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The role of a positioned nucleosome at the *Drosophila melanogaster hsp26* promoter

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The regulatory region of *Drosophila melanogaster hsp26* includes a positioned nucleosome located between the two DNase I hypersensitive (DH) sites that encompass the critical heat shock elements (HSEs). To test the role of this nucleosome in regulated expression, transgenic flies containing *hsp26–lacZ* fusion genes with alterations in the nucleosome-associated region have been generated. The positioned nucleosome is associated with a DNA sequence that does not itself contain any critical regulatory elements for heat shock-inducible expression. The nucleosome-associated sequence can be deleted, reversed, duplicated or replaced by a random sequence with no significant effect on DH site formation and gene expression. Analyses of *hsp26* and *hsp70* transgenes with spacing changes within the promoter region indicate that the location of the $(CT)_n$•$(GA)_n$ elements dictates the location of DH site formation. Wrapping the DNA between the regulatory elements around a nucleosome is as effective for gene expression as placing the regulatory elements close to each other. A loss of inducible gene expression was observed when the nucleosome-associated DNA was replaced with sequences which appear to misdirect nucleosome placement. The results indicate considerable flexibility in the spacing between DH regulatory sites.

Keywords: chromatin structure/$(CT)_n$•$(GA)_n$ repeats/DNase I hypersensitive sites/DNA with anisotropic flexibility/GAGA factor

Introduction

Promoter/enhancer regions of eukaryotic genes can be quite large, with important regulatory elements separated by regions of DNA with no apparent function. Packaging of such regions into nucleosomes might facilitate transcription by bringing into immediate contact regulatory elements otherwise separated. This has been suggested specifically in the case of the *Drosophila melanogaster hsp26* gene. Previous chromatin structure analyses have shown that the heat shock elements (HSEs) which bind heat shock factor (HSF) lie within proximal and distal nucleosome-free regions, DNase I hypersensitive (DH) sites, centered at –20 and –340 bp (see Figure 1) (Cartwright and Elgin, 1986; Thomas and Elgin, 1988). This nucleosome-free environment presumably allows for the rapid binding of HSF and induction of transcription following heat shock (Wu, 1984; Zimarino and Wu, 1987; Thomas and Elgin, 1988; O'Brien and Lis, 1993). Most of the DNA sequence between the two DH sites in the *hsp26* promoter is occupied by a positioned nucleosome (Thomas and Elgin, 1988). The folding of DNA around this nucleosome might facilitate interactions between the HSFs bound to the two HSEs upon induction (Thomas and Elgin, 1988). Nucleosomes positioned in the promoter region between regulatory elements have been proposed to play a similar role in other genes, including the *Adh* gene of *D.melanogaster* and the *vitellogenin B1* gene of *Xenopus* (Jackson and Benyajati, 1993; Schild *et al.*, 1993; reviewed by Wolffe, 1994).

To investigate the role of nucleosomes in promoter regions, we have constructed a series of transgenes using the *hsp26* promoter. We find that the nucleosome region is not essential for heat shock induction of transcription. Wrapping the DNA between the regulatory elements around a nucleosome is as effective for gene expression as placing the regulatory elements close to each other. In the majority of the altered transgenes, DH sites are generated over the HSE/$(CT)_n$•$(GA)_n$ elements, implying that these sequence elements dictate the open chromatin configuration. In cases where the nucleosome-associated sequence is substituted with an anisotropically flexible repetitive DNA sequence, there is a shift in the chromatin structure and a loss of gene activation.

Results

The nucleosome-associated sequence in the promoter region of hsp26 is non-essential for heat shock-induced expression

The *hsp26* gene in *D.melanogaster* provides an excellent system for studying the chromatin structure of a regulated gene *in vivo*. *hsp26* is activated in virtually all cells within minutes after heat shock; thus nuclei can be prepared from the whole organism for chromatin analysis of this gene in the 'on' and 'off' states. Altered *hsp26* sequences have been fused in-frame to the *Escherichia coli lacZ* gene in a P-element vector, and the constructs introduced into the *D.melanogaster* genome by P-element transformation (Rubin and Spradling, 1982). Inducible expression of the *hsp26–lacZ* transgenes can be monitored by measuring the levels of β-galactosidase activity following heat shock; the chromatin structure of the transgenes can be analyzed by nuclease digestion, using the unique probes shown in Figure 1 for indirect end-labeling experiments (see Lu *et al.*, 1993a and Materials and methods for details).

To test the functional role of the positioned nucleosome at the *hsp26* promoter and of the associated DNA, we have constructed *hsp26–lacZ* transgenes in which the

