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Promoter Sequence Containing $(CT)_n \cdot (GA)_n$ Repeats is Critical for the Formation of the DNase I Hypersensitive Sites in the *Drosophila hsp26* Gene

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We have analyzed P-element-transformed lines carrying *hsp26/lacZ* transgenes with various deletions and substitutions within the *Drosophila melanogaster hsp26* promoter region in order to identify the sequences required for the formation of the DNase I hypersensitive sites (DH sites). DH sites are generally found associated with promoters and enhancer elements of active and inducible eukaryotic genes, and are thought to be nucleosome-free regions of DNA that interact with regulatory proteins and the transcriptional machinery. There are two major DH sites located within the promoter region of the *hsp26* gene, centered at −50 and at −350 (relative to the *hsp26* transcription start site). The sequences from −135 to −85, which contain $(CT)_n \cdot (GA)_n$ repeats, contribute significantly to the formation of the DH sites in the *hsp26* promoter region. Deletion or substitution of this $(CT)_n$ region drastically reduces the accessibility of the DNA at these sites to DNase I. This reduction in accessibility was quantified by measuring the susceptibility of the DNA within nuclei to cleavage at a restriction site within the DH site. In addition to the $(CT)_n$ region and the promoter at −85 to +11 (region P), one of two other regions must be present for effective creation of the DH sites: sequences between −351 and −135 (region A), or sequences between +11 and +632 (region D). Disruption of the wild-type chromatin structure, as assayed by the loss of accessibility to the DH sites, is correlated with a decrease in inducible transcriptional activity, even when the TATA box and heat shock regulatory elements are present in their normal positions.

Keywords: chromatin structure; DH sites; gene expression; CT-binding protein/GAGA transcription factor; heat shock genes

1. Introduction

Gene expression in the eukaryotic cell is highly constrained by the packaging of DNA into a complex chromatin structure. Upon replication, DNA and histone are rapidly assembled into a nucleosome array, forming the 100 Å (1 Å = 0·1 nm) chromatin fiber, and are subsequently packaged into higher-order structures. Early studies on the organization of nucleosome arrays demonstrated that sites hypersensitive to digestion by nucleases such as DNaseI (DH‡ sites) were generally observed in association with active and inducible genes (Varshavsky *et al.*, 1978; Wu *et al.*, 1979). DH sites are commonly found at the promoters, upstream regulatory sites, and enhancers (whether 5′ or 3′) of these genes (Wu, 1980; for a review, see Gross & Garrard, 1988). Biochemical analyses of DH site regions (McGhee *et al.*, 1981), as well as footprinting of their chromatin structure in isolated nuclei

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‡ Abbreviations used: DH sites, DNase I hypersensitive sites; HSE, heat shock element; HSF, heat shock factor; kb, 10^3 bases or base-pair(s); bp, base-pair(s).

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(Thomas & Elgin, 1988), support the conclusion that these regions are free of nucleosomes (for a review, see Eissenberg *et al.*, 1985).

The features of chromatin that dictate the position of DH sites, gaps in the nucleosome array, may play critical roles in permitting certain protein–DNA interactions and in blocking others. How then are DH sites specified? The heat shock genes of *Drosophila melanogaster* are activated within minutes in response to a temperature shift or other stress; they exhibit a chromatin structure poised for transcriptional induction (Rougvie & Lis, 1988). Studies utilizing both ultraviolet photocrosslinking and run-on transcription assays have shown that RNA polymerase II is associated with the *hsp70* and *hsp26* promoter regions in *Drosophila* tissue culture cells prior to gene activation (Gilmour & Lis, 1986; Rougvie & Lis, 1988, 1990). Identified regulatory elements of *hsp26* located upstream from the transcription start site are the TATA box, proximal and distal heat shock elements (HSE) at positions −60 (HSE 1 and 2) and −350 (HSE 6), and the $(CT)_n \cdot (GA)_n$ repeats (Fig. 1). Extensive analyses of the upstream regulatory region of *hsp26* has demonstrated that the sequences from −351 to +632 are sufficient to mediate a heat shock response similar to that of the wild-type gene. Both the proximal HSE (HSE 1 and 2) and the distal HSE (HSE 6) are critical for heat shock induction; however, other recognizable elements with weak homology to HSE (HSE 3 to 5) located between −100 and −300, and HSE 7, do not appear to contribute significantly to heat shock induction (Cohen & Meselson, 1985; Pauli *et al.*, 1986; Simon & Lis, 1987; Glaser *et al.*, 1990).

High-resolution genomic footprinting analysis has revealed that within the broad proximal DH site, the TATA and $(CT)_n$ regions are both protected in non-heat-shocked nulei isolated from embryos, indicating that proteins are bound specifically to these regulatory elements prior to induction (Thomas & Elgin, 1988). In contrast, prior to heat shock, the two HSEs show a pattern of DNase I cleavage similar to that of naked DNA (Thomas & Elgin, 1988), indicating that these sites are completely accessible. After heat shock, DNase I footprinting indicates that these two HSEs are occupied by the heat shock factor (HSF; Topol *et al.*, 1985; Wu *et al.*, 1987; Thomas & Elgin, 1988). Analysis of the chromatin structure of *hsp26* with methidium-propyl-EDTA·Fe(II) and DNase I has revealed that the promoter region of *hsp26* is organized such that the HSEs lie within proximal and distal DH sites, while much of the remaining region between the DH sites is occupied by a positioned nucleosome (Fig. 1; Cartwright & Elgin, 1986; Thomas & Elgin, 1988). The wrapping of the DNA around this nucleosome may bring the proximal and distal HSEs into close proximity, facilitating interaction between the HSFs and the TATA-binding complex after heat shock induction.

While the roles of the various *cis*-acting sequence elements discussed above in heat-induced expression of *hsp26* have been extensively studied, the sequence requirements for setting up the DH sites, which maintain the HSEs in an open, accessible conformation, have not been delineated. Interactions of DNA with histones and non-histone chromosomal proteins are clearly involved in the process of DH site formation. *In vitro* studies of chromatin assembly and transcription have suggested that in some instances, binding of TFIID to the TATA box is sufficient to drive formation of an appropriate transcription complex in competition with nucleosome formation, while in other instances, ancillary proteins are necessary (Workman *et al.*, 1990; Becker *et al.*, 1991). We have investigated the sequence requirements for the formation of DH sites in the *hsp26* promoter by analyzing the chromatin structure generated *in vivo* for 12 variant *hsp26/lacZ* constructs with permutations within the promoter. These constructs were inserted into the *Drosophila* genome by P-element-mediated transformation (Rubin & Spradling, 1982). Chromatin structure of the transgenes was determined using larval nuclei from at least two independently transformed lines. Our results indi-

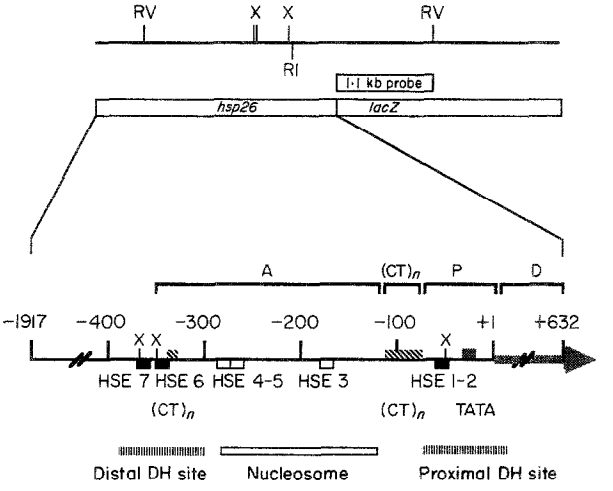


Figure 1. Map of *hsp26/lacZ* fusion gene. The structure of cP26Z-2kb is shown. The *hsp26* sequence from −1917 to +632 is fused in-frame to the *E. coli lacZ* gene as described (Glaser & Lis, 1990). Restriction sites shown on the top map are those sites giving marker fragments used in the chromatin structure analysis. The 1·1 kb DNA fragment used as probe in the indirect end-labeling analysis is the *Eco*RI–*Eco*RV fragment isolated from plasmid pMC1403, indicated as a box (Casadaban *et al.*, 1983). The partial restriction map of the *hsp26* sequence (−1917 to +632) is enlarged below with the $(CT)_n$ regions, the TATA box and the 2 required HSEs (numbers 1, 2 and 6, filled boxes) diagrammed. HSEs 3 to 5 (shown as open boxes) are not involved in heat shock induction. HSE 7 is accessible in isolated nuclei, but is not required for heat shock induction (Pauli *et al.*, 1986; Simon & Lis, 1987; Thomas & Elgin, 1988; Glaser *et al.*, 1990). Functional regions discussed in this paper are marked by brackets above the map, while chromatin structural features determined earlier are marked below. RI, *Eco*RI; RV, *Eco*RV; X, *Xba*I.

cate that the $(CT)_n$ region plays a central role in generating the DH sites; adjacent stretches of DNA are also required for appropriate DH site formation. Generation of the DH sites appears to be essential for subsequent gene activation.

