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Heterochromatic silencing of *Drosophila* heat shock genes acts at the level of promoter potentiation

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ABSTRACT

In a variety of organisms, genes placed near heterochromatin are transcriptionally silenced. In order to understand the molecular mechanisms responsible for this block in transcription, high resolution *in vivo* chromatin structure analysis was performed on two heat shock genes, *hsp26* and *hsp70*. These genes normally reside in euchromatin where GAGA factor and RNA Pol II are present on the promoter prior to heat shock induction. P-element transformation experiments led to the identification of stocks in which these two genes were inserted within heterochromatin of the chromosome 4 telomeric region. These transgenes exhibit silencing that is partially suppressed by mutations in the gene encoding HP1. Micrococcal nuclease analysis revealed that the heterochromatic transgenes were packaged in a more regular nucleosome array than when located in euchromatin. High resolution DNase I analysis demonstrated that GAGA factor and TFIID were not associated with these promoters in heterochromatin; potassium permanganate experiments showed a loss of Pol II association. Taken together, these data suggest that occlusion of *trans*-acting factors from their *cis*-acting regulatory elements leading to a block in promoter potentiation is a mechanism for heterochromatin gene silencing.

INTRODUCTION

Chromosomal position effects often arise when genes are introduced at new locations within the genome. These effects are frequently due to novel enhancer–promoter interactions. In many cases, a weak promoter falls under the control of nearby enhancers providing tissue and developmentally regulated gene expression. In other instances transcription is down-regulated due to the negative action of neighboring silencing elements. These types of position effects are due to the action of *cis*-regulatory elements located within gene-rich euchromatic regions of the genome.

A different type of position effect is associated with gene-poor heterochromatic regions of the genome. In these cases, transgenes exhibit position effect variegation (PEV) (1), gene silencing in a subset of cells in which the gene is normally expressed. For cell-autonomous proteins, PEV leads to a mosaic pattern of expression within a given tissue. For non-cell-autonomous proteins, a uniform reduction in expression can be observed. The classic example of PEV in *Drosophila* involves the *white* gene, which is required for red eye pigmentation. When the *white* gene is brought into juxtaposition with heterochromatin the resulting phenotype is a red and white speckled eye. In *Drosophila*, centric, telomeric and chromosome 4 locations have been shown to induce PEV of an *hsp70–white* transgene (2). Consistent with this discovery, centric and telomeric regions are known to be heterochromatic in a variety of eukaryotes. Chromosome 4 in *Drosophila melanogaster* is thought to be interspersed with heterochromatic domains (3–5). Regions of the *Drosophila* genome that cause PEV are late replicating and consist of repetitive DNA sequences, characteristic features of heterochromatin (1).

PEV is not specific for *Drosophila*: it has been observed for genes placed near silent mating type loci and telomeric regions in *Saccharomyces cerevisiae* (6,7), centric and telomeric regions in *Schizosaccharomyces pombe* (8,9), telomeric regions in *Trypanosoma brucei* (10,11) and centric regions in mammals (12,13). Details regarding the molecular mechanisms of gene silencing are poorly understood. In general, sequences within repressive chromatin domains are less accessible to digestion by nucleases and are packaged into regular nucleosome arrays (2,14,15). Thus, a ‘closed’ chromatin configuration correlates with the loss of gene expression.

To gain further insight into the mechanism of gene silencing associated with heterochromatin packaging in *Drosophila*, high resolution chromatin structure analysis was performed on transgenes inserted within heterochromatin. Two heat shock genes, *hsp26* and *hsp70*, which normally reside in euchromatin, are particularly useful for this analysis. The chromatin structure of the promoter region of these genes is ‘potentiated’, meaning that the transcriptional machinery is engaged and the heat shock regulatory elements are assembled into an accessible configuration prior to gene activation (16–21). More specifically, GAGA factor, known to be involved in establishing accessible regions of DNA in chromatin (21–23), is bound at several positions upstream of the transcription start site of *hsp26* and *hsp70* (21,24). GAGA factor-dependent nucleosome-free regions are present over the heat shock elements (HSEs). Prior to heat shock induction, RNA polymerase II is paused downstream of the transcription start site (25,26). As expected from the presence of polymerase, TFIID is

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also associated with the *hsp70* and *hsp26* promoters (16,20,27). This detailed information regarding the protein–DNA interactions that occur at the promoter regions of these euchromatic genes make these promoters ideal candidates for identifying changes caused upon heterochromatin packaging. The data presented here suggest that heterochromatin-induced gene silencing occurs at the level of promoter potentiation.