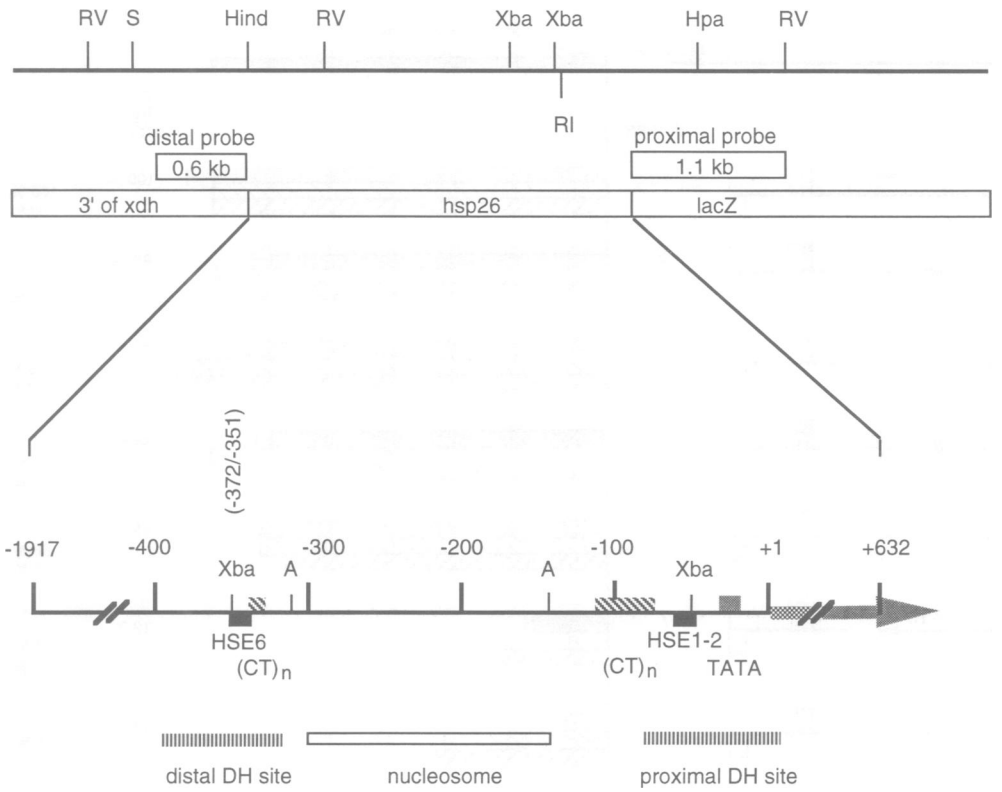

Fig. 1. Map of the *hsp26–lacZ* construct and the probes used for chromatin structure analyses. The structure of CarX is shown. *hsp26* sequences from –1917 to +632 (with the exception of sequences from –371 to –352, which are deleted) are fused in-frame to the *E.coli lacZ* gene. Restriction sites shown on the top line are those sites giving marker fragments or those used for mapping the chromatin structure in indirect end-labeling experiments. The 1.1 kb *lacZ* fragment, isolated from pMC1403 (Casadaban *et al.*, 1983), is used as a probe in experiments to map the DH sites and to quantitate access to the proximal DH site. The 0.6 kb fragment, containing 3′ sequences of the *D.melanogaster xdh* gene, is used as a probe for quantitating access to the distal DH site. The partial restriction map of *hsp26* sequences (–1917 to +632) is enlarged below with the $(CT)_n$ regions (striped boxes), the TATA box (stippled box) and the two required HSEs (HSE 1–2 and HSE 6, filled boxes) diagramed. Chromatin structural features of the *hsp26* gene are marked below. A, *Alu*I; RV, *Eco*RV; Hind, *Hin*dIII; Hpa, *Hpa*I; S, *Sma*I; Xba, *Xba*I.

nucleosome-associated region (–312 to –144, the 168 bp *Alu*I fragment in Figure 1) has been either deleted (transgene CarXΔV), inverted (transgene CarXRV), or replaced by a 168 bp fragment (considered to be random sequence) from bacteriophage λ DNA [transgene CarXΔV(Ri)]. V has been used here to symbolize υ, for nucleosome. In each case multiple independent lines possessing a single copy of the transgene have been recovered and characterized. β-galactosidase activity measurements from these transgenes indicate that the heat shock inducibility in each case is virtually identical to that of the wild-type control transgene (Figure 2). The fact that the nucleosome-associated region can be deleted, inverted, or replaced by a random sequence indicates that this segment of DNA does not contain any critical positive or negative elements regulating the heat shock response.

Examination of the chromatin structure of the two altered transgenes discussed above that do not contain spacing changes of the regulatory elements, CarXΔV(Ri) and CarXRV, revealed strong DH sites at the same locations as in the control transgene CarX (Figure 3). The region between the two DH sites remains protected, indicating the presence of a nucleosome. *Xba*I restriction sites are located within the HSEs of these DH sites; consequently, the accessibility of these elements within nuclei can be assessed by the effectiveness of restriction enzyme cleavage at these sites (Jack *et al.*, 1991; Lu *et al.*, 1993a). The accessibility of the *Xba*I sites for transgene CarXRV is 101% (proximal *Xba*I site) and 99% (distal *Xba*I site) compared with CarX; the accessibility of the *Xba*I sites for CarXΔV(Ri) is 86% (proximal *Xba*I site) and 81% (distal *Xba*I site) of CarX (Figures 2 and 4). Thus, the wild-type chromatin configuration is maintained in transgenes with an inversion or replacement of the nucleosome-associated sequence, implying that no information within this region is required for generating this defined structure.

The position of the $(CT)_n$•$(GA)_n$ elements dictates the position of the DH sites

The two DH sites of *hsp26*, each encompassing an essential HSE, are separated by ~290 bp of DNA, sufficient length to bind a histone core to generate a nucleosome. To determine whether this spacing is a critical structural feature of the promoter, we analyzed the expression and chromatin structure of transgenes in which the 168 bp nucleosome-associated sequence had been deleted or tandemly duplicated [transgenes CarXΔV and CarX(V-V)]. These changes have relatively little effect, resulting in heat shock-inducible levels of β-galactosidase activity that are 95% and 84% that of the wild-type transgene CarX (Figure 2). Thus the presence of intervening nucleosomes between the two HSEs neither facilitates nor hinders expression relative to a promoter with adjacent HSEs. As shown in Figure 3, when the nucleosome-associated sequence is deleted (transgene CarXΔV), the distal DH