2. Materials and Methods

(a) *Plasmid construction and germline transformation*

The plasmid constructs and P-element-transformed fly lines containing cP26Z-2kb (cP26Z-36A and cP26Z-84D), cP26ZΔX, cP26Z-70a and cP26Z-70b transgenes have been described by Simon & Lis (1987). All other constructs used in this study are described by Glaser *et al.* (1990) and Glaser & Lis (1990). Two independent lines showing average β-galactosidase activity for a particular construct (as quantified by CPRG assays) were used for chromatin structure determination. CPRG assays were carried out as described (Simon & Lis, 1987). The structures of the various *hsp26/lacZ* transgenes were confirmed by genomic restriction mapping of the DNA from the transformed lines (data not shown).

(b) *Preparation of larval nuclei, DNase I partial digestion and indirect end-labeling analyses*

Transformed fly lines were raised at 23 °C. Mid-third instar larvae were collected from the walls of the bottles. Non-heat-shocked larvae were collected, frozen in liquid nitrogen and then stored at −70 °C. For heat shock induction, the collected larvae were transferred to a 15 ml glass beaker kept humid with a disk of wet filter paper. The beaker was covered with aluminum foil and placed in an incubator at 37 °C for 45 min. The heat-shocked larvae were frozen in liquid nitrogen and stored at −70 °C.

Larvae (1 g) were pulverized in liquid nitrogen using a mortar and pestle. Then 3 ml of buffer A^+ solution (60 mM-KCl, 15 mM-NaCl, 1 mM-EDTA, 0·1 mM-EGTA, 15 mM-Tris·HCl (pH 7·4), 0·15 mM-spermine, 0·5 mM-spermidine, 0·5 mM-dithiothreitol, 0·5% (v/v) Nonidet P-40, 12 mM-EDTA) was added, the homogenate was transferred to a 7 ml ground glass homogenizer and disrupted with 5 strokes of the pestle. The homogenate was then transferred to a 15 ml Dounce homogenizer and disrupted with 10 strokes with a pestle (type B). The homogenate was filtered through 2 layers of 89 mm nylon mesh cloth. Then 2 ml of buffer A^+ was used to rinse the ground glass homogenizer and the Dounce homogenizer and filtered through the same 2 layers of nylon mesh cloth. The mixture was loaded over 1 ml of buffer AS (60 mM-KCl, 15 mM-NaCl, 1 mM-EDTA, 0·1 mM-EGTA, 15 mM-Tris·HCl (pH 7·4), 0·15 mM-spermine, 0·5 mM-spermidine, 0·5 mM-dithiothreitol, 0·3 M-sucrose) and centrifuged in a Sorval HB4 swinging bucket rotor at 4 °C, 3000 revs/min for 5 min. The recovered pellet was dispersed in 3 ml of buffer A^+ by 5 strokes with a pestle (type A) in a 7 ml Dounce homogenizer, and the mixture was loaded over 1 ml of buffer AS, and centrifuged as before. The recovered pellet was dispersed in 3 ml of buffer A (60 mM-KCl, 15 mM-NaCl, 1 mM-EDTA, 0·1 mM-EGTA, 15 mM-Tris·HCl (pH 7·4), 0·15 mM-spermine, 0·5 mM-spermidine, 0·5 mM-dithiothreitol) by 5 strokes in a 7 ml Dounce homogenizer with a pestle (type B). After pelleting at 2000 revs//min for 5 min the nuclei were resuspended in 1 ml of DNase I digestion buffer (60 mM-KCl, 15 mM-NaCl, 15 mM-Tris·HCl (pH 7·4), 0·25 M-sucrose, 3 mM-$MgCl_2 \cdot 6H_2O$, 0·05 mM-$CaCl_2 \cdot 2H_2O$, 0·5 mM-dithiothreitol). Portions of this suspension (250 μl each) were incubated with 0, 1, 2 or 5 μl of DNase I (20,000 units/ml; Worthington Biochemical Corporation, NJ, U.S.A.) on ice for 3 min with agitation. The digestion was stopped by the addition of EDTA to a final concentration of 8 mM. The DNA was purified as described by Wu *et al.* (1979).

Purified DNA from each sample was fractionated on an agarose gel to visualize the extent of digestion by staining with ethidium bromide. For the comparative studies shown in Figs 3 to 5, samples that showed equivalent extents of digestion from different transformed lines were used. Appropriate sample DNAs (8 μg) were cut to completion with *Eco*RV, fractionated on a 1·2% (w/v) agarose gel and transferred to Nytran membrane (Schleicher & Schuell, Inc.) by Southern blotting (Maniatis *et al.*, 1982). The membrane was hybridized with a 1·1 kb *lacZ* DNA fragment (Fig. 1) labeled with [α-^{32}P]dCTP using random hexamer priming (Feinberg & Vogelstein, 1983). The conditions for hybridization and washing were as described (Cartwright & Elgin, 1986). Membranes were exposed to preflashed XAR5 X-ray film (Kodak) for 12 to 24 h at −70 °C using 2 intensifying screens.

(c) *Restriction enzyme treatment of isolated nuclei and indirect end-labeling analyses*

Nuclei were isolated from non-heat-shocked larvae as described for DNase I analysis. The final pellet was resuspended in 1·47 ml of 10 mM-Tris·HCl (pH 7·5), 10 mM-$MgCl_2$, 50 mM-NaCl, 1 mM-dithiothreitol. Phenylmethylsulfonyl fluoride, aprotinin and leupeptin were added to final concentrations of 0·5 mM, 5 mg/ml and 0·1 unit/ml, respectively. Then 200 units of *Xba*I (New England Biolabs) were added to 250 μl portions of suspended nuclei and the mixture incubated at 37 °C for 45 min. Since the extent of cleavage at the proximal *Xba*I site of the *hsp26* promoter was used to quantify the accessibility of the sequences within the DH site, it was important to insure that the conditions allowed for complete restriction by the enzyme at all available sites. Using increasing amounts of *Xba*I, we previously determined that incubation with 200 units of *Xba*I under these conditions gave maximum restriction enzyme digestion (data not shown). The restriction enzyme digestion was terminated by the addition of EDTA to a final conentration of 8 mM and the DNA was purified according to Wu *et al.* (1979). One-half of each purified sample was cut to completion with 60 units of *Eco*RV and fractionated on a 1% (w/v) agarose gel, transferred to Nytran membrane and probed with the 1·1 kb fragment of the *E. coli lacZ* gene (Fig. 1) labeled as described above. Conditions for the Southern analysis were as published (Wallrath *et al.*, 1990), except 0·8 M-NaCl was used in the prehybridization and hybridization solution rather than 0·1 M-NaCl. Southern filters were exposed to preflashed XAR5 X-ray film (Kodak) for 36 h at −70 °C using 2 intensifying screens. The intensities of the bands on the Southern autoradiographs were measured using a scanning densitometer (Molecular Dynamics).

