp70$ gene, or, as mentioned in the initial test above, a pentamer [UPlx5] of this region fused to yp1 sequence generates an even higher level (Fig. 2). Thus, $hsp70$ sequences that reside entirely upstream of the TATA box can specify the formation of paused polymerase when fused upstream of the yp1 gene.

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**hsp70 sequences in the transcriptional start region can complement upstream sequences in setting up paused polymerase**

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Generating paused polymerase on hsp70

**Constructs**

- **UP1**
  - HSP70
  - -89
  - HSE
  - GAGA
  - TATA
  - YP1

- **UP2**
  - -256
  - -89
  - -38

- **UP1x2**
  - -89
  - -38/-89

- **UP1x5**
  - -89

**Paused Polymerase**

| Constructs | YP1/hsp70 |
|------------|-----------|
| UP1        | <0.12     |
| UP2        | 0.78      |
| UP1x2      | 0.55      |
| UP1x5      | 2.08      |

**Heat-Shock Transcription**

| Constructs | YP1/hsp70 |
|------------|-----------|
| UP1        | <0.002    |
| UP2        | 0.23      |
| UP1x2      | 0.24      |
| UP1x5      | 0.79      |

**Figure 2.** hsp70 upstream sequences can set up paused polymerase on the yp1 promoter. (A) The four different constructs shown (UP1, UP2, UP1x2, UP1x5) were analyzed for their levels of engaged RNA polymerase in both uninduced and heat-induced transgenic flies. The yp1 gene portion is illustrated as a stippled bar, the hsp70 sequence is shown as a thinner solid line. The coordinates above the construct refer to the ends of the hsp70 segment where +1 is the start of transcription in hsp70, the number below (-38) refers to the fusion point in yp1 relative to its start site. The positions of the HSE and GAGA sequences in the hsp70 sequence are shown by the wide hatched rectangles and solid triangles, respectively. The position of the TATA sequence is indicated by an oval. The number of paused RNA polymerase II complexes on the hybrid constructs was calculated from run-on assays with nuclei of uninduced transgenic male flies, as described in Fig. 1, using nuclear run-on data, an example of which is shown in B. The average value of arrested polymerase of each construct was obtained by averaging three to four independent run-on experiments using two or more independent transformants. The individual run-on results are shown in parenthesis below the average value, and the letters designate a particular transgenic line. Heat-induced transcription was determined by nuclear run-on assays using nuclei isolated from heat-shocked, male, transgenic adults. The heat-induced transcription of each hybrid gene was expressed as a ratio of signal from each hybrid gene to that from an endogenous hsp70 gene after correcting for the differences in copy number and fragment length. (B) Southern blots of restriction-cut plasmids containing the yp1 or hsp70 genes that were probed with radiolabeled run-on RNAs from uninduced males. Run-on reactions were performed in the presence of Sarkosyl. (C) Southern blots of restriction-cut plasmids containing the yp1 or hsp70 genes that were probed with radiolabeled run-on RNAs from heat shock-induced males.

It is intriguing that two very different types of additions to the -89 to -38 sequence of hsp70, either more upstream sequences (additional copies of the -89 to -38 region or sequences upstream of -89) or sequences around the transcription start, can generate paused polymerase. The upstream and start region may provide redundant functions such that the deletion of one region is complemented by the presence of the other region. Fig-
Figure 3. The hsp70 start and leader region can complement upstream sequences in setting up paused polymerase. (A) The four different constructs shown (UP1, L1 - 12, L1 + 23, L1 + 30, and L1) that have progressively more of the hsp70 start and leader sequence. The symbols and analysis are as in Fig. 2. (B) The three constructs (L2 + 23, L2 + 30, and L2) are related to the L1 constructs described in A, but have an additional copy of the hsp70 sequence - 89 to -38. The transcription starts on the parental L1 and L2 constructs at the normal hsp70 start site [Kraus et al. 1988].
Generating paused polymerase on \textit{hsp70}

Deletion of the pause region in \textit{hsp70} does not result in constitutive expression