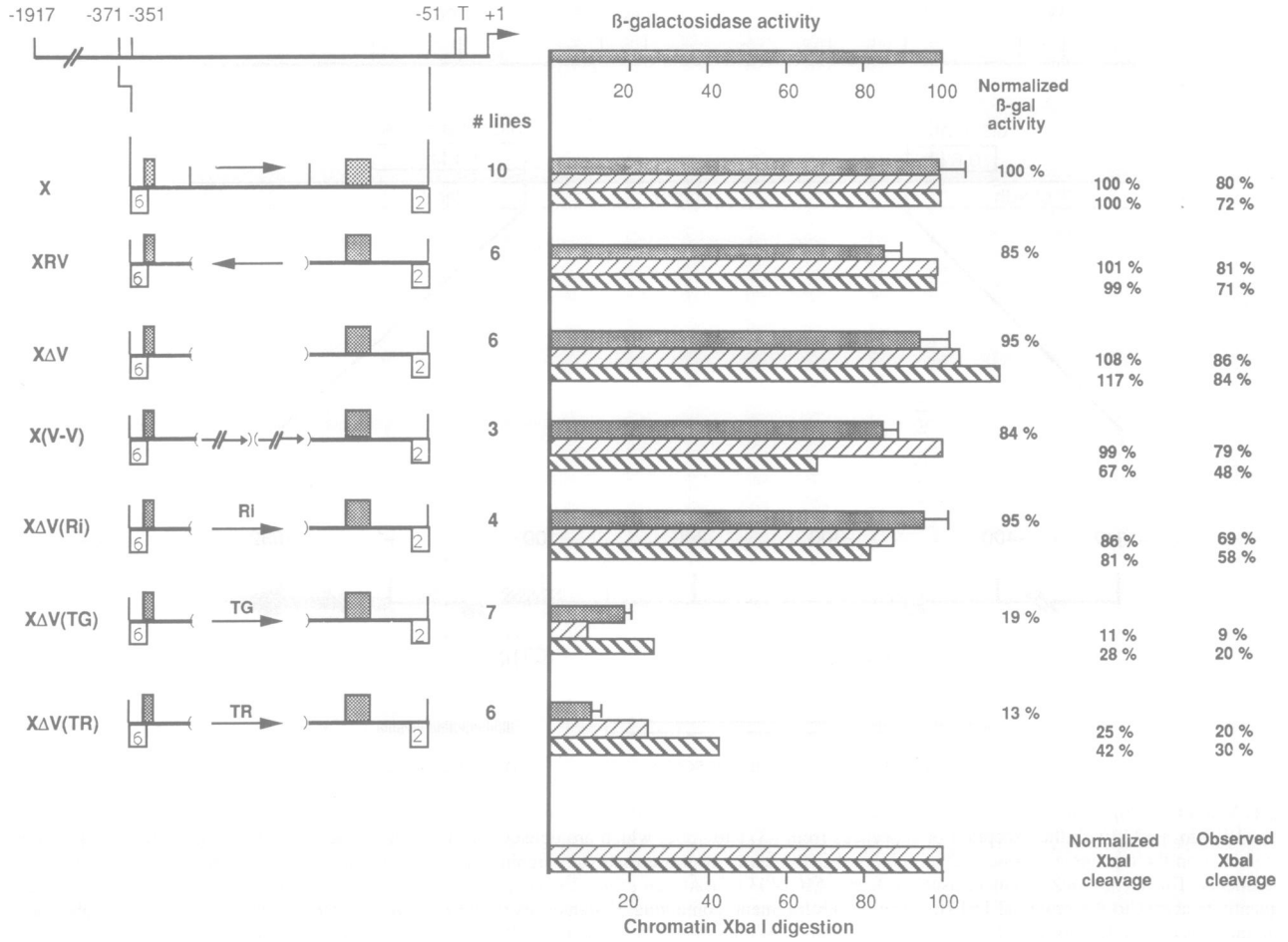

Fig. 2. Summary of inducible expression and *Xba*I accessibility of *hsp26–lacZ* transgenes. The nucleosome-associated sequence is designated as V, representing υ, the symbol for a nucleosome. Transgenes with alterations in the nucleosome-associated region are schematically presented on the left side of the figure. Functional HSEs (HSE 1–2 and HSE 6, numbered boxes below the line) and sequences containing $(CT)_n$•$(GA)_n$ repeats (boxes above the line) are positioned as indicated. 'T' (top line) indicates the TATA box. The wild-type nucleosome-associated sequence is indicated by an arrow, with alternative sequences diagramed by a labeled arrow. The number of independent transformed lines used to determine heat shock-inducible β-galactosidase activity is shown. Two lines giving responses closest to the mean were used for chromatin structure analysis. Within the bar graph, the first bar for each transgene shows relative levels of heat shock-induced expression; the second bar (thin, left-diagonal lines) shows relative values of accessibility of the proximal DH site (from results shown in Figure 4A); the third bar (thick, right-diagonal lines) shows relative values of accessibility of the distal DH site (from results shown in Figure 4B). The percentage values represent average levels of heat shock-induced β-galactosidase activity and accessibility of the *Xba*I sites for each transgene, shown normalized to the values obtained for CarX. Standard errors of the mean are given by the thin line.

site maps closer to the proximal site (generating one large hypersensitive region), as expected if the distal DH site forms on the same DNA sequences as before, just shifted downstream by the 168 bp deletion. When the nucleosome-associated sequence is tandemly duplicated [transgene CarX(V-V)], the distal DH site maps further from the proximal site, as expected if it were formed on the same DNA sequence shifted upstream by the 168 bp insertion. For transgene CarXΔV, the accessibility of the *Xba*I sites is 108% (proximal *Xba*I site) and 117% (distal *Xba*I site) compared with that of the wild-type control transgene (CarX). For transgene CarX(V-V), the accessibility of the *Xba*I sites is 99% (proximal *Xba*I site) and 67% (distal *Xba*I site) compared with that of CarX (Figures 2 and 4). These results indicate that the local sequence is the major determinant for DH site formation, although proximity to the start site may have some effect (note the difference between the values for the accessibility of the distal DH site). The results are consistent with earlier studies indicating a critical role for the $(CT)_n$•$(GA)_n$ repeats in DH site formation; these sites appear to function through binding of GAGA factor (Lu *et al.*, 1993b).

The distance between the two essential $(CT)_n$•$(GA)_n$ repeats in transgene CarXΔV has been shortened to ~50 bp due to deletion of the nucleosome-associated sequence; this is too short to accommodate a nucleosome without covering the $(CT)_n$•$(GA)_n$ repeats and the HSEs. The observations that the transgene responds fully to heat shock induction (95%) and that the *Xba*I sites show normal accessibility (118% and 117%) indicate that the regulatory regions are nucleosome free. In fact, the promoter region of CarXΔV resembles that of the *Drosophila hsp70* gene, in which the HSEs and $(CT)_n$•$(GA)_n$ repeats are dispersed throughout the promoter region. DH sites are detected over the region between –200 and +100 of *hsp70* (Wu, 1980; Figure 5A). To examine whether DH sites co-map with the $(CT)_n$•$(GA)_n$ repeats and associated HSEs in altered *hsp70* transgenes, we analyzed the chromatin structure of transgenes cBs+127Z and cBs+331Z, in which a 127 bp or a 331 bp random sequence has been

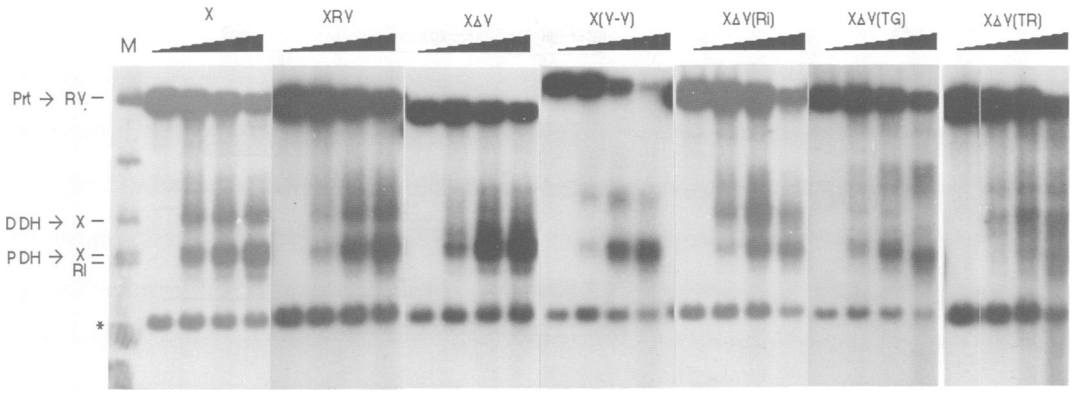

Fig. 3. DNase I hypersensitive site analyses of the *hsp26–lacZ* transgenes. Nuclei were isolated from non-heat-shocked larvae, treated briefly with DNase I and the purified DNA was digested to completion with *Eco*RV. In addition, these samples were further digested to completion with *Sma*I, which does not cleave within the parental *Eco*RV fragment of interest, but does cut an unrelated DNA fragment into the observed band (*). The samples were subjected to indirect end-labeling analysis using the 1.1 kb *lacZ* fragment as a probe (Figure 1). DH sites are indicated by arrows labeled 'P DH' (proximal DH site) and 'D DH' (distal DH site). The parental band, which is created by *Eco*RV digestion of DNA not cleaved in nuclei by DNase I, is indicated by an arrow labeled 'Prt'. * denotes an unrelated band derived from the P-element vector that cross hybridizes to *lacZ* sequences. Transgenes analyzed are identified above the appropriate lanes. Wedge shapes represent increasing amounts of DNase I used. M, markers; RI, *Eco*RI; RV, *Eco*RV; X, *Xba*I.