(d) *Northern blot analysis*

Total RNA was extracted from larvae by the hot phenol method (Jowett, 1986). DNA was removed by digestion with RNase-free DNase I (Boehringer-Mannheim Biochemicals). RNA samples were fractionated on a 1% (w/v) agarose gel with formaldehyde using standard methods (Maniatis *et al.*, 1982). RNA was transferred onto Nytran membrane in 20× SSC (SSC is 0·15 M-

NaCl, 0·015 M-sodium citrate (pH 7·0)) and the membrane baked at 80 °C in a vacuum oven for 30 min. Prehybridization was carried out in 50% formamide, 5× Denhardt's solution (Denhardt's is 0·02% (w/v) Ficoll, 0·02% (w/v) polyvinypyrollidone, 0·02% (w/v) bovine serum albumin), 0·1% (w/v) SDS, 5× SSPE (SSPE is 0·18 M-NaCl, 10 mM-$NaPO_4$ (pH 7·7), 1 mM-EDTA), with 150 μg salmon sperm DNA/ml at 42 °C for 1 h. Hybridization was carried out at 42 °C for more than 8 h in 50% formamide, 2·5× Denhardt's, 0·1% SDS, 5× SSPE, 150 μg salmon sperm DNA/ml and 10^6 cts of probe DNA/ml. The probe used was the 1·1 kb *lacZ* fragment (Fig. 1) labeled as described above. The membrane was washed twice in 6× SSPE, 0·1% SDS for 15 min at room temperature, and twice in SSPE, 0·1% SDS for 15 min at 37 °C. The filter was exposed to preflashed XAR5 X-ray film (Kodak) at −70 °C with 2 intensifying screens. Subsequently, the membrane was stripped by incubating in 300 ml of stripping solution (5 mM-Tris·HCl (pH 8·0), 0·2 mM-EDTA, 0·05% pyrophosphate, 0·1× Denhardt's) at 65 °C for 2 h, and then briefly rinsed in 2× SSPE. The membrane was reprobed under the same conditions with an *hsp70* promoter fragment (−254 to +87) labeled as described above.

3. Results

(a) *The TATA box and downstream sequences, in the absence of sequences from −52 to −372, are insufficient for normal DH site formation*

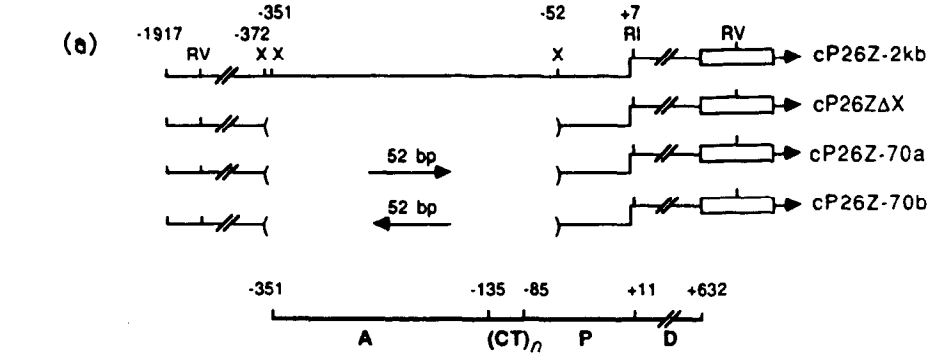
We have analyzed the DH sites of a series of constructs reintroduced into *D. melanogaster* by P-element-mediated germline transformation (Rubin & Spradling, 1982). To evaluate the role of the TATA box and downstream sequences in forming the DH sites in the *hsp26* promoter region, we analyzed a series of transformants containing *hsp26/lacZ* constructs with an upstream internal deletion or reinsertion of regulatory elements (Simon & Lis, 1987). To demonstrate the DH site pattern of the chimeric gene, nuclei isolated from non-heat-shocked larvae of the various transformed lines were treated briefly with DNase I, the DNA was purified and cut to completion with *Eco*RV, fractionated on an agarose gel, Southern blotted and the membrane probed with a *lacZ* fragment (Fig. 1). As shown in Figure 2(c), the control construct, with 1917 bp of upstream sequences (cP26Z-2kb), shows the characteristic wild-type pattern of two DH sites, one centered over the proximal HSEs (HSE 1 and 2) and the other centered over the distal HSEs (HSE 6 and 7). In cP26ZΔX, sequences from −372 to −52 are deleted, which removes the HSEs and $(CT)_n \cdot (GA)_n$ sequences, but leaves the TATA box, downstream sequences and sequences upstream from −372 (to −1917) of *hsp26* intact (Fig. 2(a)). In transformants carrying the cP26ZΔX transgene, the distal DH site was not detectable and the proximal DH site was severely reduced in intensity (Fig. 2(c)). Therefore, in the absence of the HSEs and $(CT)_n \cdot (GA)_n$ sequences, the TATA box and downstream sequences are clearly insufficient for the formation of normal DH sites.

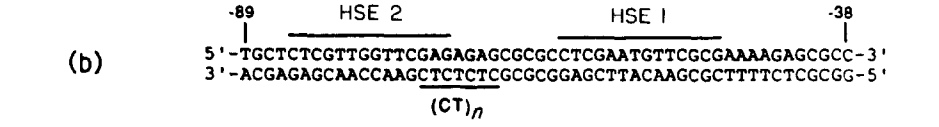
While heat shock inducibility of the cP26ZΔX transgene is abolished, insertion of a 52 bp sequence containing the *hsp70* regulatory region (−38 to −89) in either orientation at the site of the deletion of cP26ZΔX (cP26ZΔX-70a and cP26ZΔCT-70b) re-established the heat shock inducibility to 35 to 40% that of wild-type control (Simon & Lis, 1987). Examination of the DNase I hypersensitivity of these transgenes indicates that the insertion of the 52 bp fragment led to formation of a strong DH site (Fig. 2(c)). This short 52 bp sequence has only two known types of elements, HSEs and a short $(CT)_n$ sequence (7 bp). *In vitro* studies (Zimarino & Wu, 1987) and *in vivo* studies (Wu, 1984; Cartwright & Elgin, 1986; Thomas & Elgin, 1988) indicate that the HSEs in *Drosophila* are unoccupied prior to heat shock induction, and therefore unlikely to contribute to DH site formation. The results suggest that the $(CT)_n$ sequence, the only element within the 52 bp fragment of *hsp70* known to bind a chromosomal protein prior to heat shock induction, might play a role in DH site formation in this chimera.

(b) *Analysis of 5′ deletion mutations suggests that the $(CT)_n$ region and other upstream sequences play a role in DH site formation*

The formation of the DH sites in a series of *hsp26/lacZ* fusion gene constructs containing 5′ deletions to −351, −135 and −85 bp was examined. These constructs retain the TATA sequence, the transcription start site and the downstream sequence of *hsp26* to +632 (Fig. 3(a)). The cP-351 transgene shows the same proximal and distal DH sites in nuclei from non-heat-shocked animals as does the wild-type *hsp26* gene (Fig. 3(c); see wild-type analysis: Cartwright & Elgin, 1986; Thomas & Elgin, 1988). Previous studies have shown that while two HSEs (HSE 6 and 7) are found in the distal region of the wild-type gene, HSE 7 (centered at −364) is not required for full activity (Cohen & Meselson, 1985; Pauli *et al.*, 1986; Thomas & Elgin, 1988). The cP-135 transgene, which lacks the distal HSEs but contains the $(CT)_n \cdot (GA)_n$ sequences, shows a strong proximal DH site. The distal DH site has essentially disappeared, while a very weak site at position about −200 is observed (Fig. 3(c)). Upon deletion to −85 (cP-85), which eliminates the $(CT)_n$ region, the proximal DH site essentially disappears (Fig. 3(c)). These results suggest that $(CT)_n$ and upstream sequences play an important role in DH site formation.

