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Polycomb Silencing Blocks Transcription Initiation

Short Article

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Summary

Polycomb (PcG) complexes maintain the silent state of target genes. The mechanism of silencing is not known but has been inferred to involve chromatin packaging to block the access of transcription factors. We have studied the effect of PcG silencing on the *hsp26* heat shock promoter. While silencing does decrease the accessibility of some restriction enzyme sites to some extent, it does not prevent the binding of TBP, RNA polymerase, or the heat shock factor to the *hsp26* promoter, as shown by chromatin immunoprecipitation. However, we find that in the repressed state, the RNA polymerase cannot initiate transcription. We conclude that, rather than altering chromatin structure to block accessibility, PcG silencing in this construct targets directly the activity of the transcriptional machinery at the promoter.

Introduction

Polycomb Group (PcG) proteins are best known for their role in maintaining the repressed state of homeotic genes. They act by assembling chromatin complexes at the Polycomb Response Element (PRE), a specific DNA sequence of several hundred base pairs that can direct silencing of one or more promoters placed in its vicinity (for review see Pirrotta, 1997; Francis and Kingston, 2001). Many components of PcG complexes have been identified, but little is known about the mechanisms by which they repress promoter activity. Early views of Polycomb silencing, based on the prevailing notions of heterochromatin, envisioned a cooperative assembly of protein complexes coating large stretches of chromatin and packaging them into a condensed form inaccessible to transcription factors. However, chromatin immunoprecipitation experiments in *Drosophila* have not shown extensive association of PcG complexes with the silenced genes of the bithorax complex. Instead, PcG proteins appear to be bound principally to PRE regions (Strutt et al., 1997; Strutt and Paro, 1997).

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In vitro, purified PcG complexes inhibit the action of the SWI/SNF complex on a nucleosome array (Shao et al., 1999; Francis et al., 2001), suggesting that PcG silencing might block the access of transcription factors by preventing nucleosome remodeling. Whether accessibility to silenced chromatin is reduced in vivo is not clear. Conflicting reports describe both decreased reactivity to the *dam* methylase (Boivin and Dura, 1998) and the failure to detect a loss of accessibility to restriction or other enzymes in PcG-silenced regions (Schlossherr et al., 1994; Fitzgerald and Bender, 2001). A different type of silencing mechanism is suggested by the fact that PcG complexes interact with promoter factors such as TATA binding protein (TBP) and TBP-associated proteins (TAFs) (Saurin et al., 2001; Breiling et al., 2001). These findings raise the possibility that, rather than generally preventing access to the DNA, PcG complexes might interfere with specific functions of the transcriptional apparatus.

To study the effects of PRE silencing on chromatin structure and function, we have assembled a construct in which the *bxd* PRE is placed immediately upstream of two tandem reporter genes, the *hsp26-lacZ* gene and the *miniwhite* gene. We chose the *hsp26* promoter as a target for repression because its activation can be directly controlled, its chromatin structure has been extensively analyzed, and it is known to have promoter factors and RNA polymerase bound in all tissues. With this system we can therefore ask if silencing alters the chromatin configuration, prevents the binding of these factors, or interferes with their function. Unfortunately, these experiments could not be done with natural PcG targets such as homeotic genes. The chromatin structure of these genes is unknown, and they are in different states of activity in different cells. Instead, the *hsp26* promoter contains multiple binding sites that bind the GAGA factor in all cells and position a nucleosome in the region preceding the transcription start site (Lu et al., 1995). This creates DNase I hypersensitive sites (DH sites) flanking the nucleosome, corresponding to the TATA box and to binding sites for the heat shock factor (HSF). GAGA factor, TFIID, and RNA polymerase cooperate to establish this preset chromatin structure: loss of any one of the three reduces the binding of the other two and the access of HSF upon induction (Shopland et al., 1995; Leibovitch et al., 2002). The preset promoter is in a state of transcriptional readiness. The RNA polymerase initiates transcription but stalls in the region between +25 and +50, with high density pausing between +28 and +47 (Rougvie and Lis, 1988; Rasmussen and Lis, 1995). Upon heat shock, the trimeric HSF binds to the heat shock elements (HSEs), made accessible by the preset chromatin structure, and recruits Mediator complexes (Park et al., 2001), resulting in the phosphorylation of the RNA polymerase C-terminal domain, recruitment of elongation factors, and the transcriptional release of the polymerase.