inserted between HSE1 and HSE2 of the *hsp70* gene promoter, respectively (Figure 5A). Both transgenes respond to heat shock induction at a level comparable with that of the wild-type control (Simon and Lis, 1987). The insertion in each case moves the HSE2/$(CT)_n$•$(GA)_n$ element further upstream of the transcription start site. Correspondingly, the DH sites also map further upstream (see Figure 5B). In transgene cBs+127Z, the sequence between HSE1/$(CT)_n$•$(GA)_n$ and HSE2/$(CT)_n$•$(GA)_n$ (~130 bp) is not of sufficient length to accommodate a nucleosome; the entire region is hypersensitive to DNase I, indicating an absence of nucleosomes (Figure 5B). In transgene cBs+331Z, the 331 bp insertion is sufficiently long to accommodate a nucleosome, and relative protection from DNase I is seen over the region (Figure 5B) as was the case for CarX(V-V). Taken together, the results indicate that the formation of the DH sites associated with the HSE/$(CT)_n$•$(GA)_n$ elements is relatively autonomous, with nucleosome formation occurring between the $(CT)_n$•$(GA)_n$ repeats where sufficient DNA is present.

Replacement of the nucleosome-associated sequence with sequences containing 10 bp periodicity alters chromatin structure and blocks heat shock-inducible expression

Shrader and Crothers (1989) have shown that stable histone–DNA complexes form *in vitro* on DNA fragments with a 10 bp sequence periodicity that results in anisotropic flexibility. We have investigated DNA fragments TG-5 and TR-5; TG-5 has five copies of a 20 bp oligonucleotide in which 5 bp of $(A/T)_3N_2$ alternate with 5 bp of $(G/C)_3N_2$, while TR-5 has five copies of a 20 bp oligonucleotide in which five bp of A_2N_3 alternate with 5 bp of T_2N_3 (see Materials and methods). *In vivo* studies have suggested that these sequences preferentially associate on one side of a nucleosome, rather than crossing the pseudodyad axis (Tanaka *et al.*, 1992). A TG-2 element (and other similar sequences with anisotropic flexibility) has been used in many instances to provide a strong translational signal for specific positioning of short DNA fragments on a histone

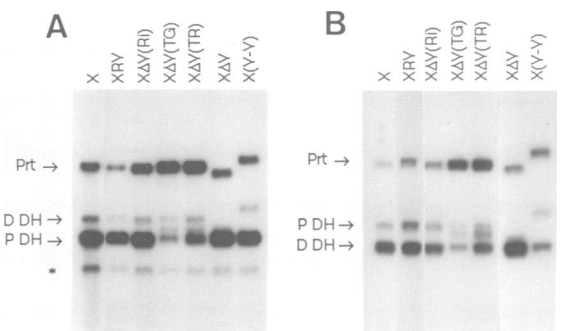

Fig. 4. Accessibility of the *Xba*I sites within the *hsp26–lacZ* transgenes. (**A**) *Xba*I accessibility within the proximal DH site. Autoradiograph of an indirect end-labeling analysis of DNA isolated from *Xba*I-treated nuclei from non-heat-shocked larvae, probed with the 1.1 kb *lacZ* fragment (Figure 1). Transgenes are indicated by the labels over each lane. The parental band, which is created by *Eco*RV digestion of the DNA not cleaved in nuclei at either *Xba*I site, is indicated by 'Prt'. The band indicated by * is unrelated to this group of fragments. The relative accessibility for each *Xba*I site in each transgene is taken as the intensity of the band representing cleavage at that site compared with the total intensity of the bands reflecting cleavage at the proximal *Xba*I site, the distal *Xba*I site, and neither site. The results are summarized in Figure 2. Note that the TG-5 and TR-5 sequences introduce an additional *Xba*I site; the intensity of the resulting band is included in the sum given above. Due to sequence deletion or insertion, the relative position of the distal DH site is shifted in transgenes CarXΔV and CarX(V-V). (**B**) *Xba*I accessibility within the distal DH site. Autoradiograph of an indirect end-labeling analysis of DNA isolated from *Xba*I-treated nuclei from non-heat-shocked larvae probed with the 0.6 kb *xdh* fragment (Figure 1). The particular transformants used are identified above each lane. The band labeled 'Prt' is derived from *Sma*I and *Hpa*I digestion of DNA not cleaved in nuclei at either *Xba*I site. No bands from the endogenous *xdh* gene are observed, since the region containing the 0.6 kb fragment has been deleted in the host stock ry^{506}. All other designations are as described for (A). The relative accessibility for each transgene is summarized in Figure 2.

core *in vitro* (Wolffe and Drew, 1989; Imbalzano *et al.*, 1994; Kwon *et al.*, 1994; reviewed by Lu *et al.*, 1994).

The fact that the nucleosome-forming sequence at *hsp26* is non-essential (see above) provides an opportunity to test the nucleosome-forming capabilities of TG-5 and of