To quantify the accessibility of the sequences within the proximal DH site, we performed restriction enzyme digestion of isolated nuclei (Fascher *et al.*, 1990; Archer *et al.*, 1991; Reik *et al.*, 1991; Jack *et al.*, 1991). Nuclei from third instar larvae containing either the cP-351, cP-135 or cP-85 transgenes were incubated with *Xba*I, which cleaves within the proximal DH site. After purification, the chromosomal DNA was restricted to completion with *Eco*RV, fractionated on an agarose gel, and probed with the 1·1 kb fragment spanning a portion





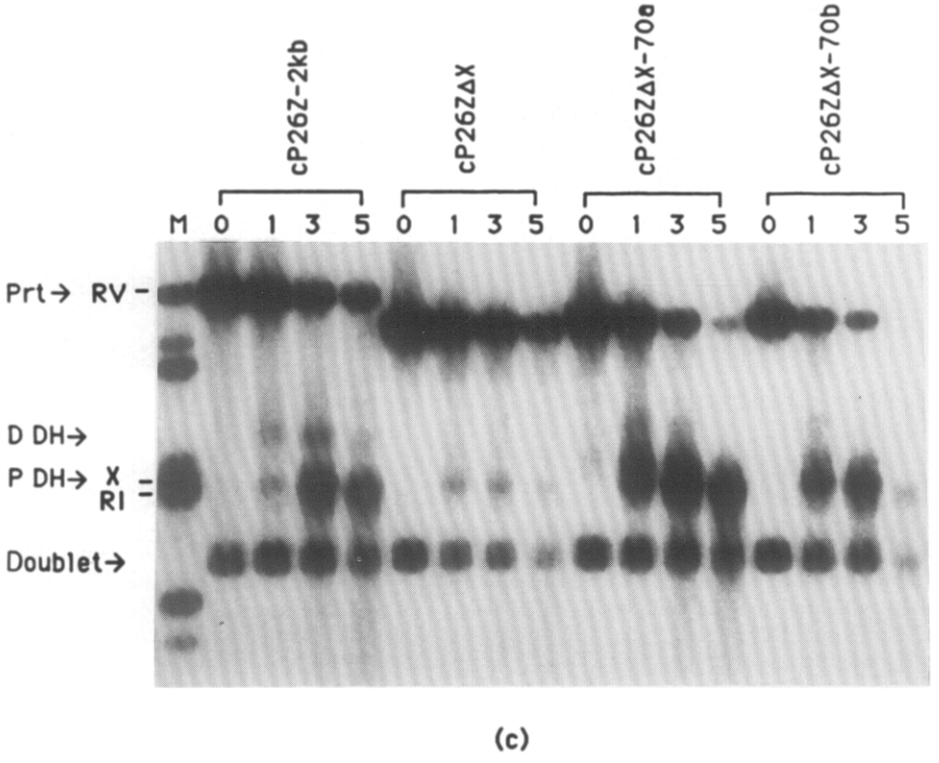


Figure 2. Chromatin structure in the presence and absence of upstream regulatory elements. Nuclei from non-heat-shocked larvae were treated briefly with DNase I and the purified DNA was subjected to indirect end-labeling analysis as described in Materials and Methods. (a) The constructs analyzed here; the transcription start site at +1 is where the line bends up; the open box indicates the *lacZ* sequence; the bottom map shows the boundaries of the functional regions discussed. (b) The 52 bp *hsp70* cassette used to make the cP26Z-70a and cP26Z-70b constructs. This sequence contains *hsp70* HSE 1 and HSE 2, and a short stretch of alternating C and T residues as indicated. The arrow indicates the natural orientation of this sequence in *hsp70*. (c) DH site formation in non-heat-shocked nuclei. Transgenes are identified above the appropriate lanes. The numbers over each lane indicate the μl of DNase I (20,000 units/ml) added to each 250 μl of nuclear suspension. 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 cut by DNase I in the initial digestion, is indicated by an arrow labeled Prt. A doublet band, which has been shown by restriction analysis to be unrelated to this group of constructs, is indicated by an arrow labeled Doublet. In addition to *Eco*RV digestion, these samples have been digested with *Ava*I. *Ava*I does not cut within the parental *Eco*RV fragment of these constructs, but does cut the unrelated DNA into the observed doublet, which does not then interfere with the DH site analysis. M, markers; RI, *Eco*RI; RV, *Eco*RV; X, *Xba*I.

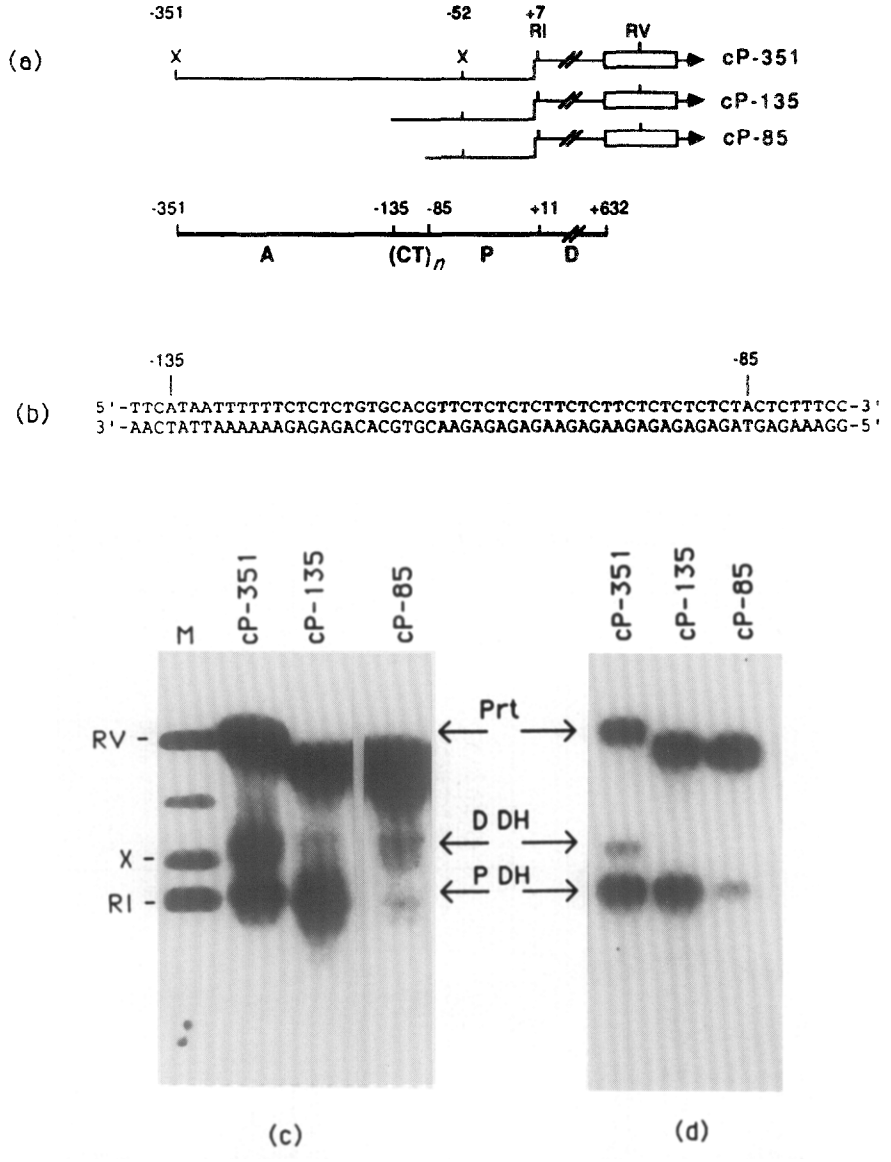


Figure 3. DH site formation in the 5′ deletion series. Nuclei from non-heat-shocked larvae were treated briefly with DNase I and the purified DNA was subjected to indirect end-labeling analysis as described in Materials and Methods. (a) The constructs used in this Figure, drawn as described for Fig. 2. (b) The sequence within the $(CT)_n$ region. The endpoints of the 5′ deletions are indicated above the sequence. (c) DH site formation in nuclei from non-heat-shocked larvae. DH sites are indicated by arrows labeled P DH (proximal DH site) and D DH (distal DH site). (d) Cleavage susceptibility of the proximal DH site to restriction enzyme *Xba*I within isolated nuclei. Nuclei were isolated from non-heat-shocked third instar larvae of transformed lines. The restricted DNA was purified and subjected to indirect end-labeling analysis as described in Materials and Methods. Transgenes are identified above the appropriate lanes; other labels are as described for Fig. 2.

of the *Escherichia coli lacZ* gene (Fig. 1). Cleavage at the *Xba*I site within the proximal DH site would produce a 1·7 kb band detected by this probe. Cleavage at a second *Xba*I site, located within the distal DH site, gives a 2·1 kb band. No restriction at the *Xba*I sites within the *hsp26* promoter sequences present in these transgenes gives a *Eco*RV parental band. The accessibility of the *Xba*I site within the proximal DH site was quantified as the fraction of the intensity of the 1·7 kb band compared to the total intensity of all the bands within a given lane on an autoradiograph. In transformants carrying the cP-351 transgene, there was 52% restriction at the *Xba*I site that lies within the proximal DH site (Fig. 3(d)). This is essentially the same as observed for the wild-type *hsp26* gene (data not shown). Transformants carrying the cP-135 transgene exhibited a moderate reduction to 38% cleavage at this site, while transformants carrying the cP-85 transgene exhibited a dramatic reduction in accessibility at the proximal *Xba*I site, showing only 4% cleavage (Fig. 3(d)). Since the majority of the sequences contained between −135 and −85 are $(CT)_n$, the reduced accessibility of the *Xba*I site within the proximal DH site of cP-85 was most likely due to the loss of the $(CT)_n \cdot (GA)_n$ repeats.