MATERIALS AND METHODS

Drosophila stocks

Drosophila stocks were raised at room temperature on standard sucrose/cornmeal medium (28). Stocks 39C-X and 39C-72 were derived from a P-element mobilization screen described previously (2). The P-element used in the mobilization contained an *hsp70–white* gene to score for PEV and the *hsp26* gene fused to barley cDNA sequences (representing the *SIP1* gene, accession no. M77475) at +490, designated *hsp26–pt* (Fig. 1). Stock 39C-X contains this P-element in euchromatin at cytological region 2D (2); stock 39C-72 contains this P-element in the telomeric region of chromosome 4 (Fig. 1). Stocks 2.11 and 2.7 were generated by standard germline transformation (29) using construct CCCA –194 containing the *xanthine dehydrogenase* (*rosy*) gene as a transformation marker and the *hsp70* gene fused to *lacZ* sequences at position +84 (Fig. 1). The TATA box in the promoter region of *hsp70* had been altered to CCCA; otherwise, this plasmid used for the transformations was identical to 70ZT (–194/+84) (30). Note that in four independent transformed stocks, the CCCA-containing transgene is induced by heat shock, despite the TATA mutation (data not shown). Stock 2.11 contains this P-element in euchromatin at cytological region 87 (data not shown); stock 2.7 contains this P-element insert at the telomeric region of chromosome 4 (Fig. 1).

Su(var)2-5^{02} contains a mutation in the gene encoding heterochromatin protein 1 (HP1) (31). Males of this stock were crossed to virgin females of stocks 39C-72 and 2.7 to test for suppression of gene silencing.

In situ hybridization

In situ hybridization to polytene chromosomes was performed using biotinylated DNA probes according to published procedures (32). For stocks 2.11 and 2.7, the P-element construct CCCA –194 (see above) was used as a probe. For stock 39C-72, the P-element construct hsp26–pt-T (2) was used as a probe. The site of hybridization was detected using streptavidin–horseradish peroxidase complex (Vector Laboratories) and 3',3'-diaminobenzidine (Sigma) (33).

β-Galactosidase expression

Females from stocks 2.7 and 2.11 were crossed to males of stock *Su(var)2-5*^{02} or *ry*^{506} as a control. Resulting third instar larvae were heat shocked for 45 min, allowed to recover for 1 h and the salivary glands were dissected and stained for β-galactosidase (34).

Micrococcal nuclease digestions

Nuclei were isolated from third instar larvae and treated with 0.16, 0.32 or 0.48 U of MNase as previously described (35). The DNA was purified and then separated by size on a 1% agarose/TAE gel (36) at 4°C. The DNA was transferred to nylon membrane (Amersham), crosslinked and hybridized to DNA fragments labeled with ^{32}P-dCTP and ^{32}P-dATP (Amersham DNA Labeling Systems). For stocks 39C-X and 39C-72, the barley DNA sequences fused to *hsp26* were used as a probe. For stocks 2.11 and 2.7, the *lacZ* sequences fused to *hsp70* were used as a probe. Following autoradiography, the bound radioactive material was removed from the membranes (36) containing the DNA isolated from the euchromatic insert stocks and the crosslinked DNA was hybridized to a 5 kb *Eco*RI restriction fragment from the 9D4 *HeT-A* retrotransposon element (accession no. X68130) labeled with ^{32}P-dATP.

DNase I genomic footprinting

For DNase I footprinting of *hsp26–pt*, nuclei were isolated from third instar larvae (35) and treated with 60 or 80 U of DNase I (Sigma). The DNA was purified and ~1 µg of DNA was subjected to ligation mediated-PCR (LM-PCR) according to a published protocol (37) with the following modifications: (i) primer P1 was annealed to the genomic DNA in amplification buffer with 2.5 mM $MgSO_4$, instead of first strand synthesis buffer; (ii) the final concentration of Tris in the ligation dilution solution was 55 mM at pH 7.0, instead of 110 mM at pH 7.5; (iii) following linker ligation the mixture was extracted with phenol:chloroform:isoamyl alcohol (25:24:1); and (iv) 21 cycles were used for amplification, instead of 18. Primer P1 (5'-CTCAAGATATGGAACATGAACAAGTGC-3') corresponds to the barley sequence fused to *hsp26*. Primers P2 (5'-GCAAAGTTGCTTTGAGTTGTTCAC-3') and P3 (5'-GCAAAGTTGCTTTGAGTTGTTCACTGCTCG-3') correspond to sequences within the coding region of the *hsp26* transgene. Half of the final LM-PCR reaction was loaded onto an 8% polyacrylamide sequencing gel.