The *miniwhite* gene confers eye pigmentation; in the presence of a PRE, it may be completely or partially silenced, often in a mosaic or variegated fashion, de-

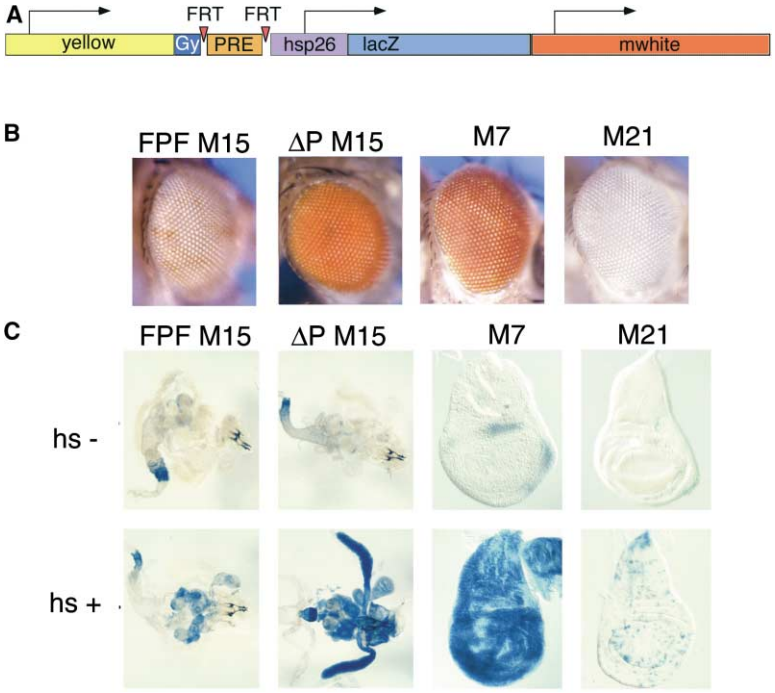

Figure 1. PcG Silencing of the *miniwhite* and *hsp26* Promoter

(A) Map of the transposon construct. The YGPhsW transposon contains a *yellow* gene marker, followed by the gypsy Su(Hw) insulator (Gy) to protect it from silencing. The *bxd* PRE is placed immediately in front of the *hsp26* promoter-*lacZ* gene, which is followed by the *miniwhite* gene. The YGFPFhsW construct has the same structure except for the addition of FRT sequences framing the PRE.

(B) Silencing of the *miniwhite* gene. The first row shows the eye pigmentation of three representative lines FPF M15, M7, and M21, and the effect of excising the PRE from FPF M15 to produce ΔP M15.

(C) Comparison of *lacZ* expression before or after heat shock. Dissected larvae stained with X-gal are shown for FPF M15 and its ΔP M15 derivative. Only the wing disc is shown for lines M7 and M21.

pending on the insertion site of the transgene construct. In this work we have used eye variegation, *lacZ* expression, and heat shock inducibility as criteria for the degree of repression to examine the changes in chromatin structure that accompany PRE-induced repression of the *hsp26* promoter. Surprisingly, we find that PcG silencing does not prevent the binding of TBP, RNA polymerase, or HSF to the *hsp26* promoter but affects specifically the ability of RNA polymerase to form the initiation complex.

Results

Effect of the PRE on the Basal Expression of *hsp26-lacZ* and *miniwhite* Genes

We constructed two related transposons containing the *bxd* PRE, the *hsp26-lacZ* gene and the *miniwhite* gene (Figure 1A). As an additional marker for transformation, the transposons contain the *yellow* gene, shielded from the repressing effects of the PRE by a Su(Hw) insulator (Sigrist and Pirrotta, 1997). The two transposons differ by the presence of FRT sites, the targets of the FLP recombinase, flanking the PRE in the YGFPFhsW but not in the YGPhsW construct. The FRT sites allow us to excise the PRE in YGFPFhsW and compare the effects of the presence and absence of the PRE in the construct at the same insertion site. The two transposon constructs are otherwise equivalent and both have been used in this study. We will refer to the YGFPFhsW lines as FPF lines and their derivatives with the PRE excised as ΔP lines.