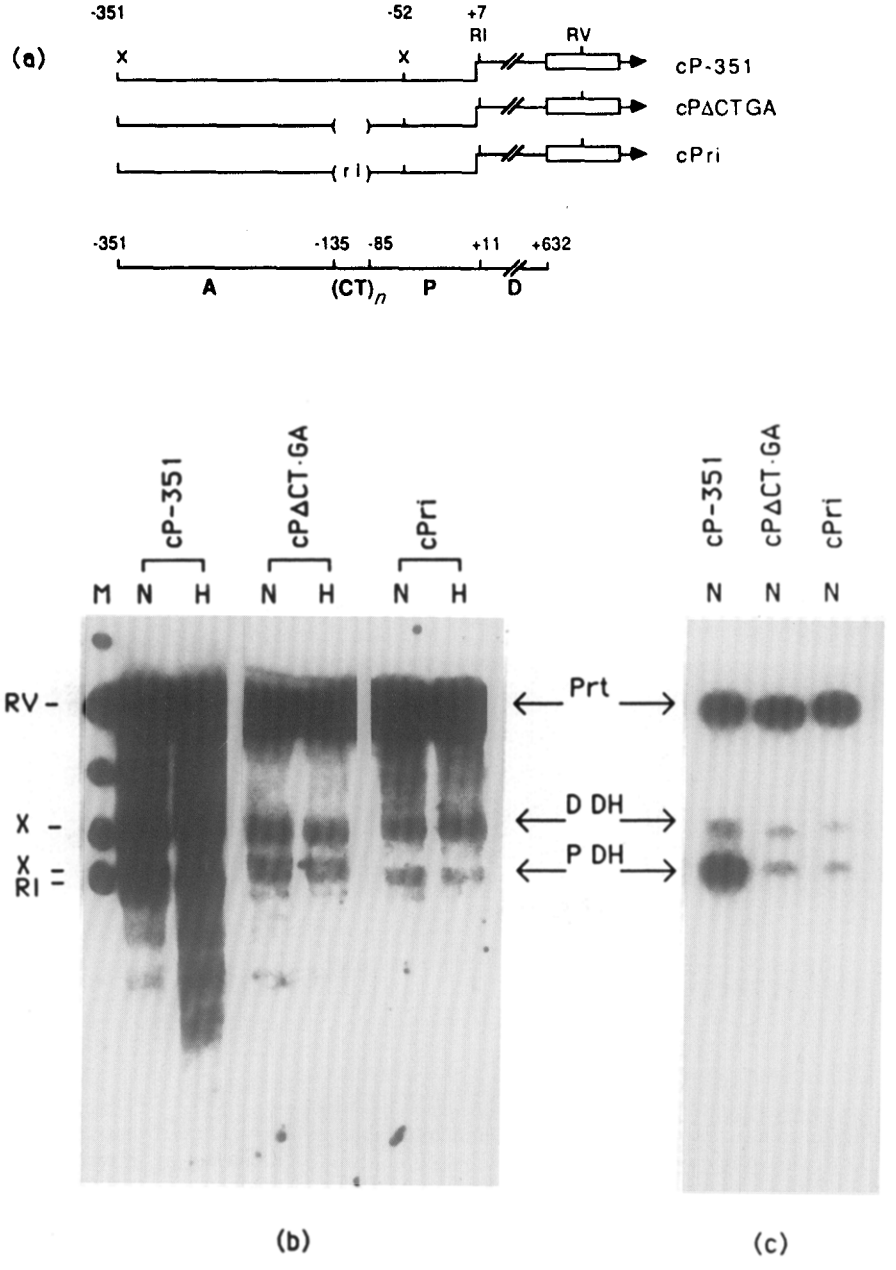


Figure 4. DH site formation in transgenes lacking the $(CT)_n$ region. Larval nuclei were treated briefly with DNase I and the purified DNA was subjected to indirect end-labeling analysis as described in Materials and Methods. (a) The constructs used in this Figure, drawn as described for Fig. 2. (b) DH site formation in non-heat-shocked (N) and heat-shocked (H) nuclei. In order to visualize the weak bands for the DH sites of transgenes containing the $(CT)_n$ deletion and substitution, the autoradiograph of this panel was overexposed. (c) Susceptibility of the proximal DH site to cleavage by the restriction enzyme *Xba*I within isolated nuclei from non-heat-shocked larvae. The DNA was purified and subjected to indirect end-labeling analysis. Transgenes are identified above the appropriate lanes. Other labels are as described for Fig. 2.

(c) *$(CT)_n \cdot (GA)_n$ sequences have a critical role in generating DH sites*

Since the above analyses suggested that the $(CT)_n \cdot (GA)_n$ sequences might play an important role in the formation of the DH sites, we undertook a chromatin structure analysis of a set of constructs that included an internal deletion of the $(CT)_n$ region (cPΔCT·GA) as well as sequence substitution at this site (Fig. 4(a)), in nuclei from either non-heat-shocked or heat-shocked larvae. Using DNase I, analysis of the cPΔCT·GA transgene showed that both the proximal and the distal DH sites were dramatically reduced compared with the cP-351 transgene under both conditions (non-heat-shocked or heat-shocked: Fig. 4(b)). (Note that the distal site appears to be slightly shifted relative to that of the wild-type control due to the removal of the $(CT)_n$ region.) When the $(CT)_n \cdot (GA)_n$ sequences were replaced with the same length of a random sequence

