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Molecular Architecture of the *hsp70* Promoter after Deletion of the TATA Box or the Upstream Regulation Region

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GAGA factor, TFIID, and paused polymerase are present on the *hsp70* promoter in *Drosophila melanogaster* prior to transcriptional activation. In order to investigate the interplay between these components, mutant constructs were analyzed after they had been transformed into flies on P elements. One construct lacked the TATA box and the other lacked the upstream regulatory region where GAGA factor binds. Transcription of each mutant during heat shock was at least 50-fold less than that of a normal promoter construct. Before and after heat shock, both mutant promoters were found to adopt a DNase I hypersensitive state that included the region downstream from the transcription start site. High-resolution analysis of the DNase I cutting pattern identified proteins that could be contributing to the hypersensitivity. GAGA factor footprints were clearly evident in the upstream region of the TATA deletion construct, and a partial footprint possibly caused by TFIID was evident on the TATA box of the upstream deletion construct. Permanganate treatment of intact salivary glands was used to further characterize each promoter construct. Paused polymerase and TFIID were readily detected on the normal promoter construct, whereas both deletions exhibited reduced levels of each of these factors. Hence both the TATA box and the upstream region are required to efficiently recruit TFIID and a paused polymerase to the promoter prior to transcriptional activation. In contrast, GAGA factor appears to be capable of binding and establishing a DNase I hypersensitive region in the absence of TFIID and polymerase. Interestingly, purified GAGA factor was found to bind near the transcription start site, and the strength of this interaction was increased by the presence of the upstream region. GAGA factor alone might be capable of establishing an open chromatin structure that encompasses the upstream regulatory region as well as the core promoter region, thus facilitating the binding of TFIID.

The promoters of protein-encoding genes are typically composed of two functionally distinct classes of sequence elements. The region surrounding the transcription start site can be thought of as a core region that is involved in associating with TFIID (10). This core region can extend from approximately 40 nucleotides upstream to 40 nucleotides downstream from the transcription start (25, 33). Often a TATA box resides approximately 30 nucleotides upstream from the start. In vitro, the core region can be sufficient to reconstitute transcription. DNA elements that regulate the expression of the promoter are often located upstream from the core promoter region. These elements interact with specific proteins which in turn regulate the activity of the core promoter region. How this regulation is achieved is just beginning to be understood. In the case of the widely studied Gal4-VP16 activator, there is evidence that this activator could influence a variety of steps including the disruption of histone-DNA interactions, assembly of a preinitiation complex, and stimulation of elongation (1, 3, 4, 14, 15, 39).

The *hsp70* promoter of *Drosophila melanogaster* has been an important paradigm for investigating the mechanism of transcriptional regulation. The *hsp70* promoter is induced by heat shock (19). Induction involves the binding of a factor called heat shock factor (HSF) to several sites located upstream from −50. The heat shock elements (HSEs) within the region from −90 to −50 are sufficient to confer heat shock induction on promoter constructs that have been stably integrated into the genome with P elements (38). Additional HSEs reside approximately 100 and 200 nucleotides further upstream. Since HSF in *Drosophila* cells does not bind DNA before heat shock, its function is likely to be restricted to the activation of the promoter.

Analysis of the structure of the *hsp70* promoter reveals that TFIID, RNA polymerase II, and another sequence-specific factor called the GAGA factor associate with the promoter prior to heat shock induction (19). The RNA polymerase initiated transcription but paused in a region 20 to 40 nucleotides downstream from the transcription start (see reference 26 and references therein). A footprint corresponding to TFIID can be detected on the TATA box (7, 35), and footprints corresponding to the GAGA factor can be detected in several locations further upstream (35). These interactions are thought to establish the transcriptional potential of the promoter so that it can be rapidly activated by HSF during times of stress.

Here, we investigate the interplay between TFIID, RNA polymerase II, and the GAGA factor by performing a detailed analysis of the molecular architecture of mutant versions of the *hsp70* promoter that have been transformed into *Drosophila* on P elements. One mutation deletes the TATA box. Another mutation eliminates the regulatory region, including the GAGA elements, located upstream from the TATA box. These severe mutations were selected for study as they were likely to greatly alter binding of GAGA factor or TFIID. The TATA deletion, for example, was shown to inhibit the binding of TFIID in vitro by at least 20-fold (5). Our results show that

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both the TATA element and the GAGA elements are required to recruit TFIID and paused polymerase to a level comparable to that of the endogenous promoter, but that non-histone protein–DNA interactions still occur even in the absence of either of these sequences.

MATERIALS AND METHODS

Transcription and DNase I analysis of transformants. Plasmids, establishment and screening of transformed lines, collection of embryos, and Southern blotting procedures were performed as previously described (35). In the −50 construct, the sequence of the junction between the −50 breakpoint and the derivative of the Car20 transformation vector is aagcttggctgcaggtcgagGCGAAAAGAGCGC CGGAGTATAAAT. The lowercase sequence corresponds to sequences derived from the Car20ZT.2 transformation vector (35), and the underlined portion corresponds to the *Hin*dIII site found in the multiple cloning site of Car20 (27). The uppercase sequence corresponds to the *hsp70* sequence: the weak GAGA element and the TATA box have been underlined. The weak GAGA element in the −50mGAGA construct has been mutated so that the sequence of the junction is aagcttggctgcaggtcgagGCGATCGCGACGCCGGAGTATAAAT. In the −194 wild-type construct, the junction region has the sequence aagcttggctgcagg tcgagggggatcctctagagTCGAGAAATTTCTCTG. The *Hin*dIII site derived from the multiple cloning site of Car20 is underlined, and the *hsp70* sequence beginning at −194 is capitalized.

For this study, all the embryos used were dechorionated and flash frozen, and heat shock was done for 1 h at 37°C. Nuclei were isolated from embryos as previously described (18). DNase I treatment of nuclei and isolation of genomic DNA was done as previously described (35). Detection of DNase I hypersensitive (DH) sites was done using indirect end-labeling with a radiolabeled *Hpa*I fragment that anneals to the region next to the *Eco*RV site located in the β-galactosidase coding sequence (see Fig. 1A, DH probe). Assays for β-galactosidase activity were done on adult males as described by Simon and Lis (29).

High-resolution mapping of the DNase I patterns was achieved by using ligation-mediated (LM)-PCR (22). Amplification of the DNA was done by using Vent polymerase. The nontranscribed strands of transformed promoters were analyzed with a nested set of primers called TR-1, TR-2, and TR-3. This series spans the region from 142 to 106 nucleotides downstream from the transcription start of the −194 construct. We used this series in previous work (35), but annealing temperatures were modified to accommodate the use of Vent polymerase. The sequence and the annealing temperature used in the PCR for each of these was as follows: TR-1, ACGACGTTGTAAAACGAC (50°C); TR-2, GTAAAACGACGGGATCGATTCC (58°C); TR-3, CGACGGGATCGATTC CAATAGGC (62°C). For Vent polymerase reactions, DNA melting was done at 95°C and polymerase extension was done at 76°C as recommended by the LM-PCR protocol (22).

Note that all DNase I genomic footprinting analyses were done on at least two independent transformants, and similar results were obtained for a given construct.