The RNA polymerase pause site in \textit{hsp70} has been mapped to approximately +25 (Rougvie and Lis 1988). Thus, the region between +23 and +30 contains or is in the immediate vicinity of the pause site. If a specific sequence element at or downstream of the pause site normally acts to block progress of RNA polymerase into the fusion gene, then the removal of such sequences by deletion should result in expression of the downstream \textit{yp1} gene sequences even in the absence of heat shock. Two independent assays of transcriptional activity demonstrate that none of the pause region deletions leads to such constitutive expression. First, the 3′-deletion constructs generate very low steady-state levels of \textit{yp1} RNA in uninduced male transgenic flies [Fig. 4A]. This reflects a low transcription rate rather than an instability of \textit{yp1} RNA in males, because we have shown previously that \textit{yp1} RNA in males is as stable as it is in females (Kraus et al. 1988). Second, run-on assays of transcriptionally engaged polymerase in isolated nuclei demonstrate that in the absence of heat shock, transcription is undetectable on the 3′ fragments of all of the \textit{hsp70–yp1} hybrid genes [Fig. 2B and data not shown]. Thus, none of the deletions that extend into the \textit{hsp70} pause region leads to constitutive transcription of the fusion gene.

\textbf{GAGA element plays a key role in setting up paused polymerase}

Because the duplication of the −89 to −38 region is sufficient to generate a paused polymerase complex, as shown by \textit{UP1x2} and \textit{UP1x5}, we analyzed the function of sequence elements in this short interval in more detail. As shown in Figure 5A, the known sequence elements in the −89 to −38 region include HSEs (for review, see Lis et al. 1990) and a GAGA element (Biggin and Tjian 1988). We have examined the effects of introducing mutations into the HSE or GAGA elements of \textit{L1} [Fig. 5A]. The introduction of a pair of point mutations into two critical bases of the promoter proximal HSE1 [located between −62 and −47] has only a modest effect, reducing paused polymerase 30% in uninduced cells [Fig. 5B]. However, this mutation reduces the level of heat shock-induced transcription by ~20-fold relative to the parental \textit{L1} gene. In contrast, the introduction of multiple point mutations into the GAGA element [located between −74 and −67] reduces the level of paused polymerase more than fourfold. This mutation also has an effect of similar magnitude on heat-induced transcription, reducing the level of polymerase on the induced gene fivefold to sixfold.

The proximal GAGA sequence element and a portion of HSE2 overlap; therefore, the introduction of multiple
mutations into GAGA must also introduce mutations into a portion of HSE2. In making the multiple mutation *mmGAGA-L1* (Fig. 5A), we attempted to minimize perturbation of the HSE2 by preserving the highly conserved and critical G at −72 of the heat shock consensus (Lis et al. 1990) and the preceding A residue at −73, which is also known to be important in binding HSF from methylation interference assays (Shuey and Parker 1986; Perisic et al. 1989). Also, we note that HSE2 is a weaker transcription element and binds HSF less tightly than HSE1 [Parker and Topol 1984]. The predicted minimal effect of this multiple mutation on HSE2 function is borne out in the heat-shock response of the *mmGAGA-L1* hybrid gene, which is much more robust than that of the hybrid containing the double mutation in HSE1 [dmHSE-L1]. The level of effect is comparable to that of a mutation of a discrete GAGA element [separate from HSEs] that resides in the regulatory region of another heat shock gene, *hsp26* (Glaser and Lis 1990). Thus, the main effect of the multiple mutation on pausing and expression appears to be through its effect on GAGA element function, rather than through the disruption of HSE2 function. Furthermore, because HSF does not appear to bind HSEs in uninduced cells (Zimarino and Wu 1987; Thomas and Elgin 1988), it is hard to envision how an HSE might serve to set up a paused polymerase.