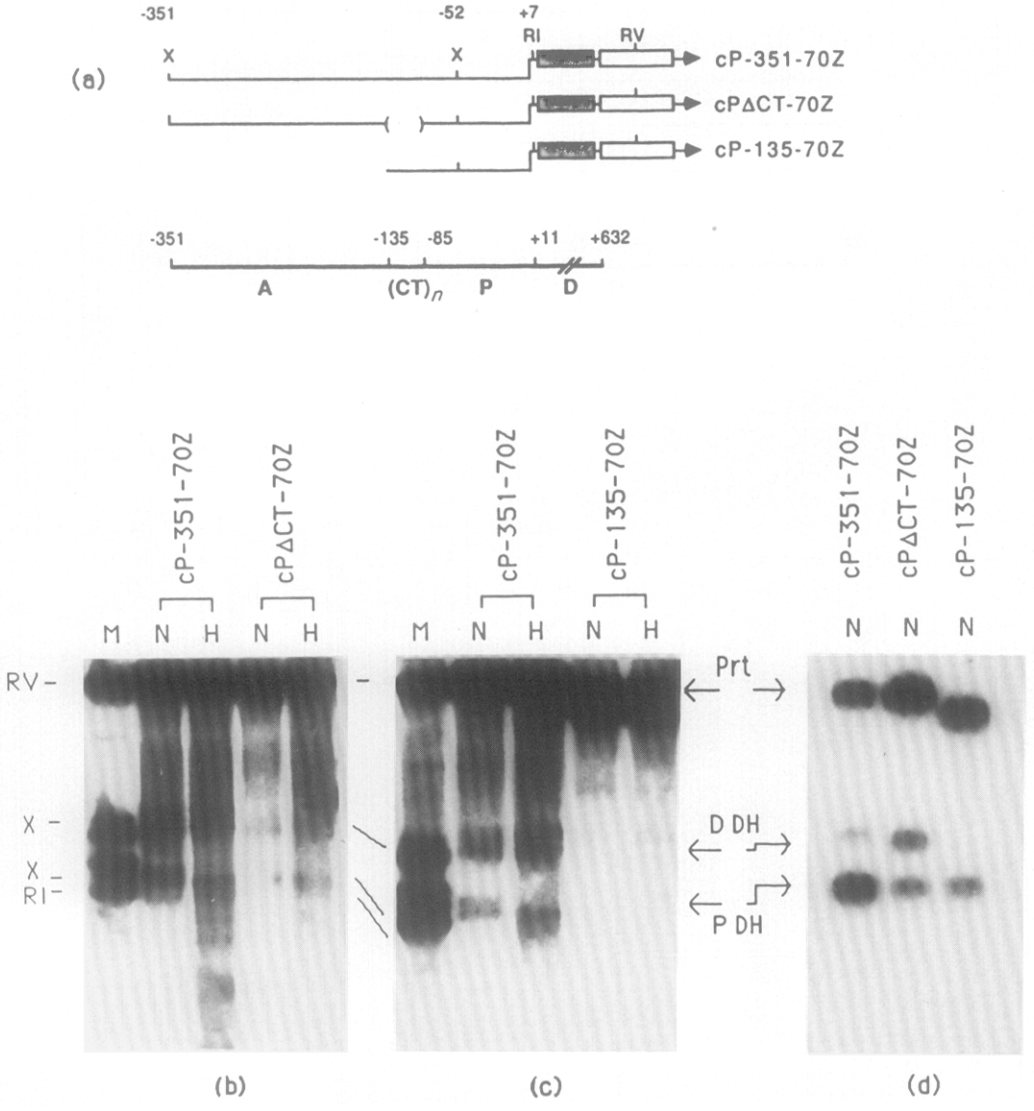


Figure 5. DH site formation in transgenes containing multiple deletions. Larval nuclei were treated briefly with DNase I and the purified DNA was subjected to indirect end-labeling analysis as described in Materials and Methods. (a) The constructs used in this Figure, drawn as described for Fig. 2, with the wild-type gene given below for reference. In these constructs the *hsp26* sequence from +11 to +632 is replaced by the *hsp70* sequence from +89 to +260 as described (Glaser & Lis, 1990). The restriction sites used in the map give marker fragments as indicated. (b) The DH site formation of the *hsp26/70/lacZ* constructs made with and without the $(CT)_n$ region. (c) The DH site formation of the *hsp26/70/lacZ* transgenes made with and without region A (from −351 to −135) of *hsp26*. (d) Cleavage susceptibility of the proximal DH site to restriction enzyme *Xba*I within isolated nuclei. The DNA was purified and subjected to indirect end-labeling analysis. Transgenes are identified above the appropriate lanes. Other labels are as described for Fig. 2.

from salmon sperm DNA (construct cPri), the DH sites appeared similar to those observed for the deletion construct cPΔCT·GA (Fig. 4(b)). Using the same strategy as described in section (b), above, restriction enzyme analyses of isolated nuclei from non-heat-shocked larvae containing either the cP-351, cPΔCT·GA or cPri transgene was performed. Transformants carrying either the cPΔCT·GA or the cPri transgenes showed dramatic reductions in the accessibility for *Xba*I within the proximal DH site, exhibiting only 8% and 6% cleavage as compared to the 52% cleavage seen for cP-351 (Fig. 4(c)). Thus, removal or replacement of the $(CT)_n$ region has a dramatic effect on DH site formation.

(d) *Additional regions that are involved in the formation of the DH sites*

The *hsp26* sequences from −351 to +632 are sufficient to mediate heat shock response similar to that of the wild-type gene (Cohen & Meselson, 1985; Pauli *et al.*, 1986; Simon & Lis, 1987; Glaser *et al.*, 1990), and to dictate formation of the wild-type chromatin structure (Fig. 2(c)). When *hsp26* sequences from −351 to +11 are fused to a different gene, such as *Adh*, this fusion gene is still heat shock inducible (Cohen & Meselson, 1985). Similarly, fusion of a fragment of *hsp26* from −351 to +11 with an *hsp70/lacZ* construct starting at +89 of *hsp70* (cP-351-70Z, Fig. 5(a)) produces a

gene that is heat shock inducible (Fig. 6). DH site analysis of transformants carrying cP-351-70Z revealed a wild-type pattern of DH site formation (Fig. 5(b)).

The proximal DH site is prominent upon analysis of transformants carrying the cP-135 transgene (Fig. 2(c)), which includes the $(CT)_n \cdot (GA)_n$ repeats and *hsp26* sequences downstream to +632; however, fusion of this construct at +11 of *hsp26* with *hsp70/lacZ* at +89 of *hsp70* (cP-135-70Z: Fig. 5(a)) results in a dramatic reduction in the formation of the proximal DH site (Fig. 5(c)). These results suggest the presence of an *hsp26* downstream element between sequences +11 to +632 (region D). When sequences from −351 to −135 (region A) are not present, region D is apparently required to co-operate with the $(CT)_n \cdot (GA)_n$ repeats and the TATA sequence to create the DH sites.

The accessibility of *Xba*I to the *Xba*I site within the proximal DH site was evaluated for transformants carrying the transgenes with region D changed as described above. Transformants carrying the cP-351-70Z transgene exhibited 46% cleavage (Fig. 5(d)), a value comparable with the similar construct possessing region D (cP-351). Transformants possessing the cP-135-70Z transgene, lacking both regions A and D, exhibited only 9% cleavage at this site (Fig. 5(d)).

The results obtained from the analysis of transformed lines carrying cPΔCT-70Z, which lacks both the *hsp26* $(CT)_n$ and the D regions, provide further support for the role of the $(CT)_n \cdot (GA)_n$ repeats in the formation of DH sites. This transgene retains the A region, HSEs and the TATA box and differs from the parental construct, cP-351-70Z, only in the deletion of the $(CT)_n$ region. DNase I has reduced access to the DH sites in lines carrying the cPΔCT-70Z transgenes relative to lines containing the control transgenes, cP-351-70Z (Fig. 5(b)). This result is consistent with those shown in Figure 4 where the deletion or substitution of the $(CT)_n$ region reduced cleavage at the DH sites even in transgenes that have both the A and D regions. Furthermore, restriction enzyme treatment of nuclei isolated from cPΔCT-70Z transformants results in only 7% cleavage by *Xba*I within the proximal DH site (Fig. 5(d)).

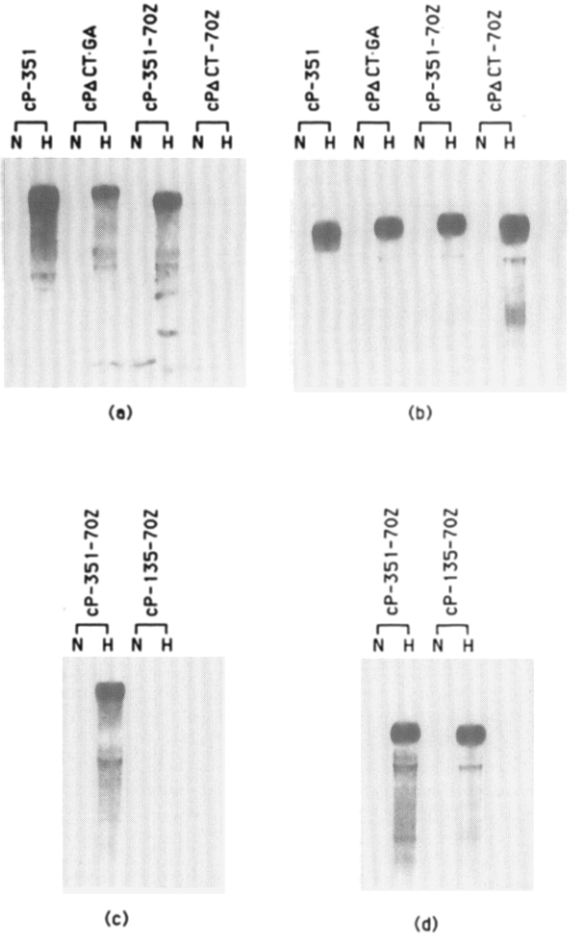


Figure 6. Northern analyses of transcripts from transgenes containing mutations within *hsp26*. Total RNA was extracted from non-heat-shocked (N) and heat-shocked (H) larvae as described in Materials and Methods. Samples containing 20 μg of total RNA were treated with RNase-free DNase I before being fractionated on a 1% agarose gel with formaldehyde. (a) and (c) The blot hybridized with the 1·1 kb *lacZ* DNA probe. (b) and (d) The same blot hybridized to an *hsp70* 5′ DNA probe (−254 to +87 of *hsp70*) as a control to confirm that a heat shock response had occurred; this detects the wild-type *hsp70* mRNA, but not the *hsp26/70/lacZ* fusion mRNA. The same blots were also hybridized to a chicken unbiquitin DNA probe to confirm that the samples were equally loaded and the RNA intact (not shown).