Permanganate analyses of transformed promoters in intact salivary glands. Salivary glands were dissected from larvae and immediately transferred to a siliconized tube containing 100 μl of ice-cold dissection buffer (130 mM NaCl, 5 mM KCl, 1.5 mM $CaCl_2$). Twelve glands were transferred to the tube over a period of 15 min. The glands were treated with permanganate by adding 100 μl of 40 mM $KMnO_4$, which was prepared by dissolving solid $KMnO_4$ in dissection buffer. The $KMnO_4$ solution was prepared fresh for each day of use and kept on ice. The permanganate reaction was incubated on ice for 2 min and then stopped by the addition of 200 μl of stop solution (20 mM Tris-HCl [pH 7.5], 20 mM NaCl, 40 mM Na_2EDTA [pH 8], 1% sodium dodecyl sulfate, 400 mM 2-mercaptoethanol). The solution was vigorously shaken until all coloration had vanished. Each sample was treated with 10 μg of proteinase K for at least 1 h and then extracted with a sequence of liquefied phenol, phenol-chloroform-isoamyl alcohol (49.5:49.5:1), and chloroform. Each extraction was done with 300 μl of the organic solution. Nucleic acid was precipitated by adding 40 μl of 0.6 M sodium acetate (pH 6) and 1 ml of ethanol. The DNA pellets were washed once with 75% ethanol and then dissolved in 20 μl of 10 mM Tris-HCl (pH 7.5)–1 mM EDTA. DNA concentrations were determined with a fluorometer. The total amount of DNA recovered from 12 glands was typically 200 to 300 ng.

Purified DNA for generating naked DNA controls and for generating molecular weight markers was isolated from the soft body tissues that remained after isolation of the salivary glands. The soft tissues from approximately six larvae were placed in 200 μl of dissection buffer and held on ice. Two-hundred microliters of stop solution was added, and the DNA was subsequently isolated as described for the DNA from permanganate-treated salivary glands. The DNA was dissolved in 50 μl of 10 mM Tris-HCl (pH 7.5)–1 mM EDTA, and the concentration of DNA was determined with a fluorometer. Approximately 2 μg of DNA was typically recovered.

Permanganate treatment of naked DNA was done by diluting 500 ng of DNA from the soft tissues to a final volume of 100 μl with ice-cold dissection buffer. One-hundred microliters of 40 mM $KMnO_4$ (dissolved in dissection buffer) was added, and the mixture was incubated on ice for 30 or 90 s. The reaction was stopped with 200 μl of stop buffer, and the DNA was precipitated with ethanol. The DNA was dissolved in 20 μl of 10 mM Tris-HCl (pH 7.5)–1 mM EDTA, and the DNA concentration was determined with a fluorometer.

To determine the pattern of permanganate reactivity, 100 ng of each DNA sample was diluted to 15 μl with 10 mM Tris-HCl (pH 7.5)–1 mM EDTA. These samples were placed in siliconized tubes. Seventy-five microliters of water and 10 μl of piperidine were added, and each sample was incubated at 90°C for 30 min. Three-hundred microliters of water was added to each sample, and then the samples were extracted three times with 700 μl of isobutanol and one time with ether. The volume of the DNA was adjusted to 100 μl with water, and then the DNA was ethanol precipitated. The DNA was dissolved in 5 μl of 10 mM Tris-HCl (pH 7.5)–1 mM EDTA and transferred to a fresh siliconized tube. Note that this last transfer was essential as something about the tube in which the piperidine reaction was performed greatly inhibited the Vent polymerase.

The DNA cleavage patterns on the nontranscribed strand of the transformed promoters were determined by LM-PCR as described above for DNA that had been cut with DNase I. LM-PCR analysis of the transcribed strand of transformed promoters was performed with a nested set of primers called Try-D, Try-A, and Try-B. This series of primers spans the region 254 to 219 nucleotides upstream from the transcription start of the −194 transformant. The sequence and annealing temperatures used in the PCR for each of these were as follows: Try-D, CGAAAATATAAGCTCAATC (46°C); Try-A, GCTCAATCAAAAGA AGC (48°C); Try-B, CAAAAGAAGCTTGGCTGC (53°C). The underlined sequence in Try-B corresponds to the *Hin*dIII site found near the junction between the Car20ZT.2 and the *hsp70* sequences (See Fig. 1A).

LM-PCR analysis of the nontranscribed strand of the endogenous *hsp70* promoter was done with a nested set of primers called Endo-A, Endo-B, and Endo-C. This series of primers spans the region 155 to 124 nucleotides downstream from the start site of *hsp70* promoters located at the 87C locus. The sequence and annealing temperatures used in the PCR for each of these was as follows: Endo-A, CAGTAGTTGCAGTTGATTTAC (53°C); Endo-B, GCAGT TGATTTACTTGGTTGC (56°C); Endo-C, GCAGTTGATTTACTTGGTTGC TGG (60°C).

DNase I footprinting analysis of recombinant GAGA factor. GAGA factor was prepared from *Escherichia coli* containing the plasmid pAR-GAGA essentially as described by Tsukiyama and Wu (32), but with the following modifications. Rifampin was added to a final concentration of 40 μg/ml 30 min after expression of the GAGA factor had been induced with isopropyl-β-D-thiogalactopyranoside. The cells were then shaken at 30°C for an additional 30 min. The final stage in the purification used a heparin-Sepharose column. A 0.4 M salt step was added to the purification procedure prior to the 0.5 M step that elutes the GAGA factor. The final preparation was judged to be greater than 80% pure based on sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Footprinting analyses were performed by incubating designated amounts of GAGA factor with 20,000 cpm of end-labeled DNA in a 25-μl volume composed of 20 mM HEPES (pH 7.6), 80 mM KCl, 5 mM $MgCl_2$, 5% glycerol, 1 mM dithiothreitol, 100 ng of acetylated bovine serum albumin/μl, and 10 ng of *Hae* III-cut *E. coli* DNA/μl. The mixture was incubated for 30 min at 25°C and then lightly digested with DNase I for 30 to 40 s. DNase I digestions were halted by the addition of 400 μl of 10 mM EDTA (pH 7.6)–0.5% Sarkosyl–50-μg/ml proteinase K–100-μg/ml sonicated salmon DNA, and incubated for 5 min at 37°C. DNA was purified by extraction with a phenol-chloroform mixture followed by ethanol precipitation, and was analyzed on an 8% sequencing gel.

RESULTS

Deletion of the TATA box or the upstream region greatly inhibits transcription under heat shock conditions. By using P-element-mediated transformation, the four promoter constructs depicted in Fig. 1A were transformed into *Drosophila*. We generated fly lines carrying single copies of each promoter construct integrated into essentially random locations in the *Drosophila* genome. The transcriptional activity and the chromatin structure of the transformed constructs were evaluated. Previous analyses showed that the −194 wild-type construct behaved similarly to the endogenous promoter (35). It was induced by heat shock, and genomic footprinting showed that it interacted with GAGA factor, TFIID, and RNA polymerase II prior to heat shock induction. The second construct, −194 ΔTATA, had a 15-nucleotide deletion that eliminated the TATA box (8). In vitro binding studies showed that this mutation reduced the binding of TFIID by at least 20-fold (5). The third promoter, −50 core, was missing all of the binding sites for HSF and numerous high-affinity binding sites for GAGA factor. Note that a weak GAGA element was still present in the −50 core promoter construct. This weak site has been ignored in some earlier studies (17, 28). The fourth pro-