The level of paused polymerase on most hybrid genes examined correlates with the potential for transcriptional activation upon heat shock (Figs. 1 and 3). The exception is the gene *dmHSE-L1*, which contains a pair of point mutations in the proximal HSE. The *dmHSE-L1* gene has an intermediate level of paused polymerase, but upon heat shock, it is transcribed at a much lower level than the *L1* gene from which it was derived [Fig. 5]. The failure of this gene to be highly expressed during heat shock is consistent with previous studies showing that
mutations in either of the proximal HSEs dramatically reduce induced expression [Xiao and Lis 1988]. Therefore, the pausing of polymerase appears to be a feature of these promoters that is necessary, but not sufficient, for high levels of heat-induced transcription of hsp70. Not surprisingly, interactions of HSF with the HSEs are also required to induce the higher transcription rates.

Discussion

The goal of this study was to identify regions of the hsp70 promoter that participate in generating the paused RNA polymerase on the 5’ end of the hsp70 gene and to examine the relationship of this pausing to heat-inducible expression. To this end, we generated and analyzed transgenic fly lines carrying a variety of fusions between hsp70 and ypl gene promoters. The endogenous ypl gene normally has no detectable paused or transcribing polymerases in the adult male. The replacement of ypl promoter sequences with sequences upstream of the hsp70 gene can, in some cases, generate a paused polymerase at the 5’ end of the ypl gene. A large segment of the hsp70 upstream sequence, from −256 to −38, when fused to position −38 of the ypl gene, can program the formation of paused polymerase, and transcription initiates at the normal start of the ypl gene [Kraus et al. 1988]. Thus, sequences upstream of the hsp70 TATA box can specify the formation of the paused polymerase on the ypl sequence, indicating that this pausing does not obligatorily require sequences from the hsp70 transcriptional start region or from the hsp70 pause site.

The sequences in the −256 to −38 interval contain multiple binding sites for heat shock transcription factor [Parker and Topol 1984] and the general GAGA factor [Gilmour et al. 1989], and perhaps other, as yet unidentified elements. The search for critical elements that specify the pause was narrowed by examining constructs that contain a shorter upstream interval, −89 to −38, containing two HSEs and one GAGA factor-binding site. This segment on its own fails to generate detectable paused polymerase when fused to −38 of the ypl gene. However, two copies of the −89 to −38 region in tandem can specify the pause. In addition, the segment from −89 to +62, which has only one copy of the −89 to −38 region plus the hsp70 start region, can also specify formation of the pause, suggesting a redundancy or a cooperation in the activities of the start region and upstream sequences. Derivatives of this L1 construct that contain multiple point mutations in either the proximal HSE or the GAGA element are telling. While the introduction of a pair of point mutations into the proximal HSE has little effect on the formation of the paused polymerase, multiple point mutations in the GAGA element reduce the level of paused polymerase by more than fourfold.

The GAGA element binds a 66-kD protein that appears to be a general transcriptional activator [Biggin and Tjian 1988; Gilmour et al. 1989], and perhaps other, as yet unidentified elements. The search for critical elements that specify the pause was narrowed by examining constructs that contain a shorter upstream interval, −89 to −38, containing two HSEs and one GAGA factor-binding site. This segment on its own fails to generate detectable paused polymerase when fused to −38 of the ypl gene. However, two copies of the −89 to −38 region in tandem can specify the pause. In addition, the segment from −89 to +62, which has only one copy of the −89 to −38 region plus the hsp70 start region, can also specify formation of the pause, suggesting a redundancy or a cooperation in the activities of the start region and upstream sequences. Derivatives of this L1 construct that contain multiple point mutations in either the proximal HSE or the GAGA element are telling. While the introduction of a pair of point mutations into the proximal HSE has little effect on the formation of the paused polymerase, multiple point mutations in the GAGA element reduce the level of paused polymerase by more than fourfold.