DNase I footprinting of the *hsp70–lacZ* transgenes was performed on salivary glands permeabilized with NP-40. Six to eight pairs of salivary glands were transferred to a 1.5 ml tube containing 100 µl of buffer M (10 mM HEPES pH 7.6, 2.5 mM KCl, 5 mM $MgCl_2$, 5% glycerol, 0.1 mM EGTA, 0.5 mM DTT). To the tube were added 2.5 µl of 20% NP-40 and the solution was gently mixed. The glands were placed on ice for 15 min and gently agitated at 3 min intervals. A fresh dilution of DNase I (DPRF grade, made up to 20 U/µl; Worthington) was prepared at 1 U/µl in buffer M lacking EGTA. For DNase I treatment, the tube containing the glands was warmed to 20°C for 1 min and then 0, 4, 8 or 16 U of freshly diluted DNase I were added. The sample was incubated at 20°C for 2 min. The DNase I digestion was stopped by adding 2 µl of 0.5 M EDTA, mixing briefly and then adding 100 µl of 20 mM Tris–HCl pH 7.4, 200 mM NaCl, 2% SDS and 200 µg/ml proteinase K. The lysed glands were incubated at 37°C overnight. Subsequently, the solution was extracted three times with phenol:chloroform:isoamyl alcohol (49.5:49.5:1). Nucleic acids were precipitated with ethanol, washed once with 70% ethanol and finally dissolved in 22 µl of 10 mM Tris–HCl pH 8, 1 mM EDTA. An aliquot of 1 µl of the DNA sample was used to evaluate the DNA concentration in a fluorometer and 1 µl of the DNA sample was evaluated for digestion on an agarose gel. Samples of 100 ng of DNA were analyzed by LM-PCR using primers TR-1, TR-2 and TR-3 as previously described (20).

Potassium permanganate genomic footprinting

Potassium permanganate genomic footprinting was performed as previously described (20). The primers TR-1, TR-2 and TR-3 were used to monitor the permanganate modification pattern on the non-transcribed strand of *hsp70–lacZ*.

RESULTS

Recovery of chromosome 4 telomeric heat shock transgenes

For high resolution chromatin structure analysis we chose two stocks with P-elements containing *Drosophila* heat shock transgenes within heterochromatin. Heat shock genes are inducible in almost every tissue at any time in development, allowing the entire organism, or dissected tissue, to be used for chromatin structure analysis. Stock 39C-72 contains a tagged version of the *hsp26* gene (designated *hsp26–pt*). This stock was recovered from a P-element mobilization screen performed to identify transgenes that variegated for an *hsp70–white* gene also present on the P-element (2). *In situ* hybridization to polytene chromosomes mapped the P-element to the last visible band of the chromosome 4 telomeric region (Fig. 1). For the purposes here, we designate the telomeric region as the most distal band observed cytologically. Stock 39C-72 was an ideal candidate for high resolution chromatin studies for several reasons. First, the *hsp70–white* transgene is completely silenced in the majority of cells within the fly's eye (Fig. 2); the linked *hsp26–pt* transgene shows 6% heat shock-inducible expression compared to that for the euchromatic insert stock 39C-X (data not shown). This low level of expression implies that the transgene is silenced in the majority of cells, minimizing 'background' bands produced from expressing cells in chromatin structure analyses. Second, the PEV in this stock is suppressed by mutations in the gene encoding HP1, a known suppressor of centric PEV (38; Fig. 2). This is in contrast to telomeric transgenes on chromosomes 2 and 3, which are unaffected by mutations in HP1 (2). Last, micrococcal nuclease experiments (see below) on several stocks with heterochromatic insertions showed that the *hsp26–pt* transgene in stock 39C-72 was packaged in the most regular nucleosome array. Taken together, these features allow high resolution chromatin structure analysis of the protein–DNA interactions that occur at a silenced promoter.

The second stock used in our analysis contains a *hsp70–lacZ* transgene. This stock was discovered among a collection of P-element transformants that were generated to investigate the function of the TATA element of *hsp70*. Several transformed stocks carrying a CCCA mutation of the TATA box had been analyzed for heat shock inducible expression of the β-galactosidase reporter gene in whole flies. All except one stock exhibited induction levels that were approximately 3- to 5-fold less than that of lines carrying the wild-type promoter sequence. This exception, designated stock 2.7, exhibited no detectable β-galactosidase activity. As shown in Figure 2, there is a striking absence of β-galactosidase activity in salivary glands upon heat shock. *In situ* hybridization to polytene chromosomes showed that the insert was located at the chromosome 4 telomere, suggesting that silencing was due to heterochromatin (Fig. 1). Consistent with this hypothesis, a mutation in the gene encoding HP1 suppresses silencing, as indicated by β-galactosidase-positive cells in the salivary glands following heat shock treatment (Fig. 2). Further evidence confirming that the silencing is due to heterochromatin, and not a secondary mutation within the transgene, was obtained by mobilizing the transgene in stock 2.7 to new locations in the genome. Upon reintegration at these sites the *hsp70–lacZ* transgene showed greater levels of expression (data not shown).