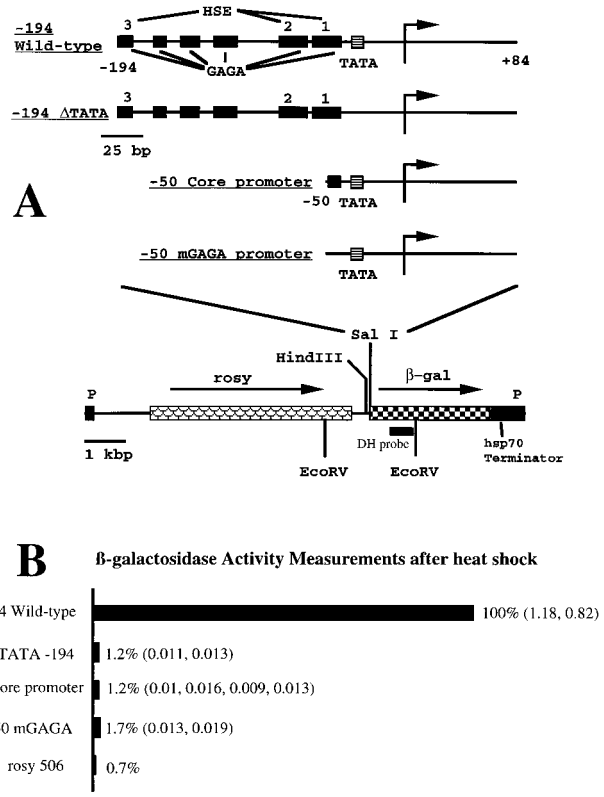

FIG. 1. *hsp70* mutant constructs and their levels of expression during heat shock. (A) Diagrams of promoter constructs. The four promoter constructs analyzed in this paper were inserted into the *Sal*I site of the transformation vector Car20ZT.2 (35). Black rectangles correspond to HSE 1, 2, and 3 (site 3 overlaps almost completely with a GAGA element). Stippled boxes refer to binding sites for the GAGA factor. The TATA box is also delineated. The rosy gene served as the selectable marker for transformation. The *Eco*RV sites and the region of the DNA probe (DH probe) are relevant to the indirect end-labeling analyses shown in Fig. 2. (B) β-Galactosidase levels after heat shock treatment of adult males. Five adult males were heat shocked at 36°C for 90 min. Extracts were prepared, and 20 μl of each extract was assayed for β-galactosidase activity. The level of β-galactosidase activity was corrected for variations in protein concentrations. The averages from the indicated number of transformants are graphed and the measurements for individual lines are provided in parentheses. The average level of β-galactosidase activity produced by the wild-type promoter was set at 100%, and all other values were adjusted accordingly.

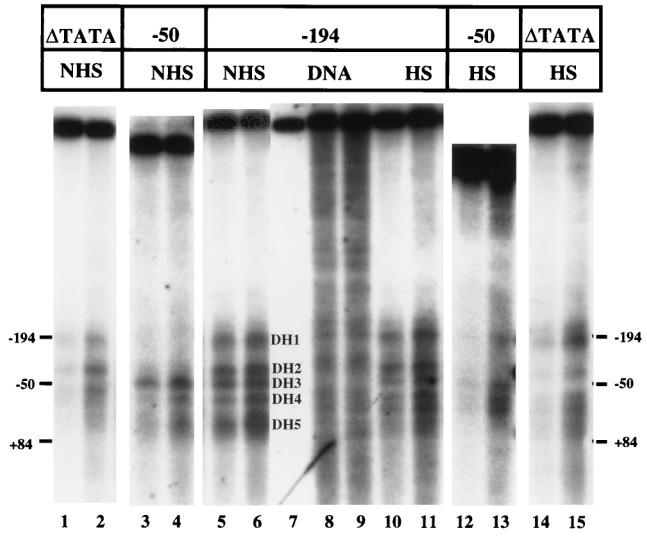

FIG. 2. Patterns of DNase I hypersensitivity associated with various *hsp70* promoter constructs. Genomic DNA was purified from DNase I-treated nuclei isolated from embryos and then digested to completion with *Eco*RV and run on a 1.2% agarose gel. The DNA was then blotted to Gene Screen Plus. The blot was probed with a fragment that hybridizes to the β-galactosidase sequences next to an *Eco*RV cut (Figure 1, DH probe). Plasmid standards were used to identify the indicated promoter positions. Lanes 1 and 2 and 14 and 15 are from a ΔTATA transformant respectively treated with 8, 16, 4, and 8 U of DNase I/ml. Lanes 3 and 4 and 12 and 13 are from a −50 transformant respectively treated with 4, 8, 8, and 16 U of DNase I/ml. Lanes 5 and 6 and 10 and 11 are from a −194 transformant respectively treated with 8, 16, 8, and 16 U of DNase I/ml. Lanes 7, 8, and 9 are purified genomic DNA from nuclei of a −194 transformant respectively treated with 0, 0.02, and 0.04 U of DNase I per μg of DNA. The labels demarcating DH1 through DH5 were placed in lane 7 because no hybridization signal was detected in this region of the blot. The intense band at the top of each lane represents the full-length *Eco*RV restriction fragment. Only subsets of the samples that provided information about the chromatin structure are presented. Although these results are a composite made from more than one gel, the positions of DH sites relative to each other are representative of situations where samples were analyzed on the same gel. NHS, no heat shock; HS, heat shock.

moter tested whether this weak GAGA element contributed to the behavior of the −50 core promoter construct. It was made late in the course of this study and has not been analyzed with DNase I. Permanganate footprinting indicates that the two versions of the −50 construct behave similarly (see Fig. 6).

The transcriptional activity of each of the promoters was assessed by measuring levels of β-galactosidase activity produced after heat shocking adult males at 36°C for 2 h. All of the mutations exhibited at least 50-fold less expression than the −194 wild-type constructs (Fig. 1B). The level of expression was only slightly more than that of the rosy 506 parent.

Analyses of DNase I hypersensitivity reveal non-histone protein interactions on the mutant promoters. The effects of the mutations on the chromatin structure of the promoters were determined by monitoring DNase I hypersensitivity. Nuclei were isolated from *Drosophila* embryos that carried the transformed constructs. The nuclei were lightly treated with DNase I, and the pattern of partial DNase I cutting was determined by using an indirect end-labeling procedure (37). Figure 2 shows the patterns of DNase I hypersensitivity detected in nuclei from non-heat-shocked and heat-shocked embryos. Five distinct DNase I hypersensitive (DH) sites are observed for the −194 wild-type construct from non-heat-shocked embryos (Fig. 2, lanes 5 and 6). Lane 7, where the labels for the DH sites have been placed, shows that no cutting is evident in samples that have not been treated with DNase I. The DH sites demarcate regions of protein binding. Several GAGA elements reside between DH 1 and 2 (9). One GAGA element resides between DH 2 and 3 (9). The TATA box resides between DH 3 and 4. The region between DH 4 and 5 corresponds to where polymerase pauses and where TFIID has been shown to contact DNA in vitro (25, 26).

Analyses of the −50 construct revealed the presence of DH sites. The pattern of DH sites over the region that was common with the −194 construct (DH sites 3, 4, and 5) was similar to that of the latter construct (Fig. 2, compare lanes 3 and 4 to lanes 5 and 6). This indicates that even in the absence of the upstream regulatory elements, TFIID and paused polymerase might still associate with the core promoter region. As expected, DH sites 1 and 2 are absent because the sequences underlying this region have been deleted. Note that there is no evidence of DH sites farther upstream from the core promoter. This suggests that there are no additional factors associated within 1 kb of the core promoter region.