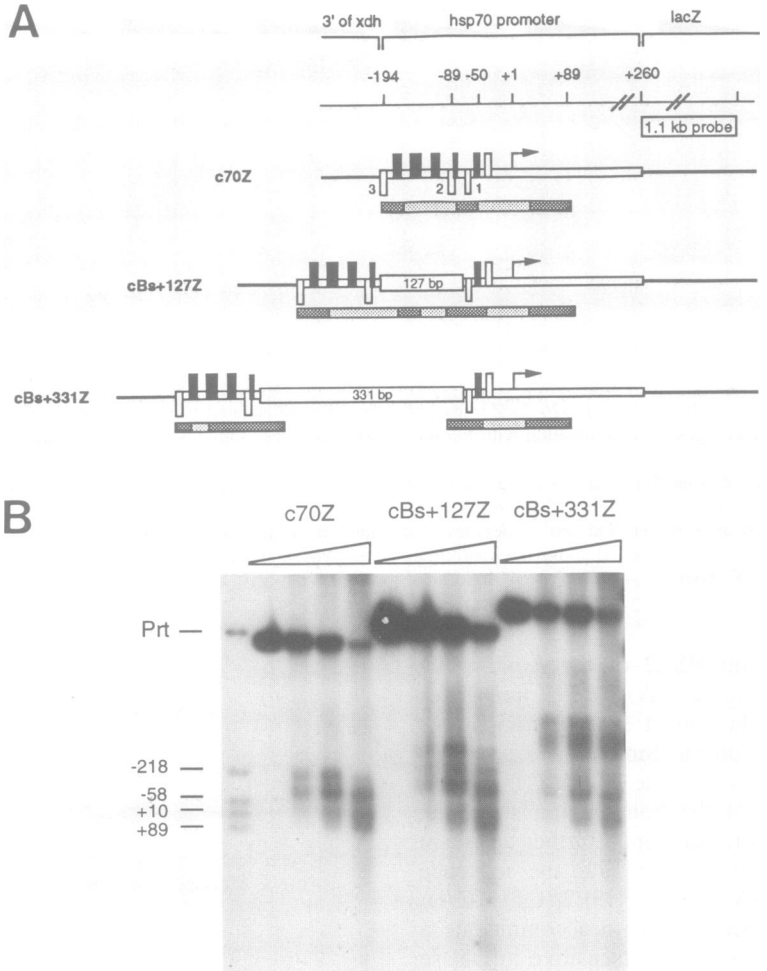

Fig. 5. DNase I hypersensitive site analyses of *hsp70–lacZ* transgenes. (**A**) Organization of the *hsp70–lacZ* transgenes and summary of the DH sites observed. Filled boxes above the lines indicate $(CT)_n$•$(GA)_n$ repeats; empty boxes below the lines indicate HSEs; an empty box above each line indicates the TATA box. Bent arrows indicate the transcriptional start site. Shaded regions below each transgene represent regions of hypersensitivity to DNase I, with the darkly shaded regions being relatively more sensitive than the lightly shaded regions. (**B**) Nuclei were isolated from non-heat-shocked larvae, treated briefly with DNase I and the purified DNA was digested to completion with *Eco*RV. The samples were subjected to indirect end-labeling analysis using the 1.1 kb *lacZ* fragment as a probe. The parental band, which is created by *Eco*RV digestion of DNA not cleaved in nuclei by DNase I, is indicated by a line labeled 'Prt'. Transgenes analyzed are identified above the appropriate lanes. Wedge shapes above each sample set represent increasing amounts of DNase I used. Numbering is from the transcription start site.

TR-5 sequences *in vivo*. A centrally positioned nucleosome should result in a fully active promoter, while formation of a dinucleosome, centered by the TG sequence, would block access to the adjacent regulatory sequences. Constructs CarXΔV(TG) and CarXΔV(TR) were made, in which the 168 bp nucleosome-associated region at the promoter of *hsp26* was replaced with a 168 bp DNA fragment containing either the 100 bp TG-5 sequence or the 100 bp TR-5 sequence in the center (Figure 2 and Materials and methods). The heat shock-inducibility of both transgenes was dramatically reduced in multiple independently isolated lines, to an average of 19% and 13% that of the wild-type control transgene (CarX) (Figure 2). Chromatin structure analysis using DNase I indicated that accessibility to the regions of the usual DH sites in these transgenes [CarXΔV(TG) and CarXΔV(TR)] was severely reduced (Figure 3). In transgene CarXΔV(TG), the accessibility of the *Xba*I sites located within the HSEs is reduced to 11% (proximal *Xba*I site) and 28% (distal *Xba*I site) compared with that of CarX (Figures 2 and 4); in transgene CarXΔV(TR), accessibility is reduced to 25% (proximal *Xba*I site) and 42% (distal *Xba*I site) compared with CarX (Figures 2 and 4). Clearly, these data indicate that replacement of the wild-type nucleosome-associated sequence with the TG-5 or TR-5 sequences favors formation of a chromatin structure that blocks heat shock-inducible expression of these transgenes.

We further characterized the chromatin structure of transgenes CarXΔV(TG) and CarXΔV(TR) using micrococcal nuclease (MNase). Nuclei were isolated from non-heat shocked mid-third instar larvae and aliquots were treated with increasing amounts of MNase. The wild-type transgene CarX exhibits enhanced MNase cleavage sites in regions around the proximal and the distal *Xba*I sites, as well as at the *Eco*RI site at +7 (indicated by open circles in Figure 6); these sites of enhanced cleavage correspond well to the DH sites. However, in both transgenes CarXΔV(TG) and CarXΔV(TR), the MNase cleavage sites around the proximal *Xba*I site (–51) are dramatically reduced, and cleavage is virtually eliminated

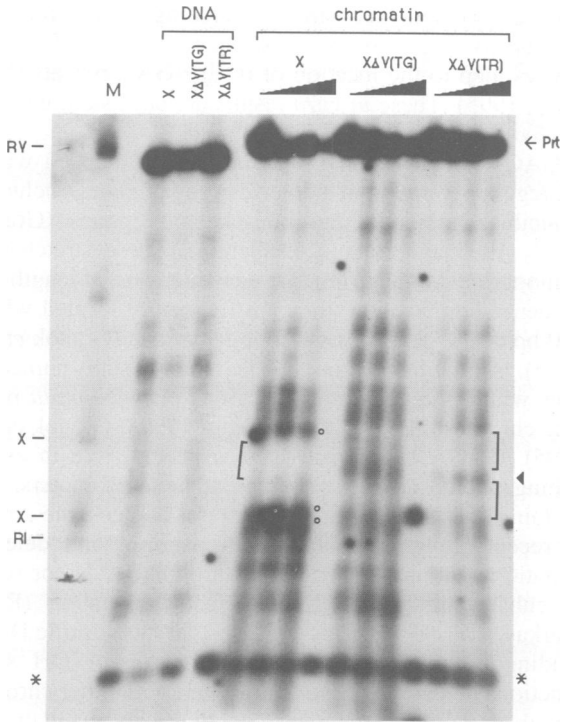

Fig. 6. Micrococcal nuclease analyses of transgenes CarXΔV(TG) and CarXΔV(TR). Nuclei were isolated from non-heat-shocked larvae, treated briefly with MNase and the purified DNA was digested to completion with *Eco*RV. These samples were further digested to completion with *Sma*I, which does not cleave within the parental *Eco*RV fragment of interest, but does cut an unrelated DNA fragment into the observed band (indicated by *). The samples were subjected to indirect end-labeling analysis using the 1.1 kb *lacZ* fragment as a probe (Figure 1). DNA and chromatin samples were run on the same gel; appropriate lanes were grouped for photographic purposes. The parental band, which is created by *Eco*RV digestion of DNA not cleaved in nuclei by MNase, is indicated by an arrow labeled 'Prt'. Transgenes analyzed are identified above the appropriate lanes. Wedge shapes represent increasing amounts of MNase used. Brackets represent the regions protected from digestion by MNase. For CarX, this region has been shown to be associated with a nucleosome (Thomas and Elgin, 1988). Enhanced MNase cleavage sites in the wild-type transgene (CarX) are indicated by open circles. An enhanced cleavage site at the center of the TG-5 and TR-5 sequences is indicated with an arrowhead. M, markers; RI, *Eco*RI; RV, *Eco*RV; X, *Xba*I.

around the distal *Xba*I site (–351) (Figure 6). Moreover a strong band, resulting from preferential MNase cleavage in the center of the TG-5 or TR-5 sequences, is observed for both of the transgenes (indicated with an arrowhead, Figure 6); the corresponding region of CarX is not sensitive to MNase cleavage (Figure 6). The results clearly show that an abnormal chromatin structure is imposed by the TG-5 or TR-5 sequences, and are consistent with the suggestion that these sequences favor formation of a dinucleosome of sufficient stability to interfere with DH site formation at the $(CT)_n$•$(GA)_n$ sites directed by GAGA factor.