The GAGA element binds a 66-kD protein that appears to be a general transcriptional activator [Biggin and Tjian 1988; Gilmour et al. 1989]. The GAGA element is found in the regulatory regions of a variety of heat shock and developmentally expressed genes and has been shown to act positively on the expression of the hsp26 gene [Glaser and Lis 1990; Glaser et al. 1990] and the Ultrabithorax (Ubx) gene [Biggin and Tjian 1988]. Furthermore, DNase I footprinting experiments have shown that GAGA factor binds strongly to the GAGA element spanning from −67 to −74 between HSE1 and HSE2 of hsp70. Recently, an analysis of transgenic lines containing hsp26 transgenes with various upstream deletions identified a CT-rich region, which contains multiple GAGA elements, as a key player in the formation of DNase I hypersensitive sites in the hsp70 promoter [Q. Lu, L. Wallrath, B. Allan, R. Glaser, J. Lis, and S. Elgin, in prep.]. Insertion of the −89 to −38 sequence of hsp70 upstream of an hsp26 gene deleted of the CT-rich region restored these hypersensitive sites. These observations are particularly intriguing because the hsp26 gene, like hsp70, has paused polymerase in uninduced cells [Rougvie and Lis 1990]. Thus, the positively acting GAGA element plays a role in specifying both the paused polymerase complex and the DNase I hypersensitive region of related heat shock promoters.

The GAGA element clearly is not the only sequence element that participates in generating a paused polymerase. The sequences around the transcriptional start region can contribute as well. This can be seen in constructs that contain one copy of hsp70 sequences that extend no farther upstream than the second HSE at position −89. In these constructs, the region of the hsp70 start between −12 and +23, which includes the initiation site, is critical for the formation of detectable paused polymerase. Initiator elements have been implicated in the assembly of a transcription complex in at least some eukaryotic genes [Smale and Baltimore 1989; Pellman et al. 1990]; thus, it is not surprising that they may play a role in generating the paused polymerase at the promoter region as well. In addition, the hsp70 sequences in the region of the pause itself, +23 to +30, also appear to contribute, albeit modestly (Fig. 3), to the formation of the pause. The precise position of the pause in the 5’ ypl sequences in the hybrid genes is not addressed in this paper, but preliminary mapping indicates that it is occurring at a specific location [C. Giardina and J. Lis, unpubl.]. It remains to be determined how dependent pausing is on the specific sequences of the hsp70 and ypl leader regions.

The level of paused polymerase on an hsp70–ypl hybrid promoter correlates, in general, with promoter activity upon heat shock. Those constructs that have the highest levels of paused RNA polymerase in uninduced cells are also expressed at the highest levels during heat shock while those with intermediate levels of paused polymerase are expressed at intermediate levels. The constructs having no detectable paused polymerase are expressed very poorly during heat shock. The one exception to this correlation is the construct with a double point mutation in the strong proximal HSE (dmHSE–L1). Although this construct has a moderately high level of arrested polymerase, its expression is only a few percent of the parental construct (L1). This apparent aberration can be easily understood if the establishment of a paused polymerase is a hallmark of the potential for activation
by heat shock. The activation also requires strong binding sites for the activator HSF and, in this construct, a pair of base substitutions disrupt the proximal HSE [Xiao and Lis 1988].

Why do some constructs have an intermediate level of paused polymerase? Forming paused polymerase may be a stochastic process in which some copies of the gene have a level of paused polymerase like that seen on the native hsp70 gene while others have none. Various factors that are involved in forming paused polymerase may compete with nucleosomes and participate in determining whether the promoter is open and has paused polymerase or is masked by histones. The intermediate level of heat-induced expression in constructs that have an intermediate level of paused polymerase may be a consequence of inducing only those genes with paused polymerase. Precedent for all or none expression of a gene has been reported recently for the inducible interleukin-2–lacZ hybrid gene. When T cells that were stably transfected with a single copy of the gene were induced and assayed individually, the resulting expression of the hybrid gene showed a clear bimodal pattern of expression (Fiering et al. 1990).