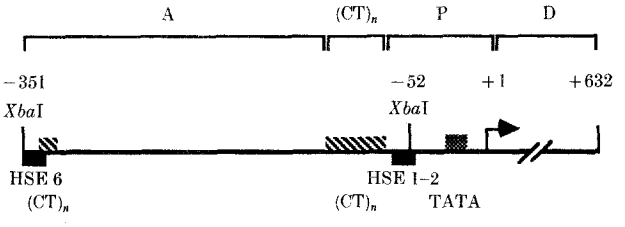
(e) *Formation of DH sites and heat-induced gene expression*

Mutations that disrupt the formation of DH sites at *hsp26* also impair its heat-induced transcriptional activation. The quantitative measurements of restriction enzyme cleavage at the *Xba*I site within the proximal DH site described in the preceding sections indicate that the deletion of the $(CT)_n$ region causes a decrease of the susceptibility of this site to 12 to 15% that of the wild-type control (compare cPΔCT·GA and cP-351: Fig. 4 and Table 1). Our previous and present measurements of the heat shock inducibility of *hsp26* transgenes show that the $(CT)_n$ region is likewise critical for the heat-induced expression of *hsp26* (Glaser *et al.*, 1990; Table 1). However, establishment of the DH sites is not sufficient to render *hsp26* heat shock inducible. When sequences that are required for transcriptional activation, such as the distal HSE, are absent, the gene is not heat shock inducible even if the DH site(s) are clearly established (see cP-135 in Figs 2 and 6). Nevertheless, if DH sites are absent, as in the $(CT)_n$ deletions, then the heat shock inducibility is severely limited, even in the presence of essential transcriptional elements such as the TATA box, transcriptional start, and HSEs (see cPΔCT·GA and cPΔCT-70Z in Figs 4 to 6 and Table 1).

Table 1

Summary of restriction enzyme accessibility at the proximal Xba*I site and relative expression of transgenes*

Construct	A	$(CT)_n$	P	D	Cleavage by *Xba*I (%)	Expression (%)
cP-351	+	+	+	+	52	100
cP-135		+	+	+	38	17
cP-85			+	+	4	4
cPΔCT·GA	+		+	+	8	23
cPri	+		+	+	6	27
cP-351-70Z	+	+	+		46	100
cP-135-70Z		+	+		9	1
cPΔCT-70Z	+		+		7	1



The percentage of cleavage by *Xba*I was measured (using a densitometer) from the autoradiographs of Figs 3(d), 4(b) and 5(d) as the fraction of DNA cleaved at the proximal *Xba*I site (see Results). The relative expression of *hsp26/lacZ* transgenes was determined by measurement of β-galactosidase activity using CPRG assays (Simon & Lis, 1987; Glaser *et al.*, 1990). Relative percentages are listed with the induced activity of cP-351 set at 100%. The relative expression of this series of transgenes has also been measured from the mRNA level with consistent results (Fig. 6; Q.L., unpublished results). The relative expression of the *hsp26/hsp70/lacZ* transgenes were determined by densitometric measurements of the autoradiograph from Northern analysis (Fig. 6), and is an average of 2 independent transformed lines. Relative percentages are listed with the expression of cP-351-70Z set at 100%. A + denotes the presence of a particular region of the *hsp26* gene. Below the Table is a diagram of the *hsp26* promoter showing specific regions with the symbols and abbreviations as described in the legend to Fig. 1.

4. Discussion

Specific protein–DNA interactions can impose striking discontinuities in chromatin structure. These discontinuities are revealed as regions of DNA that are accessible to nucleases, such as DNase I. These DH sites have been shown to be free of nucleosomes and are often located in the promoter and regulatory regions of genes. The *hsp26* gene has prominent DH sites that reside at defined positions in its regulatory region; here we have investigated the effect of various mutations in the *hsp26* promoter/regulatory region on the formation of these DH sites. The chromatin structure was examined in transformed *Drosophila* lines bearing single inserts of variant *hsp26/lacZ* transgenes. In order to quantify the accessibility of the DNA in the DH sites, we measured the susceptibility of the DH sites to cleavage by a specific restriction enzyme. The $(CT)_n$ region, which contains 32 bp of alternating C and T residues, appears to play a central and critical role in DH site formation (Table 1). In addition to the $(CT)_n$ region, one of the other regions, A or D, is required for effective formation of the appropriate DH sites (Table 1).

(a) *The $(CT)_n$ region is essential for DH site formation*

To assess the role the $(CT)_n$ region plays in transcriptional control of the *hsp26* gene, a number of constructs have been made and analyzed (Glaser *et al.*, 1990; Glaser & Lis, 1990). The results from these analyses have demonstrated that $(CT)_n \cdot (GA)_n$ is a positive regulatory element for both heat shock and developmental expression of this gene. A 66,000 M_r protein has been identified that binds to and protects the alternating C and T residues in the $(CT)_n$ region *in vitro* (Gilmour *et al.*, 1989). This CT binding protein appears to be identical with the GAGA factor, a transcription factor associated with *Ubx* and other promoters (Biggin & Tjian, 1988; Gilmour *et al.*, 1989; H. Granok & S.C.R.E., unpublished results). Footprinting experiments with isolated nuclei show that this region is protected *in vivo* prior to heat shock (Thomas & Elgin, 1988). Thus, protein–DNA interaction at this site may be a critical step in DH site formation. Alternatively, the importance of the $(CT)_n$ sequence may be due to the structure of the $(CT)_n$ sequence itself. *In vitro*, these $(CT)_n$ sequences, when present on a supercoiled plasmid, form a triple helix at low pH (Siegfried *et al.*, 1986; J. M. Teare & S.C.R.E., unpublished results). However, this triplex structure could not be detected by DEPC treatment of isolated nuclei (Glaser *et al.*, 1990); whether or not such triplexes form *in vivo* is a matter of current debate and investigation.

It should be noted that while deletion or substitution of the $(CT)_n$ sequence severely reduces accessibility of the HSE (*Xba*I site), it does not eliminate the DH site entirely (cPΔCT·GA and cPri: Fig. 4). The residual DNase I hypersensitivity might be due to a different structure, perhaps dictated by the presence of regions A and D, since these regions contribute to the formation of the DH sites; alternatively, it might indicate a random assembly process.

The *hsp70* gene also has $(CT)_n$ regions, one of which is located between the two proximal HSEs. This $(CT)_n$ region has also been shown to interact with the CT-binding protein *in vitro* (Gilmour *et al.*, 1989). A 52 bp cassette that contains the $(CT)_n$ region bordered by two HSEs was shown to rescue heat-induced expression of an *hsp26* gene deleted of its regulatory region, cP26ZΔX, when the cassette was inserted at the deletion site (Simon & Lis, 1987). This cassette likewise reinstates a major DH site in these *hsp26* transgenes (cP26Z-70a, cP26Z-70b: Fig. 2). Since HSEs appear to be unoccupied prior to heat shock in *Drosophila*, interaction of the *hsp70* $(CT)_n$ region with the CT-binding protein is a likely mechanism for dictating the formation of DH sites in the uninduced state. This hypothesis is consistent with the results of analyses of *hsp26/lacZ* transgenes with 5′ deletions (Fig. 3) or

with deletion or substitution of the $(CT)_n$ region (Fig. 4).

Previous studies on the $(CT)_n$ region (Glaser *et al.*, 1990; Glaser & Lis, 1990) did not specify whether the $(CT)_n$ sequence was involved in the formation of the DH sites or simply in transcriptional activation of *hsp26*. Our results here indicate that the $(CT)_n$ sequence clearly has a role in DH site formation, but do not exclude the possibility of an additional role in transcriptional activation.

(b) *The A and D regions contribute to the formation of the DH site*

The boundaries of region A include the sequences from −351 to −135; this region contains HSE 6, an essential heat shock regulatory element, which lies in the distal DH site and is required for heat shock induction (Cohen & Meselson, 1985; Pauli *et al.*, 1986, Simon & Lis, 1987). HSF binds *in vivo* to this sequence after, but not before, heat shock (Thomas & Elgin, 1988). A short (7 bp) stretch of alternating C and T residues is observed at the proximal side of HSE 6; this could be a critical part of region A. Mutation of this distal $(CT)_n$ sequence affects the transcriptional activity of the gene (Q.L., unpublished results). Region A also contains three sequence elements earlier designated HSE 3, 4 and 5, which are recognizable but poor matches to the HSE consensus sequence (Bienz & Pelham, 1986; Lis *et al.*, 1990). Genetic analysis indicates that these sequences do not significantly contribute to the heat shock response (Pauli *et al.*, 1986; Simon & Lis, 1987; Glaser *et al.*, 1990). Genomic footprinting has shown that *in vivo* HSE 3, 4 and 5 are packaged in a precisely positioned nucleosome; this packaging is not altered upon gene activation by heat shock (Thomas & Elgin, 1988).