Discussion

Nucleosome folding of intervening DNA neither facilitates nor hinders expression

Eukaryotic genomes have an abundance of DNA with no apparent function. Regulatory elements are often located at considerable distances from the promoters they affect. Packaging of the DNA in nucleosomes and higher order structures accomplishes a structural looping, bringing distant sequences closer together. Laybourn and Kadonaga (1992) have demonstrated that gene activation from distal GAL4 regulatory elements (1300 bp upstream) is facilitated by packaging of the template into chromatin (one nucleosome per 200 bp), presumably by bringing GAL–VP16 closer to the transcriptional machinery at the start site. Reducing the distance between regulatory elements confers an obvious advantage where protein–protein interactions are required. Packaging has also been reported to confer an advantage over short distances (~200 bp) *in vitro*. Schild *et al.* (1993) investigated the role of a nucleosome positioned between the estrogen response element (ERE) and the promoter of the *Xenopus vitellogenin B1* gene. Both packaging the DNA between the ERE and the promoter into a nucleosome and deleting this region enhanced transcription 5- to 10-fold. Interestingly, when the deletion template was assembled into chromatin, expression was down-regulated, suggesting that correct packaging was preferred to the simple spatial proximity achieved by the deletion. In contrast, transgene CarXΔV, with the normal nucleosome-associated region deleted, is expressed at a level 95% that of the wild-type transgene CarX (Figure 2). For *hsp26*, deletion of the nucleosome-associated sequence in a chromatin context does not appear to have any negative or positive effects on heat shock-induced expression. There is no advantage *in vivo* in having this region wrapped around a nucleosome compared with a deletion that provides spatial proximity of the regulatory elements.

A key difference between the *vitellogenin B1* and *hsp26* systems may lie in the mechanism used for nucleosome positioning. At *hsp26*, the nucleosome is flanked by $(CT)_n$•$(GA)_n$ repeats; in creating DH sites, these repeats define the nucleosome position precisely. *In vitro* assembly of histones on this DNA does not recreate the original preferred position (J.J.Hayes and A.P.Wolffe, personal communication). The nucleosome in the *vitellogenin B1* promoter does reassemble at the same position as seen *in vivo*; a conserved sequence near the 5′ boundary has been suggested to function as a positioning element (Schild *et al.*, 1993). The deletion constructs of the *vitellogenin B1* gene which were studied lack this sequence, and appear to be assembled in a random nucleosome array, repressing expression. In contrast, the *hsp26* deletion constructs retained all of the $(CT)_n$•$(GA)_n$ repeats critical for organization of the DH sites. Thus, these transgenes were correctly assembled and normally expressed in the absence of the nucleosome sequence. We infer that nucleosomes in promoter regions may be established by neighboring positioning signals, as appears to be the case for *hsp26*, and in other instances, by signals within the nucleosome-associated sequence itself, as appears to be the case for *vitellogenin B1*.

In addition to spacing changes, a second consideration in the interaction of regulatory factors is their position on one side or the other of the double helix. Cohen and Meselson (1988) performed a series of experiments to address this issue by making insertions of various sizes between the two most proximal HSEs and between the proximal HSE and the TATA box of the *D.melanogaster*

hsp70 promoter. They discovered that insertions of an integral multiple of 10.5, one turn of the B form DNA helix, ±1 bp showed a small reduction in heat shock-induced expression. Insertions that were at least 3 bp different from an integral multiple of 10.5 bp showed more severe reductions in heat shock-induced expression. These data imply that the *trans*-acting factors require a specific rotational setting for complete activation of *hsp70;* however, such requirements were evident only when the distance between the two heat shock elements was <40 bp and were not evident at distances of ~300 bp. In similar experiments using *hsp26*, a 5 bp insertion between the proximal $(CT)_n$•$(GA)_n$ element and the proximal HSE, which alters the position of the distal HSE (261 bp upstream) by 5 bp, showed nearly wild-type levels of heat shock-induced expression (Elgin *et al.,* 1993). These data, taken together with the spacing changes described here, demonstrate that the protein–protein interactions at *hsp26* required for transcriptional activation exhibit considerable flexibility.

$(CT)_n$•$(GA)_n$ elements drive the formation of DH sites

The *hsp26* gene is packaged in a 'preset' chromatin configuration (reviewed by Lu *et al.*, 1994; Wallrath *et al.*, 1994); there are no major changes in the chromatin structure upon activation of transcription. This is in contrast to promoters that undergo 'remodeling' upon activation such as those that regulate transcription from *PHO5, GAL1, Sgs3, TAT,* the MMTV LTR and the HIV type 1 5′ LTR. In these cases, nucleosomes positioned over the regulatory elements must be perturbed to allow for the binding of *trans*-acting regulators (Archer *et al.*, 1991; Reik *et al.*, 1991; Straka and Hörz, 1991; Axelrod *et al.*, 1993; Georgel *et al.*, 1993; Verdin *et al.*, 1993).

Studies both *in vivo* and *in vitro* indicate that the GAGA factor plays a key role in generating the preset chromatin structure of heat shock genes. Analysis of *hsp26–lacZ* transgenes with deletions or alterations of the $(CT)_n$•$(GA)_n$ repeats have shown that these sequences are needed to set up the correct chromatin structure; most likely these sites function through binding of GAGA factor (Lu *et al.*, 1992, 1993b). The analysis shown here of transgenes CarXΔV, CarX(V-V), cBs+127Z and cBs+331Z (Figures 3 and 5) finds that in all cases the DH sites co-map to the locations of the HSE/$(CT)_n$•$(GA)_n$ elements, regardless of the distance between the two HSE/$(CT)_n$•$(GA)_n$ elements or of the distance between the distal HSE/$(CT)_n$•$(GA)_n$ element and the transcription start site. These data suggest that GAGA factor has the ability to establish a local nucleosome-free environment. This is consistent with *in vitro* studies using purified GAGA factor and *Drosophila* chromatin assembly systems (Kerrigan *et al.*, 1991; Tsukiyama *et al.*, 1994). In *in vitro* nucleosome assembly and transcription reactions, GAGA factor has been shown to counteract the effects of histone-mediated repression (Kerrigan *et al.*, 1991). Tsukiyama *et al.* (1994) have shown that GAGA factor can disrupt nucleosome organization in an ATP-dependent reaction on an *hsp70* template assembled into nucleosomes. This disruption lead to a reorganization of the nucleosomes *in vitro*, with the generation of DH sites over the HSE/$(CT)_n$•$(GA)_n$ elements, a chromatin structure that parallels what is seen *in vivo*. Further *in vitro* reconstitution studies with *hsp26* deletion constructs (including one that removes the nucleosome-associated sequence) show that DH sites always map to the location of $(CT)_n$•$(GA)_n$ repeats (Wall *et al.*, 1995). These *in vitro* results are consistent with the *in vivo* findings reported here.