Telomeric heat shock genes are packaged in a regular nucleosome array

We examined the pattern of MNase cleavage that occurred on euchromatic and heterochromatic transgenes. MNase cleaves in the linker region between nucleosomes. Nuclei were isolated from non-heat shocked third instar larvae and treated with increasing amounts of MNase. The DNA was isolated from each sample and analyzed by Southern hybridization. Fragments from the *hsp26* transgene were detected with a probe representing the barley sequences and fragments from the *hsp70* transgene were detected with a probe representing the *lacZ* sequences. A

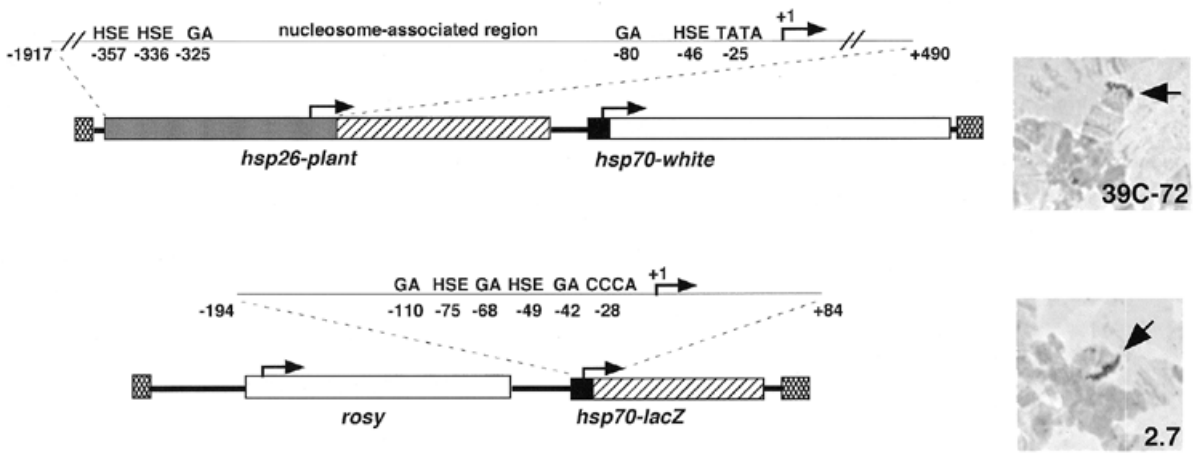

Figure 1. Diagram of the P-element constructs and chromosomal localization of the *hsp26–pt* and *hsp70–lacZ* transgenes. The P-element constructs containing the *hsp26–pt* and *hsp70–lacZ* transgenes are shown. *rosy* and *hsp70–white* served as reporter genes. The location of the heat shock elements (HSE), GAGA factor (GA) binding sites and the TATA or the mutated TATA box (CCCA) (see Materials and Methods for details) are indicated. P-element inverted repeats are indicated by stippled boxes. The chromosomal locations of the P-element inserts were determined by *in situ* hybridization to polytene chromosomes with probes corresponding to the P-element constructs. Both inserts map to the most distal band of chromosome 4 (arrow). For stock 2.11, the hybridization signal shows ectopic association with pericentric heterochromatin.

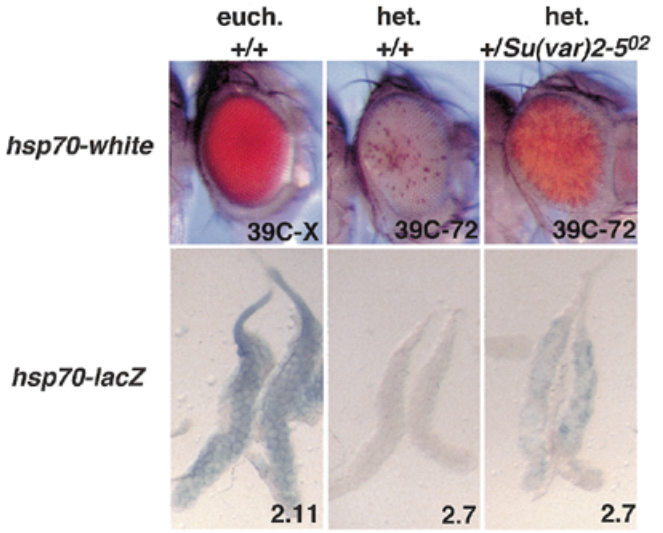

Figure 2. Expression of the *hsp70–white* and *hsp70–lacZ* transgenes. (Top row, left to right) The eye phenotype of stocks 39C-X, 39C-72 and 39C-72 with the *Su(var)2-5*^{02} mutation without heat shock treatment. Expression of a euchromatic *hsp70–white* transgene is sufficient to provide a strong red eye phenotype even in the absence of heat shock. (Bottom row) Salivary glands from stocks 2.11, 2.7 and 2.7 with the *Su(var)2-5*^{02} mutation dissected from heat shocked third instar larvae stained for β-galactosidase. euch., euchromatin; het., heterochromatin; +, wild-type chromosome.