Native hsp70 genes appear to have approximately one paused polymerase per gene in the uninduced state, as calculated by comparison with the number of elongating RNA polymerases on hsp70 genes during heat shock (Gilmour and Lis 1986; O’Brien and Lis 1991). Thus, all copies of the hsp70 gene in a cell appear poised for activation. The level of paused polymerase on hybrid genes L2, L2+30, and UP1x5 was calculated to be about twice that of the native hsp70 gene (Figs. 2 and 3). This is not a consequence of multiple, well-separated start sites, as Kraus et al. (1988) examined the transcription initiation site of the construct L2 by primer extension experiments and found that transcription initiates from one site only. Perhaps, these constructs have more than one paused polymerase that reside different distances from a common initiation site. Alternatively, the amount of RNA polymerase on our standard gene, hsp70, may be overestimated. Although in cell cultures the average level of paused polymerase per hsp70 gene has been estimated to be one (O’Brien and Lis 1991), perhaps not all tissues of the adult flies, which are used in this study, have an arrested polymerase on each copy of the hsp70 gene. In fact, at least one cell type, primary spermatocytes in adults, does not have heat-inducible hsp70 expression [Bonner et al. 1984].

The formation of the paused polymerase on the hsp70 gene occurs in the absence of heat shock and is dependent on the GAGA element and the transcriptional start region sequences, both of which interact with constitutively active factors [Parker and Topol 1984; Wu 1984; Gilmour et al. 1989]. The importance of the GAGA element in pausing suggests that the constitutively active GAGA factor may provide a key function to hsp70 in the uninduced cell. Two mechanisms for the GAGA element/factor interaction can be readily envisioned in the recruitment of RNA polymerase to the hsp70 promoter. The binding of GAGA factor may exclude nucleosomes or histone H1, rendering the promoter accessible for the assembly of the transcription complex [Crosston et al. 1991; Workman et al. 1991]. Alternatively, GAGA-binding proteins may actively facilitate assembly of the uninduced transcription complex by interacting with proteins that bind TATA or other components of the start region complex.

We speculate that the features of the promoter that attract RNA polymerase are also responsible for holding the polymerase at the promoter even after it has initiated transcription. Therefore, RNA polymerase can initiate transcription and transcribe two or three turns of the DNA helix but pauses at approximately +25, perhaps without having broken all of its contacts with the proteins and sequences of the strong hsp70 promoter. In such a model, the escape of RNA polymerase in response to heat shock is facilitated by the binding of activated HSF.

The potential advantage of generating a paused polymerase in uninduced cells can be understood in terms of having the gene poised, ready to immediately release a transcribing polymerase in response to heat shock or other cellular stress signals. In addition, the combination of constitutively bound transcription factors, such as GAGA and TFIID, and the large, paused RNA polymerase II together can create highly accessible regions, detected as hypersensitive sites, that allow the binding of the activator HSF to HSEs upon heat shock. Maintenance of the heat shock regulatory regions in an accessible configuration is particularly important, because it is known that HSF (at least human HSF) fails to bind HSEs if the HSEs are associated with nucleosomes [Taylor et al. 1991]. Once active HSF binds, we envision that it accelerates the rate at which RNA polymerase breaks its contacts with the promoter and escapes into a productive elongation mode. This escape may be tightly coupled to the recruitment of the next polymerase, because the pause site remains occupied even when the gene is induced [O’Brien and Lis 1991; C. Giardina and J. Lis, unpubl.]. The paused polymerase may act as the “foot in the door” that maintains the promoter/regulatory region accessible to the RNA polymerase and factors required for both initial and subsequent rounds of transcription.

Materials and methods

Construction of plasmids

Except where mentioned, standard cloning methods were used [Berger and Kimmel 1987; Sambrook et al. 1989]. To create UP1, L1-12, L1+23, L1+30, and L1 (Figs. 2 and 3), 5’ deletions of the yp1 gene from its unique StuI site of pYP1 [Barnett et al. 1980] and 3’ deletions of the hsp70 gene from its unique PstI site at +89 of pXT1 [Xiao and Lis 1986] were made using Bal 31 nuclease [Legerski et al. 1978; Kraus et al. 1988]. SauI linkers were added to the ends of each deleted gene. The hsp70 3’ deletions were excised from the pXT1 derivatives as XhoI–SauI fragments [XhoI is at -89 of hsp70 in pXT1] and ligated into the SauI site of the P-element transformation vector cp20.1, a Carnegie 20 derivative [Simon et al. 1985]. This leaves a SauI site at the 3’ end of the hsp70 deletion. The 5’-deleted yp1 gene was then
moved as a SaI fragment (using the newly generated SaI site and the other SaI site of pYP1, which is −2.2 kb past the 3' end of the yp1 gene) into the SaI site at the 3' end of the hs70 deletion.