GAGA factor binding sites have been mapped within the regulatory region of a diverse group of genes, including inducible, homeotic and housekeeping genes (Granok *et al.,* 1995 and references therein). These stretches of homopurine–homopyrimidine elements vary in length and sequence composition; they are frequently located within 200 bp of the gene's transcription start site (Granok *et al.,* 1995). Antibodies against GAGA factor stain numerous sites within the euchromatic regions of *Drosophila* polytene chromosomes (Tsukiyama *et al.*, 1994; Granok *et al.*, 1995). GAGA factor may have a general role in establishing nucleosome-free regions at gene promoters, and in doing so, serving as a boundary for nucleosome arrays. A recent study has found that insertion and deletion mutations within the gene encoding GAGA factor result in enhancement of position effect variegation (PEV) (Farkas *et al.*, 1994). This suggests that this specific DNA-binding protein, which drives formation of DH sites, functions in a competitive equilibrium with other chromosomal proteins (suppressors of PEV) to maintain the accessible chromatin structure utilized for transcription.

A shift in chromatin structure imposed by the TG-5/TR-5 sequences represses gene activation in the presence of all known necessary regulatory elements

The observation that stable nucleosomes are formed with the TG-5 or TR-5 sequences preferentially on one side of the pseudodyad axis has been reported from studies of these sequences in yeast (Tanaka *et al.*, 1992). Thoma and his colleagues introduced the TG-5 sequence into various regions of a circular yeast plasmid: within the region of a nucleosome, on the edge of a nucleosome, and in a nuclease sensitive region. Their results show that the TG-5 sequences within long stretches of DNA do not center a nucleosome; rather, the TG-5 sequence usually positions a nucleosome on one side or the other, suggesting that the 10 bp repeat is not favored in crossing the pseudodyad axis under these circumstances (Tanaka *et al.*, 1992). The propensity of sequences with anisotropic flexibility to position nucleosomes is sufficiently strong that such sequences are now used routinely to set the position of DNA fragments on histone octamers for *in vitro* binding studies (see Wolffe and Drew, 1989; Imbalzano *et al.*, 1994; Kwon *et al.*, 1994). This positioning, achieved *in vitro,* is clearly a function of DNA–histone interactions.

The results of our studies on transgenes CarXΔV(TG) and CarXΔV(TR) indicate that these repetitive sequences, put in place of an otherwise non-essential region at the *hsp26* promoter, can drive a significant change in chromatin structure as shown by several methods. First, the DH sites covering the two essential HSEs, which are evident in the wild-type control transgene CarX, are significantly diminished in CarXΔV(TG) and CarXΔV(TR) (Figure 3). Second, the accessibility of the *Xba*I sites (at –51 and –351) in nuclei is significantly reduced compared with that in the wild-type control transgene CarX (Figures 2 and 4). Third, MNase analysis

reveals that the central portion of the TG-5 and TR-5 sequences are preferentially sensitive to MNase digestion, while the surrounding sequences are protected, presumably packaged into nucleosomes (Figure 6). This suggests formation of a dinucleosome at the insert, extending over the adjacent regulatory regions. Collectively, the above assays demonstrate that both the TG-5 or TR-5 sequences cause structural changes at the gene promoter which significantly reduce the accessibility of the HSEs and the extent of transcriptional activation.

In transgenes CarXΔV(TG) and CarXΔV(TR), the HSE/$(GA)_n$•$(CT)_n$ elements have lost sensitivity to DNase I, *Xba*I and micrococcal nuclease (Figures 3, 4 and 6). For this reason, we infer that the juxtaposition of the HSE/$(GA)_n$•$(CT)_n$ elements with either the TG-5 or TR-5 sequence (Figure 6) creates an unfavorable situation for GAGA factor binding. No doubt this reflects the very stable nucleosome structure observed *in vitro* with TG-5 and TR-5. The results suggest a direct competition for binding between GAGA factor and nucleosomes. Alternatively, GAGA factor may remodel chromatin by a nucleosome 'sliding' mechanism proposed by Wall *et al.* (1995). Under this assumption, GAGA factor would normally cause nucleosomes to move away from the $(CT)_n$•$(GA)_n$ elements into neutral sequence environments, generating a DH site. Nucleosomes may remain positioned on TG-5 and TR-5 because GAGA factor is unable to promote dissociation of these very stable complexes. It will be of interest to test these constructs further, both in *in vitro* assembly systems and *in vivo* with modifiers of PEV, to better establish the nature of the competition to set a chromatin structure that allows or blocks gene expression.

Materials and methods

DNA constructs

The numerical assignment of nucleotides and the transcription start site of the *Drosophila hsp26* gene is based on the description of Ingolia and Craig (1981). Plasmid CarX has been described previously (Lu *et al.*, 1992). CarX contains the *xanthine dehydrogenase* gene as a marker, and *hsp26* sequences from −1917 to +632 (except that sequences from −371 to −352 are deleted), fused in-frame to the *E.coli lacZ* gene. Plasmids CarXΔV, CarXRV and CarX(V-V) were made as follows. The 299 bp *Xba*I fragment of *hsp26* (−351 to −52) was isolated, and digested with *Alu*I which cleaves at −312 and −144. The *Alu*I-digested *Xba*I fragment was mixed with *Xba*I-digested M13mp18 in the presence of T4 DNA ligase. Recombinant M13 clones were verified by sequencing. Clones containing the *Xba*I fragment with the internal *Alu*I fragment (−144 to −312) deleted, reversed or doubled were selected, and designated as M13mp18XΔV, M13mp18XRV or M13mp18(V-V), respectively. The corresponding *Xba*I fragment was isolated from the replicative form of M13mp18XΔV, M13mp18XRV or M13mp18V-V, and used to replace the *Xba*I fragment in CarX, forming CarXΔV, CarXRV or CarX(V-V), respectively.