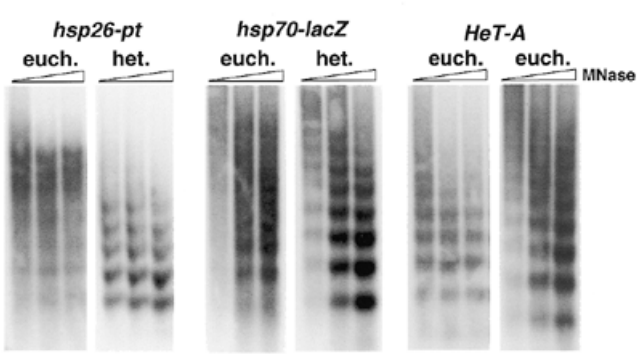

Figure 3. MNase digestion of *hsp26–pt* and *hsp70–lacZ* transgenes. Nuclei from third instar larvae containing either a euchromatic (stocks 39C-X and 2.11) or a heterochromatic (stocks 39C-72 and 2.7) transgene and treated with 0.16, 0.32 or 0.48 U of MNase. Digestion products were hybridized with probes corresponding to the barley sequences for *hsp26* and the *lacZ* sequences for *hsp70*. For both transgenes, the heterochromatic location results in a more regular nucleosome array than the euchromatic location. The radioactive probe was removed from the membranes containing DNA from the euchromatic insert stocks and then hybridized to *HeT-A* retrotransposon sequences. Regular nucleosome arrays apparent over the *HeT-A* sequences confirmed the integrity of the DNA in these samples. Abbreviations are as for Figure 2.

random distribution of nucleosomes over a given DNA fragment on a cell-by-cell basis leads to a smear of different length DNA fragments after MNase digestion. In contrast, alignment of nucleosomes into evenly spaced arrays over a given DNA fragment within the majority of cells leads to a ladder of nucleosome length fragments. For both *hsp26* and *hsp70* the MNase cleavage patterns for the heterochromatic transgenes (39C-72 and 2.7, respectively) showed a highly regular nucleosome array compared to the corresponding euchromatic transgene (stocks 39C-X and 2.11, respectively) (Fig. 3). The regular array of nucleosomes over the heterochromatic transgenes extended at least 1 kb, as five to seven 'rungs' in the MNase ladder could be observed.

To demonstrate that the nucleosomal pattern over the euchromatic transgenes was due to irregular packaging, and not degradation of the DNA during sample preparation, membranes containing the DNA from the euchromatic insert stocks were stripped of bound probe and then hybridized to *HeT-A* sequences. *HeT-A* is a retrotransposon present at *Drosophila* telomeres (39,40) that we previously discovered was packaged into regular nucleosome arrays (D.E.Cryderman and L.L.Wallrath, unpublished data). The resulting autoradiograph showed that the *HeT-A* sequences were indeed packaged into regular nucleosome arrays, verifying that the smear observed for the heat shock euchromatic transgenes was due to irregular nucleosome packaging.

Heterochromatic heat shock genes are not associated with TFIID or GAGA factor

The heat shock genes are preprogrammed prior to heat shock such that all the necessary transcription factors are bound except for heat shock factor. The correlation of the loss of heat shock inducible expression and the presence of a highly regular nucleosome array suggested that transcription factors may be excluded from the heterochromatic heat shock gene promoters. High resolution DNase I analysis was used to analyze the protein–DNA interactions occurring on the heterochromatic heat shock promoters. Nuclei were isolated from non-heat shocked third instar larvae and treated with DNase I. The DNA was purified and subjected to LM-PCR (37). Primers specific for the barley sequences permitted examination of the protein–DNA contacts along the non-ranscribed strand of the *hsp26–pt* transgene. The patterns of cleavage between the euchromatic, heterochromatic and deproteinized DNA samples were compared. Sequences between –20 and –43, the region expected to be associated with TFIID, are relatively protected (Fig. 4). The hypersensitive sites at positions –44 and –47 are also indicative of TFIID association. This protection/cleavage profile is similar to that observed for *hsp26 in vitro* footprinting experiments using purified TFIID (41). In contrast, a different pattern is observed over the heterochromatic *hsp26–pt* transgene. Changes in the cleavage pattern of the TATA box sequences (–25 to –31) could not be ascertained due to their relative insensitivity to cleavage in naked DNA (Fig. 4).