Deletion of the TATA box has little effect on DH sites 1 and 2 (Fig. 2, lanes 1 and 2). This suggests that the protein-DNA

interactions in this region are similar to those found in the normal promoter. A third site in the TATA deletion is shifted slightly lower than DH site 3 of the −194 construct. A change in the pattern is expected, since the TATA box located between DH sites 3 and 4 in the −194 construct was removed. The appearance of a patch of protection centered at the −50 marker indicated possible binding of protein. Unlike the −194 construct, the region downstream from the transcription start adopts a broad region of hypersensitivity. The appearance of a broad region of DNase I hypersensitivity suggests that the DNA is more accessible than DNA located outside the *hsp70* promoter and that the DNA is probably not packaged into a nucleosome.

Heat shock alters the pattern of DH sites on each promoter construct. Both the −194 and −50 constructs lose the sharpness of the banding patterns that were seen prior to heat shock (Fig. 2, lanes 11 and 13). The blurring of the bands in the heat-shocked samples might reflect a heterogeneous state that both the promoters adopt during heat shock. The heat-shock-induced change in the −50 construct was not expected, since this construct lacks HSEs. This issue will be addressed later on, when we present evidence that polymerase is still recruited to this construct after heat shock (see Fig. 6). The TATA deletion also exhibits differences between the heat-shocked and non-heat-shocked samples but these are not as pronounced as those that were observed for the −50 and −194 constructs.

The −50 mG construct has not been analyzed with DNase I, but subsequent results from permanganate footprinting indicate that its chromatin structure is likely to be very similar to the −50 deletion. Both constructs exhibit low levels of paused polymerase prior to heat shock and a detectable increase in paused polymerase after heat shock (see Fig. 6).

Analysis of the DNase I cutting pattern at nucleotide resolution identifies interactions that can account for some DH sites. The pattern of DNase I hypersensitivity provides little indication of what factors are associated with the DNA. More information can be obtained by analyzing the pattern of DNase I cutting at nucleotide resolution. Previously, we had detected protein interactions at the TATA box and at GAGA elements located upstream (35). Modifications to our original procedure have greatly improved the quality of the data (see Materials and Methods). Figure 3 shows the pattern of cutting on the nontranscribed strand of the −194 wild-type construct that was detected by LM-PCR. Comparison of the cutting patterns in nuclei and in naked DNA showed that there was clear protection from DNase I cutting over the TATA box and over several GAGA elements. In addition, there was a broad region of partial protection that extended downstream from the transcription start. Weak bands in the uncut samples (lanes 3 and 8) are most likely due to the action of contaminating nucleases that have attacked the DNA during the preparation of the samples. For the most part, this pattern of weak bands is distinct from the cutting pattern produced by DNase I.

The pattern of DNase I cutting that was evident at the nucleotide level aligned well with the pattern of double-stranded cuts that was detected on the Southern blot shown in Fig. 2. The regions on the sequencing gel shown in Fig. 3 that appear to correspond with DH sites 1 through 5 in Fig. 2 are indicated at the right of lane 5. This suggests that the appearance of the DH region reflects the binding of GAGA factor, TFIID, and paused polymerase.

Similarities in the DH sites between the −50 and −194 constructs led us to suspect that TFIID might still be interacting with the −50 deletion. This was borne out by analysis of the DNase I cutting pattern at nucleotide resolution (Fig. 4A). Comparison of the DNase I cutting pattern in naked DNA to

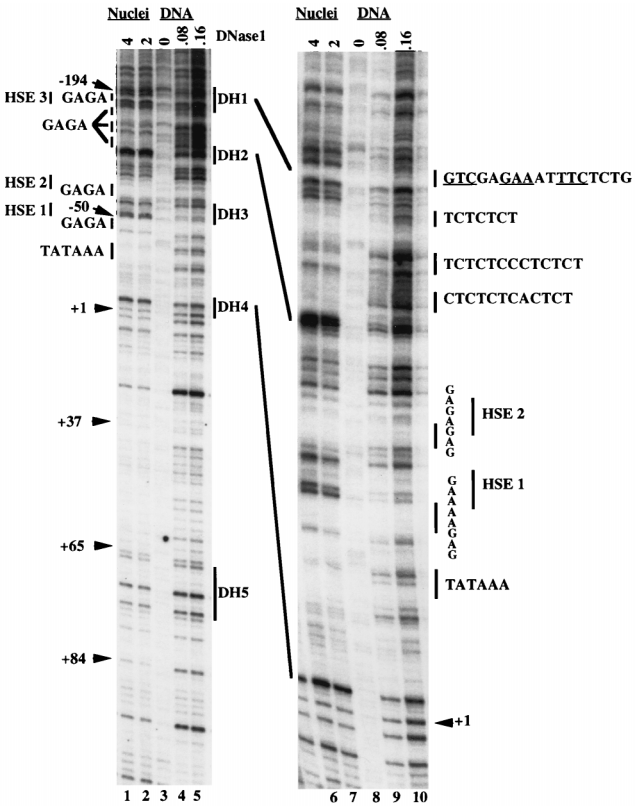

FIG. 3. High-resolution analysis of the DNase I cutting pattern that occurred on the nontranscribed strand of the −194 wild-type promoter in nuclei derived from non-heat-shocked embryos. Short (left) and long (right) runs of LM-PCR products derived from the nontranscribed strand of the −194 promoter construct are shown. The designations to the right of lane 10 provide some of the sequences of elements that have been shown to bind GAGA factor, HSF, or TFIID. The three triplets that are underlined at the top correspond to HSE 3. The locations of the binding sites were determined with molecular weight markers that were present on the original gel. Regions corresponding to DH sites 1 through 5 in Fig. 2 are delineated at the right of lane 5. Note that the naked DNAs (lanes 3, 4, 5, 8, 9, and 10) were prepared by treating genomic DNA isolated from nuclei.

that in nuclei clearly indicates that there was protection of the TATA box (Fig. 4A, compare lanes 6 and 7 to lanes 3 and 4). Moreover, the region between the TATA box and the GAGA element was hypersensitive to DNase I cutting. Hypersensitivity in this region also occurs when purified TFIID binds the promoter (5, 25). The cutting patterns downstream from the TATA box exhibited only minor differences from the cutting pattern on naked DNA, and no distinct protein-DNA interactions were evident. This was somewhat surprising because DH sites 4 and 5 were apparent in Fig. 2 (lanes 3 and 4). One possible explanation for this discrepancy is that the subtle differences in the cutting at the nucleotide level lead to more apparent differences in the pattern of double-stranded cuts detected with a Southern blot.

Similarities in the DH sites in the upstream region between the TATA deletion and the −194 construct led us to suspect that GAGA factor interactions in the upstream region were similar. Analysis of the DNase I cutting pattern at nucleotide resolution confirmed this (Fig. 4B, lane 2). Protection of several GAGA elements was clearly observed in the upstream region. Inspection of the region downstream from the transcription start site provided little evidence for strong protein-DNA interactions in this region.