The transposon insertion site affects the level of expression and the degree of silencing/variegation effected by the PRE on the two reporter genes. Among the transgenic lines, some, like M7, show very weak repression of either reporter; one, M21, displays virtually complete silencing (Figure 1B), while the majority have highly variegated eye phenotypes and are poorly inducible by heat shocks (e.g., FPF M15). In the lines used in this work, the repression of the *miniwhite* gene is reduced by PcG mutations (*Pc*, *Pcl*, *Psc*, *Scm*) but is not affected by *Su(var)* mutations in the HP1 gene, indicating that the variegation is due to the PRE and not to heterochromatic position effects (data not shown). To determine the specific effects of the presence and absence of the PRE, the FPF lines were crossed to flies expressing the FLP recombinase under control of a heat shock promoter. The excision of the PRE, detected by changes in eye pigmentation and/or variegation, was verified by Southern blot hybridization and the corresponding ΔP lines were established.

Some degree of inducibility of the *hsp26-lacZ* gene can be observed even in the most repressed lines (Figure 1C). After heat shock, even in line M21 some weak *lacZ* expression is induced in patches of cells but both the number of *lacZ*-expressing cells and the level of expression are strongly reduced. In the ΔP lines, excision of the PRE has restored efficient and abundant heat shock inducibility in most tissues. An assay of β-galactosidase activity in lines FPF M15 and ΔP M15 shows that the presence of the PRE decreases the inducible expression of the *hsp26-lacZ* gene by a factor of 10 overall (data not shown).

Effects of Silencing on Chromatin Structure

In the *hsp26* promoter, a positioned nucleosome, flanked on either side by clusters of GAGA factor binding sites, creates two DNase I hypersensitive regions (DH regions) corresponding to the highly accessible HSEs, the binding sites of HSF (Thomas and Elgin, 1988), and the TATA box. The DNase I pattern within the transposon chromatin (Figure 2A) shows two DH sites, corresponding to