UP2 (Fig. 2) was constructed by ligating the XhoI–BssHII fragment (containing the sequences from −256 to −65 of the hs70 gene) to the BssHII–SaI fragment from the hs70–yp1 UP1 hybrid gene (containing the sequences from −65 of the hs70 gene to 2.2 kb downstream of the 3' end of the yp1 gene) and cloning into XhoI–SaI-cut P201.1.

UP1x2, L2 + 23, L2 + 30, and L2 were generated by first moving the XhoI–MspI fragment (−89 to −38) of pXM1 (Xiao and Lis 1986) into the XhoI–SaI site of pUC13X (Xiao and Lis 1986). The XhoI–SaI fragment from this latter plasmid was moved into the SaI site of Carnegie 20. The hs70 fragment containing the sequences from −89 to various 3'-deletion points was then added as an XhoI–SaI fragment. Finally, the SaI fragment containing 5'-deleted yp1 gene (as described in the UP1 construct) was then ligated into the SaI site downstream of the hs70 promoter region.

To create UP1x5 (Fig. 2), the XhoI–MspI fragment (−89 to −38) of pXM1 was cloned into the XhoI–SaI site of pUC13X. The XhoI–SaI fragment (containing −89 to −38) from this latter plasmid was then polymerized and characterized (Xiao and Lis 1986). A XhoI–SaI fragment containing five copies of −89 to −38 sequence arranged in head-to-tail orientation was then ligated into the SaI site of Carnegie 20. The SaI fragment containing 5'-deleted yp1 gene (as described in the UP1 construct) was then ligated into the SaI site downstream of the hs70 promoter region.

mmGAGA–L1 (Fig. 5) was generated by mutating 4 nucleotides of the GAGA consensus sequence in the L1 construct (Fig. 3). Two complementary 28-base oligonucleotides were synthesized which, when annealed, contained the region from −89 to −64 of the hs70 gene, including the mutated GAGA consensus sequence (Fig. 5A). The 28-base oligonucleotide has XhoI and BssHII sites on its ends. A plasmid containing hs70 sequences from −89 to +62 of the hs70 (used in L1) was cut with XhoI and BssHII, and the 28-mer was cloned into these sites. The 5'-deleted yp1 gene (the 5' end is at −9) was cloned as a SaI fragment into the SaI site (at the 3' end of the hs70 sequence) of the plasmid that contains the inserted 28-nucleotide synthetic DNA fragment. The resulting hs70–yp1 fusion gene was cut with Sacl (one Sacl site is in the vector, the other is 1.6 kb downstream of the 3' end of the yp1 gene), the ends of the fragments were filled in by Klenow treatment, and the fragment containing the fusion gene was then moved into the Hpfl site of the Carnegie 20 vector to generate mmGAGA–L1.

dmHSE–L1 (Fig. 5) was generated by mutating 2 specific nucleotides of HSE1 of construct L1. It was shown previously that a double point mutation of HSE1 abolished heat shock inducibility of the promoter when it was fused to the lacZ gene (Xiao and Lis 1988). Two complementary oligonucleotides, 13 bases (spanning from −51 to −63 of the hs70 gene) and 17 bases (spanning from −67 to −51) in length, which include the double-mutated HSE1, were synthesized. The 13-mer and 17-mer were annealed to produce a fragment that has Hpal and BssHII sites on its ends. A plasmid of hs70 3' deletions containing −89 to +62 sequence of the hs70 (used in L1) was cut with NruI and BssHII, and the annealed 13-mer/17-mer were cloned into the site. The 5'-deleted yp1 gene (5' end is at −9) was cloned as a SaI fragment into the SaI site (at the 3' end of the hs70 deletion) of the latter plasmid, which contains the newly cloned mutated HSE sequence. The hs70–yp1 fusion gene was moved into Carnegie 20 vector, as described for mmGAGA–L1, to generate dmHSE–L1.