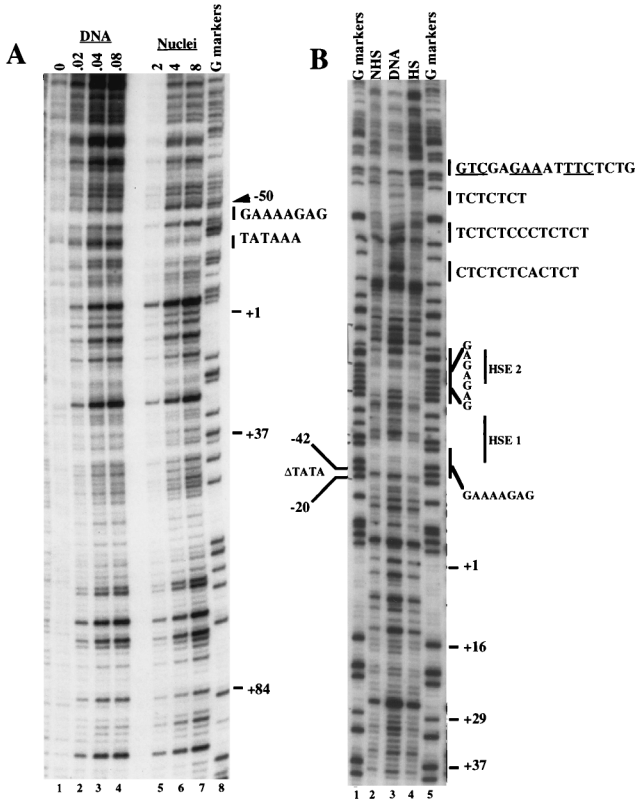

FIG. 4. High-resolution analysis of the DNase I cutting pattern that occurs on the nontranscribed strand of the −50 deletion and the TATA deletion in nuclei derived from embryos. (A) LM-PCR products derived from the nontranscribed strand of the −50 deletion construct. Nuclei were derived from non-heat-shocked embryos. Lanes 1 to 4 show naked DNA that was treated with increasing amounts of DNase I (0 to 0.08 U per μg of DNA). Lanes 5 to 7 show DNA from nuclei that were treated with increasing amounts of DNase I (2 to 8 U per ml of nuclei). Lane 8 shows a cleavage pattern at guanines generated by treating naked DNA with dimethylsulfate. (B) LM-PCR products derived from the nontranscribed strand of the TATA deletion construct. Nuclei from non-heat-shocked and heat-shocked embryos were lightly digested with DNase I (lanes 2 and 4, respectively). The DNase I cutting pattern for naked DNA is shown in lane 3. Lanes 1 and 5 show cleavage patterns at guanines. The downstream region has not been shown as there were no clearly discernible differences between the cutting pattern in nuclei and the cutting pattern in naked DNA. NHS, no heat shock; HS, heat shock.

We also analyzed the pattern of DNase I cutting for samples from heat-shocked embryos. This analysis did not reveal any major differences from the pattern observed on non-heat shocked samples so we have not presented most of this data. An example of what was typically observed is shown in Fig. 4B, lane 4. Heat-shock-dependent effects were very subtle and somewhat irreproducible. This could be due to the dynamic state of the promoters in the cell or to rearrangements in protein-DNA interactions that occur when isolating nuclei. The overlap between GAGA factor and HSF binding sites in the −194 and TATA deletion constructs might render the promoter structure quite unstable during the nuclear isolation procedure.

Detection of paused polymerase and TFIID on the *hsp70* promoter in intact salivary glands. Permanganate has been used to detect the interactions of TFIID and polymerase at the *hsp70* promoter (6, 7). Paused polymerase causes thymines in the region 20 to 40 nucleotides downstream from the transcription start to be hyperreactive to oxidation, whereas TFIID protects thymines in the TATA box. All previously reported structural analyses of transformed promoter constructs have been done on isolated nuclei. We wanted to analyze the transformed constructs in intact cells in order to avoid the risks of altering protein-DNA interactions during the isolation procedure. The salivary glands from third-instar larvae were selected because genomic footprinting with dimethylsulfate had been successfully performed on the *Adh* promoter in intact glands (13).

We first analyzed the endogenous *hsp70* promoter in order to test the feasibility of using permanganate on intact salivary glands. The results in Fig. 5 show that the interactions of TFIID and RNA polymerase II are readily apparent. The endogenous *hsp70* promoter was examined in two fly lines in order to assess the reproducibility of the approach. Thymine residues located at +22, +30, and +34 were significantly more reactive to permanganate in salivary glands from non-heat-shocked larvae (lanes 2 and 7) than in naked DNA (lanes 5 and 10). This is the region where the paused polymerase resides. Thymine residues located in the TATA element were less reactive in salivary glands from non-heat-shocked larvae than in naked DNA. This is consistent with binding of TFIID. Unfortunately, the interaction of GAGA factor does not seem to be detected with permanganate.

One will note that numerous bands in each lane do not correspond to thymines, and that they are present even in DNA samples which have not been treated with permanganate (Fig. 5, lanes 4 and 9). Fortunately, these bands map to guanines and do not interfere with the detection of the permanganate-dependent cleavages. This type of background has been observed by others, and it appears to be a consequence of the piperidine treatment (21). The two sets of data (Fig. 5, lanes 2 to 6 and lanes 7 to 11) are derived from separate fly lines containing P element insertions of either the −194 wild-type promoter or the TATA deletion. The reproducibility of the results for the endogenous *hsp70* promoter was quite striking.

We have also analyzed salivary glands from heat-shocked larvae. Heat shock caused all of the thymine residues on the nontranscribed strand between +2 and +76 to become hyperreactive (Fig. 5, lane 3 and 8). Similar results were observed by Giardina et al. (6, 7) when they analyzed the endogenous gene in intact tissue culture cells. We suspect that this hyperreactivity was caused by a train of polymerase molecules that was associated with the active gene. As expected, the association of TFIID with the TATA box was also readily apparent in glands from heat-shocked larvae. Unfortunately, as was the case for GAGA factor, permanganate provided no evidence for the interaction of HSF.

Permanganate analysis of intact salivary glands shows that RNA polymerase II and TFIID interactions on the promoter were reduced when the upstream regulatory region was deleted. We next used permanganate to monitor the interactions of polymerase and TFIID on various transformed *hsp70* promoter constructs. The −194 wild-type construct was strikingly similar in appearance to the endogenous *hsp70* promoter (Fig. 6, lanes 1 to 4). Hyperreactivity was detected at +22, +30, and +34 in salivary glands from non-heat-shocked larvae and from +2 to +67 in glands from heat-shocked larvae. In addition, good protection of the TATA box was observed in glands from both larvae preparations.

Deletion of the upstream region resulted in markedly reduced levels of permanganate reactivity at +22, +30, and +34 and reduced levels of protection in the region of the TATA box (Fig. 6). We examined four different insertions of the −50 construct (Fig. 6, lanes 12, 14, 16, and 18) and two insertions of a construct that also had the weak GAGA element between −50 and −40 mutated (lanes 7 and 9). These results indicate