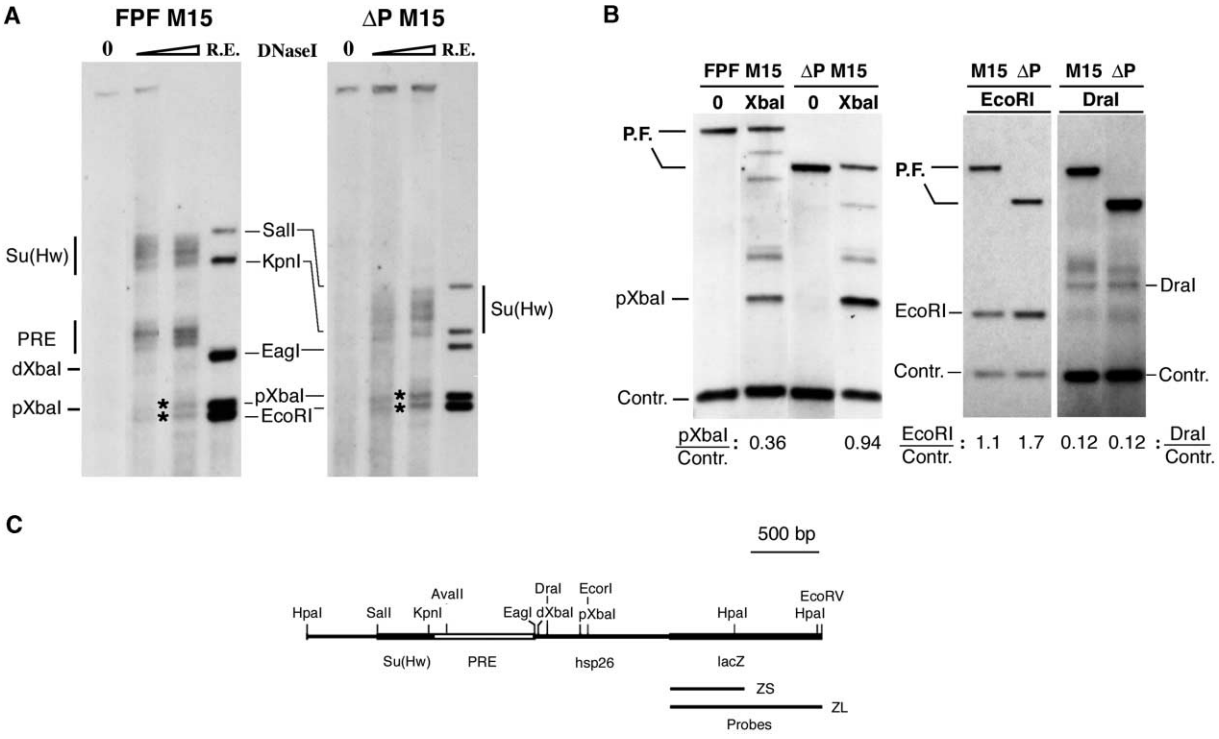

Figure 2. DNase I and Restriction Enzyme Accessibility

(A) DNase I hypersensitive sites in the FPF M15 line and its derivative, ΔP M15. Southern blot hybridization of chromatin samples of third instar larvae incubated without (0) or with DNase I. The purified DNA was then cleaved with EcoRV and hybridized with probe ZL. Lane R.E. shows restriction sites marking the functional elements within the construct. DH sites in *hsp26* are indicated by asterisks.

(B) Effect of PRE excision on restriction enzyme accessibility. Nuclei from third instar larvae of line FPF M15 and ΔP M15 were treated without (0) or with an excess of XbaI, EcoRI, or DraI. The purified DNA was cleaved with HpaI and AvaII or HpaI alone and hybridized with probe ZS. PF, parental fragment. The quantitation of the pXbaI, EcoRI, or DraI bands is expressed as a ratio to the corresponding HpaI control band (Contr.) that contains no XbaI, EcoRI, or DraI sites.

(C) Restriction map of the relevant part of the YGFPFhsW construct (also see Figure 3 for details).

the proximal HSE and TATA box in the *hsp26* promoter, while the distal DH region is very weak both in the presence and absence of the PRE, probably because the upstream *hsp26* region is truncated in our construct. At least three very prominent DH sites within the PRE are characteristically found at all developmental stages and are independent of the degree of silencing (Dellino et al., 2002). In addition, a series of three to four DH sites, detected across the Su(Hw) insulator element, appears weak in embryonic chromatin but becomes prominent in larvae. The excision of the PRE from the FPF lines does not result in any observable difference in the *hsp26* DH sites.

Two XbaI restriction sites, pXbaI and dXbaI, are located in the proximal and distal HSE regions, respectively. We used the promoter-proximal restriction site, pXbaI, for a quantitative comparison of chromatin accessibility in repressed and nonrepressed lines. A small but reproducible decrease in pXbaI cleavage was observed comparing the FPF lines with their ΔP derivatives, indicating a shift in chromatin structure or plasticity (Figure 2B). Similarly, the EcoR1 site at position +8 relative to the transcriptional start becomes more accessible when the PRE is excised, but a DraI site within the positioned nucleosome region is very poorly cleaved in the presence or in the absence of the PRE. In contrast, BglI and PstI sites in the PRE core are always hyperaccessible and are unaffected by the degree of silencing (results not shown). We conclude that PcG silencing does not prevent the positioning of the nucleosome but it does cause some decrease in the degree of accessibility to the flanking sites, possibly suggesting a greater degree of wobble in position.

TFIID and RNA Polymerase Are Present at the Repressed Promoter

Like other heat shock promoters, the *hsp26* promoter is programmed prior to heat shock by the cooperative binding of GAGA factor, TFIID, and RNA polymerase. By interfering with the binding of GAGA factor, TFIID, or RNA polymerase II, PcG silencing could prevent the establishment of the preset chromatin conformation and inhibit heat shock induction.