The base sequence of modified regulatory regions was confirmed by DNA sequencing. Restriction fragments were cloned into M13 and sequenced by the dye-deoxy chain termination method using Sequenase (U.S. Biochemical).

Isolation of nuclei from adult flies

The protocol is a modification of that of Love and Minton (1985). Everything that came into contact with the flies or their extracts was prechilled at the start. The 1–1.5 gram of adult flies were collected in a prechilled bottle and transferred into an Omni mixer (Du Pont). Then, 15 ml of ice-cold buffer A [0.3 M sucrose, 2 mM MgOAc, 3 mM CaCl2, 10 mM Tris-HCl (pH 8), 0.3% Triton X-100, 0.5 mM DTT] was added, and the flies were ground in the buffer for 1–2 min at the maximum speed. This procedure took place on ice. The mixture was filtered through two layers of 100-μm nylon mesh. The filtrate was put into a 15-ml glass/Teflon homogenizer and stirred −40 times while on ice. The mix was filtered through two layers of 35-μm nylon mesh twice and mixed with an equal volume of buffer B [1.75 M sucrose, 5 mM MgOAc, 10 mM Tris-HCl (pH 8), 0.5 mM DTT]. The mixture was layered over a sucrose gradient containing 8 ml of buffer B over 4 ml of buffer B' [1.9 M sucrose, 5 mM MgOAc, 10 mM Tris-HCl (pH 8), 0.5 mM DTT], and spun down for 20 min at 15,000 rpm using the SW28 rotor at 4°C. After the supernatant was discarded, the walls of the centrifuge tubes were wiped with Kimwipes. The pellet was resuspended in 1.5 ml of buffer C [25% glycerol, 5 mM MgOAc, 50 mM Tris-HCl (pH 8), 5 mM DTT, 0.1 mM EDTA] and spun down for 1 min in a cold microcentrifuge. After the supernatant was discarded, the pellet was frozen quickly in liquid nitrogen and stored at −70°C.

Nuclear run-on transcription assays

Nuclear run-on transcription assays were performed essentially as described previously (Love and Minton 1985). The 100 μl of nuclei in buffer C [25% glycerol, 5 mM MgOAc, 50 mM Tris-HCl (pH 8), 5 mM DTT, 0.1 mM EDTA] were mixed with 117 μl of buffer D [1 mM ATP, 1 mM CTP, 1 mM GTP, 24 μM UTP, 4.4 mM MgOAc, 1.7 mM MnCl2, 7.2 mM Tris-HCl (pH 8), 150 mM KCl, 1 μl of RNasin (40 U/μl), 10 μl of [α-32P]UTP (100 μCi at 3000 Ci/m mole)]. For reactions performed in the presence of Sarkosyl, this detergent was added to buffer D to 1.2% [wt/vol]. Unlabeled nucleoside triphosphates were purchased from Pharmacia or Sigma, and [α-32P]UTP was from Amersham.

After incubation (from 2 to 5 min) at room temperature, reactions were stopped by the addition of 1 ml of stop buffer [2% SDS, 7 M urea, 350 mM NaCl, 1 mM EDTA, 10 mM Tris-HCl (pH 8)], transferred to a 15-ml tube containing an additional 4-ml stop buffer, extracted three times with Leder phenol (phenol/chloroform/isoamyl alcohol at 50 : 50 : 1), and ethanol-precipitated. Pellets were rinsed with 70% ethanol, dried, and resuspended in H2O. CaCl2 and MgCl2 were added to 6 mM, and the DNA was digested with 1 unit of RNase-free DNase (RNQ1 DNase; Promega) for 20 min at 37°C. After ethanol precipitation, the pellets were resuspended in H2O. The radiolabeled RNA was added to a hybridization solution for probing Southern blots.