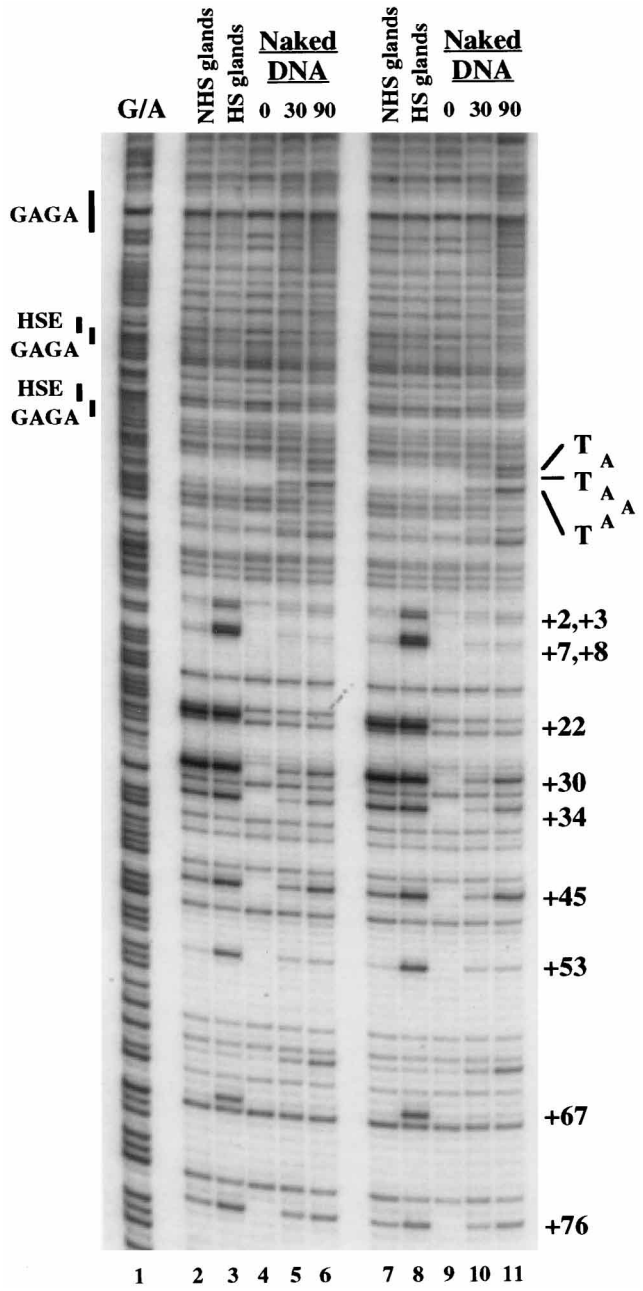

FIG. 5. Permanganate analysis of the nontranscribed strand of the endogenous *hsp70* promoter in intact salivary glands. The pattern of permanganate reactivity that occurred on the endogenous copies of the *hsp70* promoter at the 87C locus was monitored. Lane 1 shows the cleavage pattern at guanines and adenines generated by lightly treating naked DNA with formic acid and then cleaving at apurinic sites with piperidine. Lanes 2 to 6 were derived from a fly line containing an insert of the −194 promoter construct, and lanes 7 to 11 were derived from a fly line containing an insert of the TATA deletion. Salivary glands were dissected from heat-shocked (HS) and non-heat-shocked (NHS) larvae and then treated for 2 min with 20 mM permanganate. Following purification of the DNA, permanganate-modified positions were cleaved with piperidine. The sites of cleavage on the nontranscribed strand were mapped by using LM-PCR. Most of the thymine residues within the core promoter are designated by the numbers at the right margin. Naked DNA samples were treated with permanganate for 0, 30, or 90 s. A background pattern of cleavage is evident even in samples that have not been treated with permanganate. These bands correspond to G residues in the sequence, and the basis for this background is not known.

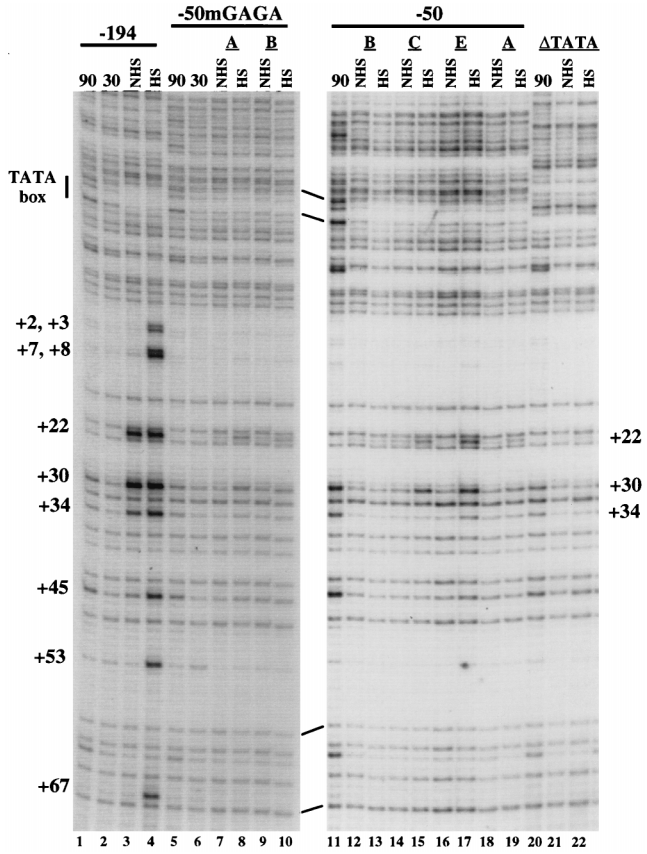

FIG. 6. Detection of TFIID and polymerase on transformed *hsp70* promoter constructs in intact salivary glands. The pattern of permanganate modification on the nontranscribed strand was determined for various insertions of the following transformed promoter constructs. Lanes 1 to 4 show a −194 wild-type construct. Lanes 5 to 10 show two different insertions of the −50 mGAGA construct. Lanes 11 to 19 show four different insertions of the −50 core promoter construct. Lanes 20 to 22 show a TATA deletion. Naked genomic DNAs from various lines were treated with 20 mM permanganate for either 30 or 90 s as designated at the top of lanes 1, 2, 5, 6, 11, and 20. Dissected salivary glands were treated with permanganate as described in Fig. 5 and in Materials and Methods. Thymine residues are indicated by numbers on the left and right. NHS, no heat shock; HS, heat shock.

that the deletion of the upstream region leads to a clear decrease in the levels of TFIID and RNA polymerase II that associate with the promoter prior to heat shock. The level of permanganate reactivity in the TATA box is very similar to that observed with the 30-s treatment of naked DNA so it is not clear from this analysis whether any TFIID associates with these deletions. This appears to contradict the previous DNase I results, which clearly indicated the presence of protein in this region (Fig. 4A). As will be discussed later, the discrepancy is probably based on the different steric constraints that DNase I and permanganate encounter when each attacks the DNA in chromatin.

We were interested in determining what happens to the pattern of permanganate reactivity during heat shock, since the DNase I cutting pattern on the −194 and −50 constructs was altered by heat shock (compare lanes 4 and 13 in Fig. 2). Interestingly, heat shock caused an increase in the level of permanganate reactivity detected at +22 and +30 for several of the −50 deletions and the two −50 mGAGA deletions. The level of permanganate reactivity was quite variable but reproducible. Such variability had not been seen on various −194

insertions (data not shown). This leads us to suspect that the heat-shock-dependent recruitment of polymerase to the different upstream deletions was influenced by sequences flanking the site of P element insertion. Immunolocalization of HSF on polytene chromosomes shows that HSF is widely distributed on the chromosome (36). It is possible that HSF associated with regions that flank the insert could be directing the association of polymerase with the −50 deletions to various extents. The recruitment of polymerase, therefore, could be causing the change in the DNase I hypersensitivity observed on the −50 constructs in response to heat shock (Fig. 2).

Paused polymerase is not detected on the promoter that lacks the TATA box. We suspected that the deletion of the TATA box would lead to reduced binding of TFIID and polymerase. Unfortunately, it was not possible to directly monitor the interaction of TFIID because the TATA element was gone. However, we were able to determine that the level of paused polymerase was significantly less than what was observed on the −194 construct. Lanes 21 and 22 of Fig. 6 show the patterns of permanganate reactivity detected before and after heat shock on the nontranscribed strand, respectively; lanes 7 and 8 of Fig. 7 show the respective patterns detected on the transcribed strand. Neither strand of the TATA deletion exhibits the hyperreactivity that is clearly evident on the corresponding strands of the −194 construct. We conclude that there is little, if any, polymerase associating with the TATA deletion under either non-heat-shocked or heat-shocked conditions. It should be noted that the pattern of permanganate reactivity detected on the transcribed strand of the −194 wild-type (Fig. 7, lanes 2 and 3) was in excellent agreement with results previously reported for tissue culture cells (7). This further validates the use of the salivary glands for analyzing the molecular architecture of transformed promoter constructs.