Region D is loated downstream from the transcription start site, and includes sequences from +11 to +632. Previous studies of heat shock genes have identified elements in this region that are necessary for efficient gene expression (see below); the present data indicate a significant role in the formation of the DH sites. The transcriptional start of the *hsp26* gene has been shown to be occupied by the RNA polymerase II complex before and after heat shock induction (Rougvie & Lis, 1990). *In vitro* footprinting analysis of the *hsp26* promoter indicates binding of the TATA box-dependent complex to the region from −38 to +35; the sequences downstream from the TATA box are important in maintaining a stable complex (Dietz, 1990). The importance of this region in a related heat shock gene, *hsp22*, has been demonstrated. In this case, transcription is reduced to 1% to 10% of that of wild-type when a region from +1 to +26 is removed. When this region is left intact, up to 86% of the 5′ untranslated sequence can be deleted without affecting transcription (Hultmark *et al.*, 1986).

In one series of constructs studied here, upstream regions of the *hsp26* gene (to +11) were fused to an *hsp70/lacZ* construct resulting in a chimeric gene containing *hsp70* sequences from +89 to +260 (+250 is the translation start). These constructs lacking the *hsp26* D region show a normal chromatin structure only with the intact −351 to +11 fragment of *hsp26*, when regions A and $(CT)_n$ are present (Fig. 5). In contrast, only the $(CT)_n$ region is needed for DH site formation in the presence of *hsp26* region D (see Figs 2 and 4). The fact that the *hsp70* sequence does not rescue DH site formation on the *hsp26* promoter in the case of cP-135-70Z implies that the *hsp70* sequences from +89 to +260 make no contribution to DH site formation in these constructs. Whether the functional element(s) of the D region lies in the sequence +11 to +89 and is missing, or whether the functional element(s) of the D region for the two genes are heterologous, remains to be determined. Nonetheless, the present findings clearly point to an important contribution to the chromatin structure of *hsp26* promoter by region D.

How do regions A and D affect the formation of DH sites? As discussed above, these two regions contain many binding sites for regulatory proteins that are likely to influence the chromatin structure of the *hsp26* promoter. These may act either directly through interactions with nucleosomes, or indirectly through promotion of assembly of the active transcription complex, to determine nucleosome position. Further analysis of *hsp26* transgenes with small deletions within regions A and D is being initiated to define these effects more precisely.

(c) *Possible molecular mechanisms for DH site formation*

What is the mechanism behind the formation of stable DH sites by the A, $(CT)_n$ and D regions? The TATA binding factor and RNA polymerase II are bound to the *hsp26* promoter region *in vivo* prior to heat shock induction (Thomas & Elgin, 1988; Rougvie & Lis, 1990), initially suggesting to us that these proteins might be sufficient to establish the chromatin structure pattern. The prior binding of TFIID has been shown to allow binding of the HSF to nucleosome templates *in vitro* (Taylor *et al.*, 1991). However, this study demonstrates that effective DH site formation for *hsp26 in vivo* requires the $(CT)_n \cdot (GA)_n$ repeats, and at least one additional sequence element (region A or D) in addition to the promoter complex. The $(CT)_n$ element might facilitate binding of TFIID *in vivo*, allowing the transcription complex to compete successfully against nucleosomes in binding to this site. This hypothesis is supported by recent observations indicating a cooperative interaction between the TATA factor and the CT binding protein *in vitro*. Binding of the TATA factor stabilizes the interaction of the CT binding protein with the $(CT)_n$ region of *hsp26*; conversely, the CT binding protein facilitates formation of the TATA box-dependent footprint on this promoter *in vitro* (Dietz, 1990). Proteins bound on regions A or D, together with the CT binding protein, might interact with each other to stabilize the

transcription complex, and therefore exclude nucleosomes from the *hsp26* promoter. In the absence of the CT binding protein, nucleosomes or other non-histone chromosomal proteins may be distributed throughout the promoter region, causing the decrease in accessibility of the proximal DH site to DNase I and *Xba*I digestion that is observed. Nucleosomes have been shown to block the accessibility of a restriction enzyme to its recognition site within isolated nuclei (Fascher *et al.*, 1990; Archer *et al.*, 1991). In contrast, transcription factors, such as HSF, appear not to block the accessibility of a given restriction site within isolated nuclei (Jack *et al.*, 1991; L.L.W., unpublished results). Thus, the results imply an alteration in the nucleosome array in the absence of the $(CT)_n$ sequence.

The ability of a specific transcription factor to facilitate the binding of TFIID has been shown in a mammalian system (Workman *et al.*, 1990). *In vitro* reconstitution of a eukaryotic RNA polymerase II transcription system has shown a competition between binding of the polymerase complex and assembly of nucleosomes in the promoter region of many genes. Formation of a stable preinitiation complex is dependent on the binding of general transcription factor TFIID to the TATA element, a process that is itself blocked by prior nucleosome assembly (Matsui, 1987; Workman & Roeder, 1987; Losa & Brown, 1987; Knezetic *et al.*, 1988). Once bound, however, TFIID can direct assembly of a transcription complex even in the presence of histones (Becker *et al.*, 1991). Proteins such as USF, a common upstream transcription factor, may function in part by facilitating the binding of TFIID, and hence favoring the formation of a stable transcription complex in preference to nucleosome binding at the promoter (Workman *et al.*, 1990). *In vitro* these events can be manipulated by the order of addition of the components, whereas *in vivo* one would infer a general competition during the chromatin assembly that must follow DNA replication. Here the $(CT)_n$ element appears to play a critical role.

Additional examples in which nucleosomes may serve as negative regulators of gene expression come from studies on the *Saccharomyces cerevisiae PHO5* gene and on the MMTV promoter. In the case of the *PHO5* gene, the promoter is packaged in nucleosomes when the gene is repressed; these nucleosomes are displaced when the gene is active (Almer *et al.*, 1986). Displacement or alteration of the nucleosomes appears to be a critical step in this gene activation (Straka & Hörz, 1991). At the MMTV promoter, a nucleosome masks a promoter proximal NF1 binding site when the promoter is inactive. Upon hormone induction, the binding of the glucocorticoid receptor to the MMTV promoter eliminates the nucleosome footprint and renders the NF1 site accessible (Cordingly *et al.*, 1987; Richard-Foy & Hagar, 1987; Piña *et al.*, 1990; Fascher *et al.* 1990; Archer *et al.*, 1991). The preformed DH sites in the heat shock regulatory regions may be particularly critical, since HSF fails to bind to HSEs that are assembled into nucleosomes *in vitro* (Taylor *et al.*, 1991).

From these studies and our work on the chromatin structure of the *hsp26* gene, it appears that the chromatin structure of inducible promoters may fall into two classes. In cases such as those described above, the inactive state of a gene is correlated with the presence of nucleosomes along the promoter, blocking access to critical *trans*-acting factor binding sites (i.e. the TATA box and the NF1 binding site). Upon activation, these nucleosomes must be altered or displaced ("remodeled") in such a way as to allow access. Alternatively, in the case of *hsp26* the chromatin structure of the promoter is specifically organized so as to allow access for the binding of the *trans*-acting factor, HSF; the "switch" is then controlled by a change in the binding affinity of HSF. Such a transition for the HSF in *Drosophila* has been demonstrated (Zimarino & Wu, 1987). This mechanism of activation allows for rapid switching. In fact, all genes reported to be associated with a "poised polymerase", including some constitutively expressed genes (Rougvie & Lis, 1990), may fall into this category of having a "pre-set" accessible promoter. Both mechanisms of gene activation require a highly defined chromatin structure as part of the regulatory apparatus. As shown here, the reduced ability to form a particular chromatin structure correlates with reduced inducible expression. Further mutagenic and biochemical analyses of the chromatin structure of other genes should lead to a better understanding of how these promoter structures are established and utilized.

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