Constructs in which the nucleosome-associated *Alu*I fragment (−312 to −144) was replaced by other sequences were made as follows. An *Xho*I linker (5′-CCTCGAGG-3′) was inserted into the *Alu*I site within the XΔV fragment of M13mp18XΔV, forming M13mp18XΔV(Xho). The replicative form of M13mp18XΔV was linearized by *Xho*I and the ends were filled in using Klenow in the presence of all four nucleotide triphosphates. To make plasmid CarXΔV(Ri), bacteriophage λ DNA was partially digested with *Alu*I and *Rsa*I, and the products were fractionated on a 2% agarose gel; gel slices containing fragments ~156 bp in length were excised and the DNA purified. The purified λ DNA fragments were ligated into the filled ends of *Xho*I-linearized M13mp18XΔV(Xho). A recombinant clone containing an insertion of 156 bp of λ DNA was identified by sequencing and designated M13mp18XΔV(Ri). An *Xba*I fragment that contains the random sequences in place of the nucleosome-associated sequences was isolated from the replicative form of M13mp18XΔV(Ri) and used to replace the *Xba*I fragment in CarX, resulting in CarXΔV(Ri). Thus in CarXΔV(Ri), the 168 bp wild-type nucleosome-associated sequence has been replaced by a 156 bp λ DNA sequence plus the *Xho*I linker sequence to give 168 bp. Plasmids CarXΔV(TG) and CarXΔV(TR) were made as follows. *Alu*I fragments (156 bp) were isolated from plasmid pTG-5 and pTR-5 (Shrader and Crothers, 1989), and separately cloned into the filled ends of *Xho*I-linearized M13mp18XΔV(Xho), forming M13mp18XΔV(TG) and M13mp18XΔV(TR), respectively. The *Xba*I fragments containing TG-5 or TR-5 sequence were isolated from the replicative form of M13mp18XΔV(TG) or M13mp18XΔV(TR), and used to replace the *Xba*I fragment in CarX, resulting in CarXΔV(TG) and CarXΔV(TR), respectively. In CarXΔV(TG) the 168 bp wild-type nucleosome-associated sequence was replaced by a 168 bp sequence 5′-TCGAGGCTCG-CCCGGCCGGGGATCGC[**TCGGTGTTAGAGCCTGTAAC**]$_5$ TCG-GGTGGATCCTCTAGAGTCGACCTGCAGCCCAAGCCTCGA-3′. In CarXΔV(TR), the 168 bp wild-type sequence was replaced by the sequence 5′-TCGAGGCTCGCCCGGCCGGGGATCGC [**TCGGAA-GACTTGTCAACTGT**]$_5$ TCGGGTGGATCCTCTAGAGTCGACCT-GCAGCCCAAGCCTC GA-3′. *Xba*I cleavage sites introduced by the insert are underlined above.

DNA constructs for the *hsp70–lacZ* transgenes (c70Z, cBs+127Z, cBs+331Z) have been described previously (Simon and Lis, 1987). c70Z is a wild-type control, which contains *hsp70* promoter sequence from −194 to +260, fused in-frame to the *E.coli lacZ* gene. Transgenes cBs+127Z and cBs+331Z are identical to c70Z except that they contain an insertion of a 127 bp or a 331 bp DNA fragment from pBR322 at position −50 (Simon and Lis, 1987).

Drosophila germline transformation

Drosophila lines with one copy of the *hsp70–lacZ* transgenes (c70Z, cBs+127Z or cBs+331Z) have been described previously (Simon and Lis, 1987). Construct CarX had also been previously introduced into the *Drosophila* genome (Lu *et al.*, 1993b). The remaining *hsp26–lacZ* constructs used here were introduced into the *D.melanogaster* germline by P-element mediated transformation (Rubin and Spradling, 1982; Spradling, 1986), using the *xanthine dehydrogenase* gene as a marker and ry^{506} as the host stock. Transformants from progeny of the injected embryos were identified by wild-type eye color. Transformants containing independent, single inserts of the P-element were identified by Southern blot analysis. Only transgenic lines containing a single insert were used in this study. The integrity of the transgenes was confirmed by genomic restriction mapping using the 1.1 kb *lacZ* sequence (Figure 1) as a probe (data not shown).

CPRG assays

Expression of *hsp26–lacZ* transgenes was assessed following heat shock by determining levels of β-galactosidase activity, using chlorophenol red-β-D-galactopyranoside (CPRG) as a substrate as described (Lu *et al.*, 1993b). Standard errors of the mean were 15% or less in each case. In the cases analyzed here, 3–10 independent lines were established for each transgene.

Indirect end-labeling chromatin structure analyses

For chromatin structure analyses, two independent transformed lines which showed average inducible β-galactosidase activity (as quantitated by CPRG assays) were used. Methods used for the isolation of nuclei, digestion of nuclei with DNase I, micrococcal nuclease (MNase) or a restriction enzyme; DNA purification, and indirect end-labeling Southern blot analysis were as described (Lu *et al.*, 1993a). Micrococcal nuclease analysis was performed using a 40 cm 1.2 % agarose gel for higher resolution of the digestion products. All other products were analyzed on a 20 cm 1.0 % agarose gel. The mapping strategy for the detection of the DH sites and the probes used for detecting the accessibility of *Xba*I sites within the proximal and distal DH sites are illustrated in Figure 1. For quantitation of the accessibility of the *Xba*I site within the proximal DH site, nuclei were treated with an excess of *Xba*I and the genomic DNA purified and restricted to completion with *Eco*RV and *Sma*I. After size fractionation by electrophoresis through a 1% agarose gel and transfer to a nylon membrane, the DNA was probed with the 1.1 kb *lacZ* fragment (Lu *et al.*, 1993b). The percent accessibility of the proximal *Xba*I site was determined by comparing the intensity of the band representing cleavage within the proximal DH site with the total intensity of the bands present (due to cleavage at the proximal DH site, the distal DH site and the parental *Eco*RV band). Measurements of the intensities of the bands on the autoradiographs were made using a densitometer (Molecular Dynamics).

For quantitation of the accessibility of the *Xba*I site within the distal DH site, DNA from nuclei treated with excess *Xba*I was purified and restricted with *Sma*I and *Hpa*I. The DNA was fractionated on a 1% agarose gel and transferred to a nylon membrane as above. The membrane was probed with a 0.6 kb DNA fragment from the 3′ region of the *xanthine dehydrogenase* gene (*xdh*), which is located upstream of the *hsp26* sequences in the constructs used here (Lu *et al.*, 1993b). The percent accessibility of the distal *Xba*I site was determined by comparing the intensity of the band representing cleavage within the distal DH site to the total intensity of the bands present (due to cleavage at the proximal DH site, the distal DH site and the parental band). The variation in accessibility at a given site is approximately ±5%.

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