Binding analyses of recombinant GAGA factor reveal interactions that could be pertinent to the chromatin structure of the mutant and wild-type promoters. During the course of our studies, we uncovered two interesting features of the GAGA factor. We observed that recombinant GAGA factor interacted with a region next to the transcription start and a second region next to the TATA box (Fig. 8). Sequences associated with these regions of protection are shown in parentheses in Fig. 8 because they were not detected in earlier studies with GAGA factor purified from embryos (9). Interestingly, the association of the GAGA factor with the core promoter region was stimulated by the presence of the upstream region. Tenfold-less GAGA factor was required to achieve protection near the transcription start when the upstream region was present than was required when this region was absent (Fig. 8, compare lanes 4 and 5 with lanes 9 and 10).

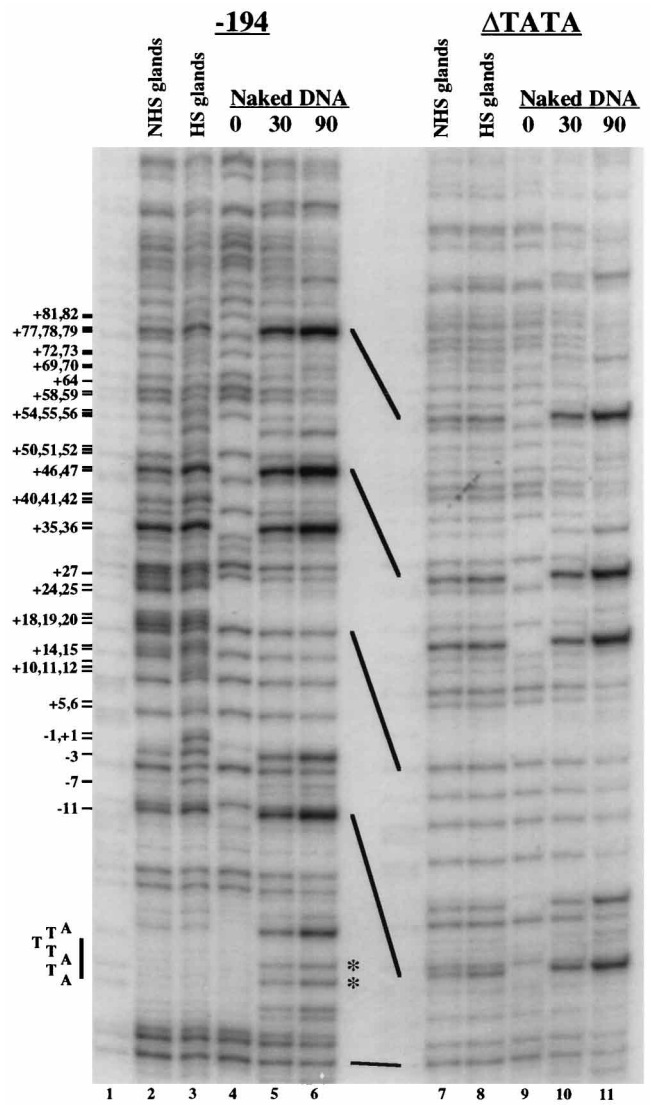

FIG. 7. Permanganate analysis of the transcribed strand of promoter constructs representing the −194 wild-type and TATA deletion. Permanganate patterns of modification on the transcribed strands of transformed promoters were detected by using a set of primers that annealed upstream from the promoter region. Thymine residues downstream from the TATA box are indicated by the numbers on the left. The sequence of the TATA box is given to match the sequence composition of the strand that was detected. The two asterisks highlight thymine residues that are protected in the −194 construct. For some unknown reason, only one of the thymines in the array of three consecutive ones appeared to react with permanganate. The TATA box region is missing from the TATA deletion. NHS, no heat shock; HS, heat shock.

DISCUSSION

We have analyzed the chromatin structures of mutant promoters in order to obtain insight into the interplay between the various factors that interact with the promoter prior to heat shock induction. Complementary methods were used to analyze the molecular architecture of the mutants after they had been transformed into flies on P elements. The promoter constructs with deletions of the TATA box or the upstream regulatory region were found to be hypersensitive to DNase I. Genomic footprinting further showed that GAGA factor was associated with the upstream region of the TATA deletion, but no paused polymerase could be detected. We could not directly establish that TFIID was absent from the TATA deletion, but we suspect that this is the case since the TATA deletion reduces the affinity of the DNA for purified TFIID by at least 20-fold (5). Hence it appears that GAGA factor can associate with the promoter independently from the rest of the transcriptional machinery.

The finding that GAGA factor associates with high-affinity sites in the TATA deletion could have significant implications beyond regulation of the heat shock genes. GAGA factor binding sites are commonly found in the promoters of genes in *Drosophila* (30), and immunofluorescence staining of polytene chromosomes shows that GAGA factor is widely distributed on the chromosomes (31). Our finding that GAGA factor can still establish an accessible region of DNA apparently in the absence of other components of the transcriptional machinery indicates that it could serve to render the DNA accessible to

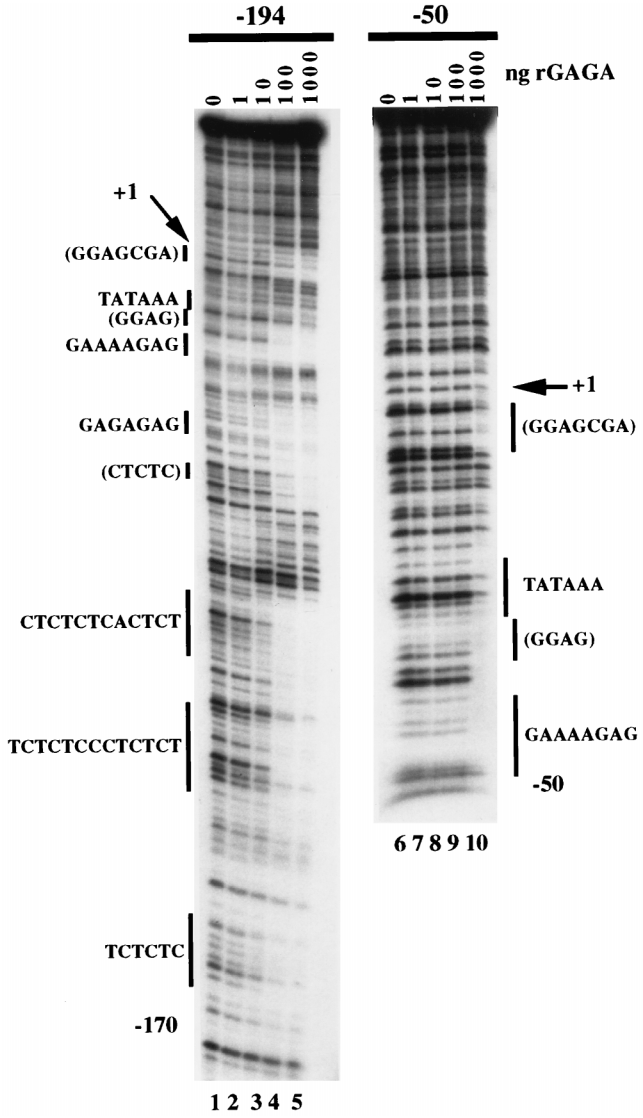

FIG. 8. DNase I footprinting analysis of recombinant GAGA factor reveals interactions within the core promoter region which are increased by the presence of the upstream region. An end-labeled fragment spanning from −194 to +84 (lanes 1 to 5) or from −50 to +84 (lanes 6 to 10) was incubated with the indicated amounts of recombinant GAGA factor. Binding was allowed to occur at room temperature for 30 min. GAGA factor binding was detected by DNase I footprinting. The cutting pattern corresponds to the nontranscribed strand. GAGA elements that were previously recognized as binding sites are indicated without parentheses (9), whereas additional GAGA elements that could be responsible for the additional interactions have been indicated with parentheses.

either the general transcriptional machinery or other DNA-binding proteins. In this respect, it is interesting that binding sites for the GAGA factor are often interdigitated with binding sites for other regulatory proteins, as is the case for the *hsp70* and *hsp26* genes (e.g., see reference 16).