To determine directly whether PcG silencing prevents the binding of TFIID and RNA polymerase, chromatin immunoprecipitation (ChIP) was carried out with chromatin from embryos of lines FPF M15 and its ΔP derivative with antibodies against Polycomb (PC), TBP, or the large subunit of RNA polymerase II. The products of the different immunoprecipitations were then analyzed by real-time PCR with primers specific for the transgenic *hsp26* promoter. For internal comparison, we also evalu-

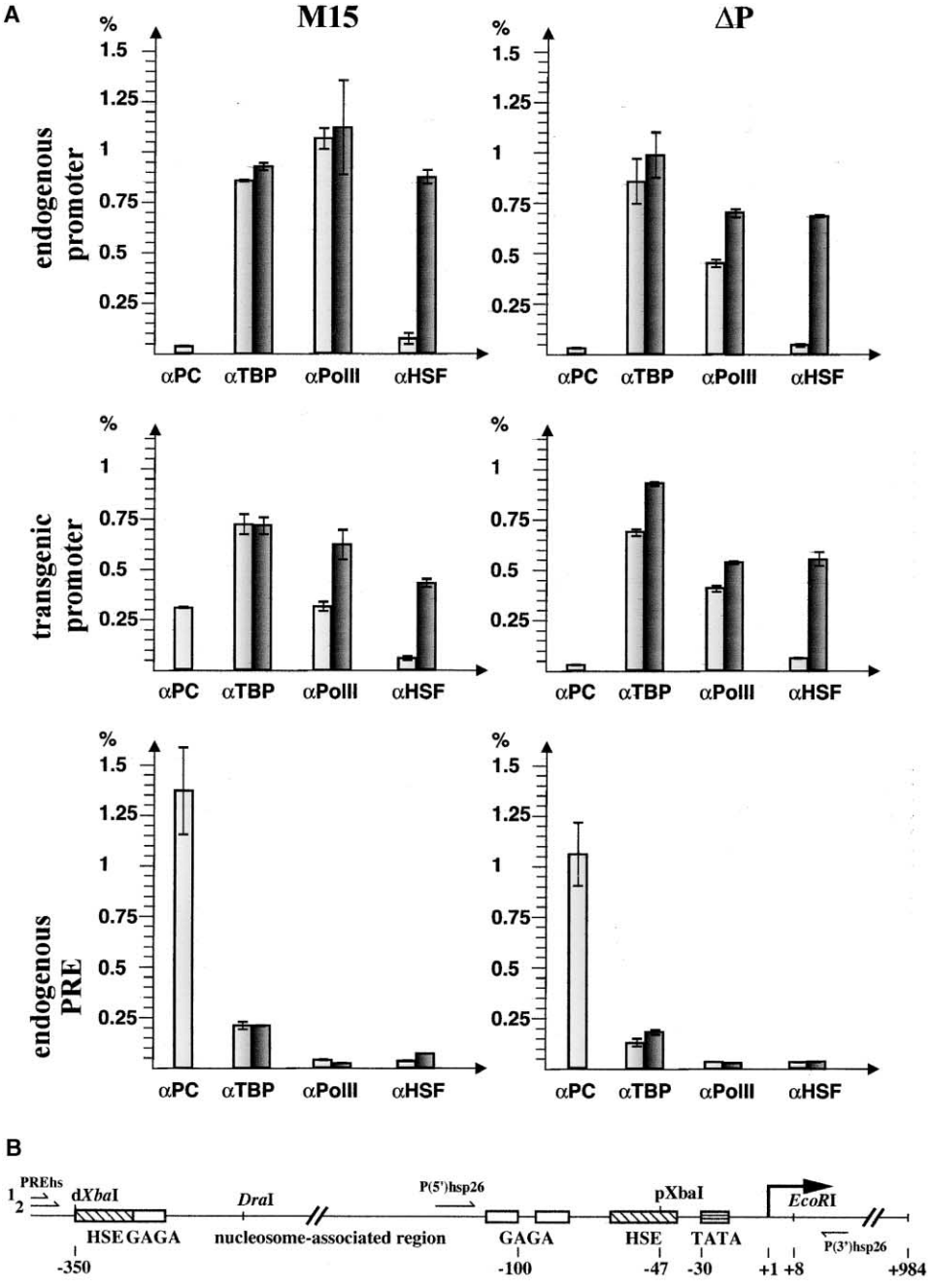

Figure 3. Chromatin Immunoprecipitation

(A) The results of real-time PCR analysis of the chromatin immunoprecipitation experiments are summarized in the histograms where the amount of chromatin precipitated is plotted as percent of input chromatin. The M15 and ΔP lines were analyzed with respect to the endogenous *hsp26* promoter, the transgenic promoter, and the endogenous PRE (as an internal control) by precipitation with antibodies directed against PC, TBP, RNA polymerase II, or HSF. Open bars, without heat shock; gray bars, with heat shock.