Similar DNase I footprinting experiments were performed on the *hsp70–lacZ* transgene using salivary gland nuclei from stocks 2.11 and 2.7. Previously, specific bases within the DNA between –50 and –200 have been shown to bind GAGA factor (42). Footprints corresponding to GAGA factor binding were readily apparent for the euchromatic *hsp70–lacZ* transgene at the two distal GAGA factor binding sites, but not for the heterochromatic transgene (Fig. 5). The footprints observed on the euchromatic transgene were strikingly similar to those produced by purified GAGA factor (42) and to those observed for a wild-type *hsp70* transgene (lacking the CCCA mutation) (20). No strong protection was detected over the mutant TATA box (designated CCCA). This was not surprising since this

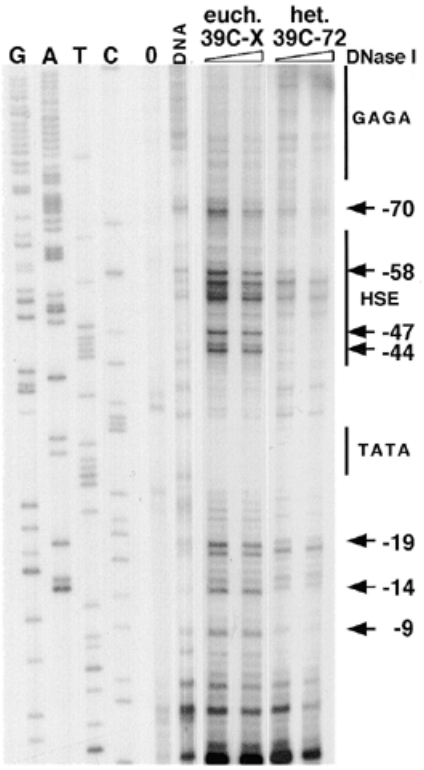

Figure 4. DNase I genomic footprinting of *hsp26–pt*. The sequence of the transcribed strand of the *hsp26* promoter is shown on the left (lanes 1–4) with the locations of regulatory elements shown on the right. Lane 5 is a sample in which no DNase I was added (labeled 0). Lane 6 contains a sample in which the DNA was deproteinized (labeled DNA). Lanes 7 and 8 contain LM-PCR reaction products from nuclei from third instar larvae of stock 39C-X treated with 60 and 80 U of DNase I, respectively. Lanes 9 and 10 contain LM-PCR reaction products from nuclei from third instar larvae of stock 39C-72 treated with 60 and 80 U of DNase I, respectively. Arrows denote regions of DNase I hypersensitivity that are protected in heterochromatin.

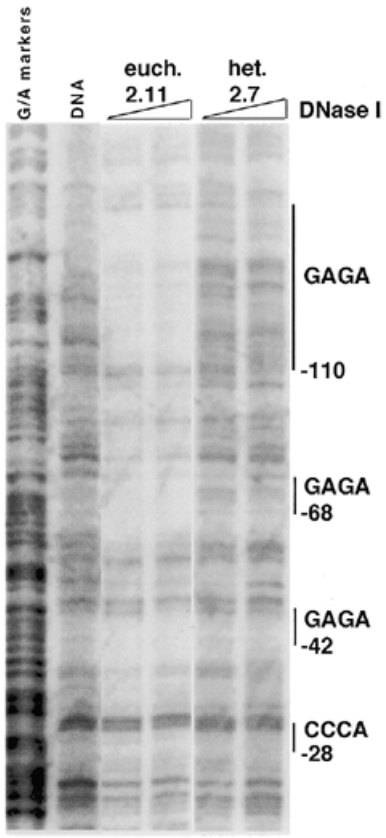

Figure 5. DNase I genomic footprinting of *hsp70–lacZ*. Lane 1 corresponds to the pattern of guanine and adenine residues generated by treating the DNA with formic acid. Lane 2 shows the LM-PCR pattern that occurs on deproteinized DNA. Lanes 3 and 4 correspond to reaction products using nuclei from salivary glands of the euchromatic insert stock 2.11 treated with 4 and 16 U of DNase I, respectively. Lanes 5 and 6 correspond to reaction products using nuclei from the heterochromatic insert stock 2.7 treated with 4 and 8 U of DNase I, respectively. Regions of protection corresponding to previously characterized interactions with GAGA factor (42) are delineated to the right of the panel. In addition, the region corresponding to the CCCA mutation in the TATA box is shown.

mutation reduced the affinity of the DNA for TFIID 3-fold (data not shown) and the corresponding mutation in the *hsp26* promoter also showed reduced levels of DNase I protection provided by purified TFIID (43).