We were surprised to find that the −50 construct also assumed a DH state. High-resolution analysis of the DNase I cutting pattern provided evidence that the promoter interacted with TFIID. Reasonably good protection of the TATA box is shown in Fig. 4A, and a hypersensitive cut which is characteristic of TFIID was detected immediately upstream from the TATA box. Our previous analysis had shown that TFIID causes each strand of the DNA immediately upstream from the TATA box to be hypersensitive to DNase I cutting (25). This feature could lead to strong double-strand cutting in the region upstream from the TATA box, and such cutting is observed in Fig. 2, lane 4. The observation that the level of permanganate cutting at +22 clearly increased in response to heat shock indicated that TFIID must associate with the −50 promoter at least under heat shock conditions.

It appears that TFIID associates with a lower proportion of the −50 constructs than is the case with the wild-type promoter. This would explain the apparent discrepancy between the DNase I cutting pattern, which indicated that TFIID was present, and the permanganate cutting pattern, which did not. DNase I and permanganate encounter different steric constraints when reacting with chromatin. DNA packaged with histones tends to resist DNase I cutting, so the pattern of DNase I cutting will be derived primarily from the subset of DNA that is bound by nonhistone proteins. Our impression is that the degree of hypersensitivity on the −50 construct is less than that found on the intact promoter. This is difficult to quantitate as many parameters can affect the level of cutting. Some of these include differences in protein interactions and batch to batch variation in the level of digestion that occurs between different nuclei preparations. Permanganate, being much smaller than DNase I, is less likely to be influenced by histones. This is certainly the case for the DNA alkylating agent, dimethylsulfate (20). Therefore, the cleavage pattern is likely to be representative of the total population of DNA molecules.

The effects of the upstream region are in agreement with the observations of Lis and colleagues. They found that mutation of a GAGA element located at −70 reduced the levels of RNA polymerase II (17) and TFIID (28) that were associated with an *hsp70* promoter construct that spanned the region from −89 to +64. These observations were made on nuclei isolated from transformed flies. Our results extend these results to intact living cells. Our results also provide information about the core promoter region in the absence of all upstream sequences known to influence the *hsp70* promoter. In particular, the GAGA element situated between −50 and −40 does not appear to make any contribution to the behavior of the core promoter region.

What is the mechanism by which the upstream region contributes to the recruitment of TFIID prior to heat shock, and why would a low level of TFIID still be able to associate with the −50 constructs in the absence of the upstream region? Our biochemical analysis of GAGA factor has identified possible answers to both of these questions. The presence of the upstream region enhances the interaction between GAGA factor and regions within the core promoter region (Fig. 8). Work by Tsukiyama et al. (31, 32) showed that GAGA factor was capable of disrupting nucleosome interactions in a process that depends on ATP and a nucleosome remodeling factor called NURF. Taking these observations together, we posit that the GAGA factor interactions that occur within the core promoter might render the DNA in this region accessible to TFIID. In the absence of the upstream region, a low level of association by the GAGA factor with the core promoter might allow a fraction of the promoters to associate with TFIID.

We have no direct proof that the GAGA factor interacts with the core promoter region in vivo, since neither DNase I nor permanganate revealed any footprints in the core promoter region that could be ascribed to GAGA factor. A combination of results concerning the behavior of our TATA deletion in cells and previous biochemical analysis of the GAGA factor, however, support our model. We observed that the region of DNase I hypersensitivity associated with the TATA

mutant extended downstream from the transcription start site (Fig. 2). The appearance of the DH region was very different from the appearance of the hypersensitive region observed on the −194 and −50 constructs. This suggests that TFIID or polymerase were not responsible. That GAGA factor could be responsible for this downstream accessibility is indicated by results from Tsukiyama et al. (31). They observed that the addition of GAGA factor at the beginning of a chromatin reconstitution reaction resulted in a broad region of hypersensitivity. This region of hypersensitivity extended from the high-affinity sites located upstream of the TATA box into the region downstream from the transcription start. Notably, the extracts used in the chromatin assembly reactions were deficient in TFIID; hence, these results parallel our finding in cells that the hypersensitivity occurs even in the absence of a TATA box.

A number of explanations could account for our inability to detect the GAGA factor on the downstream sequences. In the case of the −50 deletion, GAGA factor interactions in the core region could be obscured or displaced by TFIID. In the case of the TATA deletion, these interactions might be transient or too weak to hold up to the nuclear isolation procedure. Recent biochemical studies with reconstituted chromatin show that a DH region can persist even after the factors causing the structure have been removed from the DNA (24).

Alternative explanations for the DNase I hypersensitivity found in the downstream region of the TATA deletion and the −50 construct must be considered. We showed that TFIID recognizes specific sequences situated throughout the region spanning from the TATA box downstream to +30 (5, 25). It is possible that these TFIID interactions might be sufficient to establish a DH region. We do not favor this hypothesis, because our TATA deletion fails to exhibit any specific interaction with purified TFIID as judged by DNase I footprinting and protein-DNA cross-linking (data not shown). It is also possible that additional GAGA factor interactions could be involved. O'Brien et al. (23) analyzed the binding of several chimeric proteins consisting of GAGA factor fused to glutathione transferase or to the maltose binding protein. They observed footprinting at an additional site located around +30. We have not observed this interaction with our preparation of GAGA factor, but the difference could be due to variations in the amino acid sequences fused to the recombinant GAGA factor. Finally, there is the possibility that an as yet undiscovered protein recognizes sequences in the downstream region.

For the past several years, in vitro analyses of transcription have outpaced in vivo analyses. There is a dire need to evaluate possible mechanisms of regulation in the normal context of the cell. Our success at analyzing transformed promoters in intact salivary glands should be viewed as an important new step towards analyzing transcriptional regulatory mechanisms in intact cells. Our −50 core promoter construct is very similar to promoter constructs that have been used to characterize a variety of *cis*-acting regulatory elements (2, 11, 12, 34). By placing the *cis*-acting elements immediately upstream from the *hsp70* TATA box, expression patterns that match the temporal and spatial pattern of the gene from which the *cis* elements were derived have often been observed. This implies that the *hsp70* core promoter region is receptive to a variety of activators. The permanganate analysis of intact salivary glands provides us with three steps in the transcription process that can be readily detected. One is binding of TFIID. This is reflected by the level of permanganate protection that occurs at the TATA box. A second concerns the paused polymerase. This phase is reflected by the level of permanganate reactivity detected in the +20 to +40 region. A third is reinitiation. At high levels of reinitiation, permanganate reactivity is detected throughout an 80-nucleotide region extending downstream from the transcription start site. It should be insightful to determine how different activators affect these phases of the transcription process in vivo.

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