DNA (Southern) blots

Restriction-digested DNA (2 μg per lane) was separated on 0.7–1.2% agarose gels in TAE buffer (Sambrook et al. 1989) and transferred to GeneScreen Plus (Du Pont) membranes or BioTrace RP (Gelman) membranes in 0.4 N NaOH for 12 hr. Following transfer, DNA was UV cross-linked to the mem-
branes by placing the membrane DNA-side down on a short-wave UV transilluminator for 45 sec. The membranes were then neutralized in 0.2 M Tris-HCl (pH 7.5) and 2× SSC, and air-dried. Filters to be probed with radiolabeled RNA were prehybridized for 2–5 hr in 50% formamide, 6× SSC, 1% SDS, and 10% dextran sulfate. Probe, which had been preincubated with blank filters overnight, was added to the prehybridized filters, and the hybridizations were incubated in roller bottles at 42°C for 24–48 hr. Following hybridization, the blots were washed twice, for 10 min each, in 2× SSC, 0.2% SDS, at room temperature, followed by two washes, for 30 min each, of 0.1× SSC, 0.1% SDS, at 55°C. After washing, the filters were dried and counted using the Betascope for quantitation.

Estimating the number of paused RNA polymerase II molecules on hsp70–ypl fusion constructs

The numbers of paused RNA polymerase II molecules on the fusion constructs were estimated by comparing the hybridization signal of ypl 5′ fragment to that of hsp70 5′ fragment. The level of polymerase on an uninduced hsp70 gene was taken as one, based on estimates from both in vivo UV cross-linking assays (Gilmour and Lis 1986) and nuclear run-on assays (O'Brien and Lis 1991). The values are expressed in Figures 2, 3, and 5 as the ratio of polymerases on the ypl sequences of the hybrid gene divided by the polymerases per copy of the hsp70 gene and, thus, are corrected for the fact that the signals for hsp70 are from five copies of this gene per genome, whereas the ypl signals are from a single copy of the hsp70–ypl gene.

In all of the hybridization assays in this paper, the 5′ fragment of ypl 1 to +62. This use of only sequences downstream of +62 avoids cross-hybridization of RNA from the γ element (Brown and Ish-Horowicz 1981; Lis et al. 1981a). The γ element is a repetitive sequence interspersed with αB repeats at the 87C locus, and γ has 99% sequence identity to the hsp70 upstream sequences and downstream sequences to +62 [Hackett and Lis 1981]. Because of this sequence identity, the γ sequence may have a paused polymerase on its promoter like the hsp70 gene. We found that the removal of the +1 to +62 sequence from the 5′ fragment of hsp70 caused significant reduction (about threefold) of the hybridization signal to the 5′ fragment, suggesting that the γ element contributes to the signal. However, the removal of the +1 to +62 sequence would also eliminate the contribution of short nascent hsp70 RNAs hybridizing upstream of the +62 region. Because Drosophila simulans flies do not have γ or αB elements in their genome, we performed run-on assays using nuclei isolated from D. simulans to determine the relative contribution of the +1 to +62 region of hsp70 to the hybridization signal. Any reduction in the hybridization signal of the 5′ fragment missing the sequences upstream of +62 would be due solely to the loss of the contribution from short hsp70 RNAs that hybridize upstream of the +62 sequence. In D. simulans, the hybridization signal decreased ~1.5-fold after removal of the +1 to +62 sequence, indicating that the signal from the hsp70 5′ fragment in D. melanogaster is under-represented ~1.5-fold as a result of the lack of that sequence in the fragment. This correction value was applied to the calculation of the number of paused polymerases on each construct.

RNA (Northern) blots

To prepare heat shock RNA, adult flies were heat shocked by immersing a glass vial containing the flies in a water bath at 36.5°C for 1 hr. RNA was prepared as described previously [Simon et al. 1985]. Non-heat-shock RNA was prepared similarly but without heat shock treatment of flies. RNA samples were electrophoresed and transferred to GeneScreen Plus (Du Pont) essentially as described in the manual. Ethidium bromide staining revealed the presence of approximately equivalent amounts of RNA in all lanes.

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