Heterochromatic *hsp70* lacks detectable levels of paused polymerase

It is well established that a molecule of RNA polymerase II resides immediately downstream of the transcription start site of the *hsp70* and *hsp26* promoters prior to heat shock induction (25,26). Paused polymerase can be detected on the *hsp70* transgene in salivary glands by treating intact glands with potassium permanganate (20). Since the CCCA mutation prevents the detection of TFIID on the heterochromatic and euchromatic *hsp70* transgene, we wondered whether paused polymerase would be present. Thymines located within the single-stranded transcription bubble are hyper-reactive to potassium permanganate; the pattern of reactivity can be determined using LM-PCR. As shown in Figure 6, potassium permanganate reactivity is observed at positions +22 and +30 for the euchromatic *hsp70* transgene. These sites are consistent with those observed for the endogenous *hsp70* gene, indicating the presence of RNA polymerase II (20,44). In contrast, this reactivity is not observed for the heterochromatic *hsp70* transgene, indicating an absence of paused polymerase. The presence of paused polymerase on the euchromatic *hsp70* transgene strongly suggests that the euchromatic transgene is associated with TFIID. Our failure to detect TFIID with DNase I probably results from incomplete binding. This conclusion is consistent with the observation that the level of permanganate reactivity at position +22 for the euchromatic CCCA mutation is less than the level of reactivity observed for a wild-type euchromatic *hsp70* transgene or the endogenous *hsp70* gene (20).

DISCUSSION

We have investigated the molecular basis for heterochromatic silencing for two heat shock genes inserted near the telomere of chromosome 4 in *Drosophila*. Data presented here suggest that

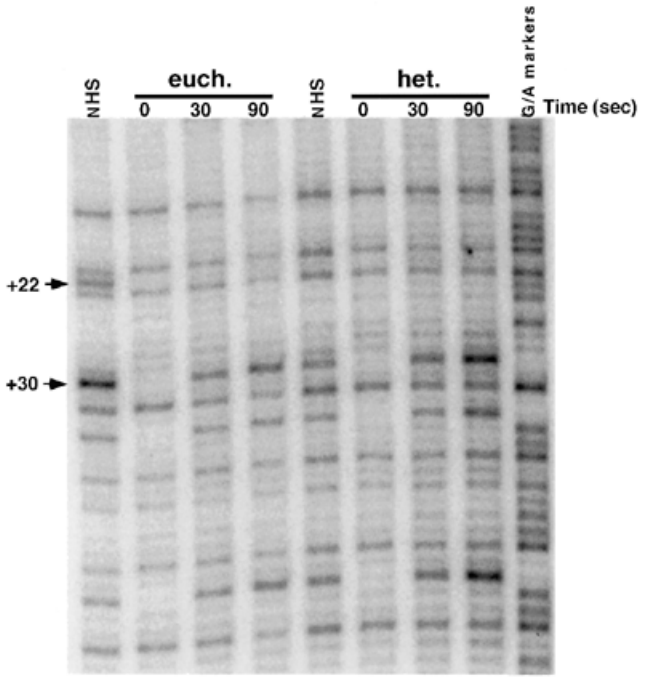

Figure 6. Potassium permanganate genomic footprinting of *hsp70–lacZ*. Lanes 1 and 5 correspond to reaction products generated from non-heat shock larvae (NHS) of stocks 2.11 and 2.7, respectively. Note that the thymine residues at +22 and +30 are more reactive in the case of the inducible transgene of stock 2.11 than for the heterochromatic transgene of stock 2.7. Lanes 2–4 and 6–8 show the patterns of reactivity for deproteinized DNA samples treated for 0, 30 or 90 s. Note that the pattern of bands detected in samples not treated with permanganate (lanes 2 and 6) has been observed previously and represents background in the analysis. These bands correspond to breaks at guanine residues which do not interfere with the evaluation of permanganate reactivity at thymine residues. Lane 9 shows the pattern of purines (G/A) generated by partial depurination and subsequent cleavage with piperidine.

heterochromatic silencing results from a block early in the transcription process, leading to a failure to form a potentiated promoter structure. Ideally, we would have liked to have discerned the interactions of GAGA factor, TFIID and polymerase II on one promoter. Instead, we found that we could best monitor GAGA factor and polymerase on the *hsp70–lacZ* transgene and TFIID on the *hsp26–pt* transgene. This should not detract from our general conclusion that these three factors are occluded by the heterochromatic structure because these two promoters function very similarly. Both promoters exhibit nearly identical interactions with purified TFIID (41) and both promoters are activated by heat shock when the upstream regulatory regions are swapped (45,46). One hypothesis is that the chromatin structure of the heterochromatic region prevents GAGA factor from interacting with the promoters and this in turn leads to a failure of TFIID and RNA polymerase II to associate. Molecular genetic analysis indicates that GAGA factor helps to establish an accessible chromatin configuration at the promoter region of heat shock genes (21,24). In the case of *hsp26*, euchromatic transgenes lacking GAGA sites show a more 'closed' chromatin configuration in which the HSEs are less accessible to restriction enzyme digestion that correlates with a loss of inducible expression (17,46). Binding of GAGA factor to multiple sites in the region upstream of *hsp70* appears to be cooperative (20,47) and this may contribute to its ability to establish an accessible region of the promoter. In the case of *hsp70*, euchromatic transgenes with mutations of the GAGA sites show reduced binding of TFIID, HSF and paused polymerase (19,20,48). *In vitro* GAGA factor recruits the ATP-dependent chromatin remodeling machinery NURF to clear nucleosomes from promoter regions (22,23,49). Thus, without GAGA factor association, the lack of TFIID and paused polymerase at the heterochromatic heat shock genes can be explained.

Another hypothesis to explain the lack of regulatory factors at the promoters of the heterochromatic heat shock genes is that heterochromatin localizes to an area of the nucleus that is relatively devoid of these factors. There is accumulating evidence to support a role for nuclear organization in gene expression (50–53). GAGA factor associates with heterochromatin throughout the cell cycle in early embryos (54); however, later in development association with GA-rich satellite sequences is only observed on condensed chromosomes (55). GAGA factor antibodies recognize hundreds of distinct bands in the euchromatic regions of interphase polytene chromosomes, but show no staining at the heterochromatin-rich chromocenter (22,24). The immunological data support our findings that genes which require GAGA factor for expression are not expressed when located in heterochromatin. For the heterochromatic heat shock transgenes, the regular nucleosomal packaging may be the 'default' state that results in the absence of GAGA factor binding.

The chromatin structure of genes within silent domains has only been evaluated in a small number of cases. *In vivo* genomic footprinting was used to compare the promoter region of the *PKG-1* gene on the active and inactive X chromosome in mammals (56). Four protected regions that are likely to interact with specific *trans*-acting regulatory factors were observed on the active X chromosome. In contrast, these footprints were not present upstream of the *PKG-1* gene on the heterochromatic inactive X chromosome; this region gave DNase I cleavage patterns suggestive of protection by two nucleosomes. In *S.cerevisiae*, high resolution chromatin structure analysis was performed to compare and contrast the transcriptionally silent *HML*α and the active *MAT*α (15). An array of positioned nucleosomes was observed over the α1 and α2 coding sequences of *HML*α. In contrast to our findings for heat shock genes, the promoter regions of the two genes were more accessible in the silent chromatin domain. However, the *HO* endonuclease recognition site showed the anticipated pattern of accessibility, hypersensitive at *MAT*α and protected at *HML*α.

The mechanisms by which heterochromatin might block transcription factor binding are unknown. In general, repressive chromatin domains contain hypoacetylated histones (57,58). *In vitro* experiments suggest that the lack of acetylation does not explain the occlusion of GAGA factor; GAGA factor can counteract the negative effects of chromatin equally in the presence of hypo- or hyperacetylated histones (59). In contrast, HSF and polymerase II bind more efficiently to DNA packaged with hyperacetylated histones (59). One common feature shared by repressive chromatin domains and silenced transgenes is the presence of highly regular nucleosome arrays (14,15; this study). This highly ordered packaging might be a reflection of the repetitive DNA sequences within these regions and flanking the silenced transgenes. Various satellite sequences and repetitive DNA elements have been shown to

position nucleosomes *in vitro* (60). The *hsp26–pt* transgene studied here is adjacent to transposable element sequences from the *F*-element family (53,61). In addition, certain non-histone proteins probably associate specifically with the heterochromatin to establish the repressive structure. In support of this hypothesis, a mutation in the gene encoding HP1 increases the level of heat shock-induced expression of the *hsp26–pt* transgene in stock 39C-72 from 6 to 11% (data not shown). Moreover, mutations in HP1 cause the accessibility of a centric *hsp26–pt* transgene to shift from 5 to 28%, allowing for increased levels of gene expression (62). It is tempting to speculate that HP1-containing complexes might exclude GAGA factor from heterochromatic promoters, given the mutually exclusive distribution of GAGA factor and HP1 on chromosomes (55).

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