(B) Map of the *hsp26-lacZ* transgene promoter. The map shows the positions of the PCR primers (see Experimental Procedures), the GAGA factor binding sites, heat shock elements (HSEs), TATA box, and the positioned nucleosome flanked by DH regions. The arrow indicates the transcription start site, and half arrows mark the positions of LM-PCR primers.

ated the immunoprecipitation of the endogenous *hsp26* promoter and of the endogenous *bxd* PRE, using corresponding specific primers. The PCR results (summarized in Figure 3A) show that PC binds abundantly to the vicinity of the FPF M15 *hsp26* promoter but not near the ΔP M15 or the endogenous *hsp26* promoters. Both TBP and RNA polymerase are found at the promoters of the endogenous *hsp26* and of the nonrepressed ΔP derivative; they are also found at the promoter of the *hsp26* transgene in the repressed FPF M15 line.

The real-time PCR quantitation of the immunoprecipitated DNA shows that the amount of RNA polymerase

bound at the transgenic *hsp26* in the FPF M15 line is only slightly lower than that bound at the ΔP derivative (Figure 3A and see Supplemental Data at http://www.molecule.org/cgi/content/full/13/6/887/DC1). In contrast, the level of expression of β-galactosidase after heat shock induction is more than ten times lower in FPF M15 than in ΔP M15. Similar results were obtained with other lines. We conclude that the repression effects are not due to interference with the binding of RNA polymerase to the promoter.

Heat shock promoters do not require SWI/SNF function and do not bind the BRAHMA complex (Armstrong et al., 2002), but the NURF remodeling complex is necessary for normal binding of HSF and for the heat shock response (Badenhorst et al., 2002). Our results show that nucleosome positioning still occurs in our repressed hsp26 reporter, and TBP and RNA polymerase are not prevented from binding at the promoter. Therefore, at least in the *hsp26* gene, PcG silencing does not block the NURF-dependent remodeling or access to the promoter. We cannot exclude the possibility that inhibition of chromatin remodeling by PcG complexes might occur at other promoters and be important for their repression.

HSF Binds to the Repressed *hsp26* Promoter

A strict test of the ability of PcG complexes to prevent the access of transcription factors would be to determine whether HSF can bind to the HSE after a heat shock. We therefore carried out chromatin immunoprecipitation using anti-HSF with non-heat-shocked and heat-shocked embryos. A control assay using the endogenous *hsp26* gene shows that HSF presence at this site increases sharply after heat shock, while the *Ubx* promoter, which is not sensitive to heat shock, does not bind HSF (data not shown). Both the FPF M15 (repressed) and the ΔP M15 promoters show a sharp increase in HSF after heat shock (Figure 3). The transgenic promoter binds about half as much HSF as the endogenous promoter, which may be due to the fact that some additional HSF binding sites are deleted in the *hsp26* transgene. We conclude that, in these experiments, the presence of the PRE has little effect on the access of HSF. If PcG silencing does not significantly affect chromatin architecture, does not prevent the binding of TBP and pol II, and does not interfere with the access of HSF upon heat shock, how then does it silence the *hsp26* promoter?

Polymerase at the Repressed Promoter Does Not Form an Initiation Complex

Repression of heat shock induction could still be achieved by interfering with the formation of the initiation complex or the recruitment of Mediator and the release of the polymerase from the promoter. To understand the status of the polymerase bound at the repressed promoter, we treated the chromatin with $KMnO_4$, and analyzed the products by high-resolution LM-PCR using primers specific for the *hsp26* transgene. At the normal *hsp26* promoter, the RNA polymerase is transcriptionally engaged, producing short RNAs of 25–50 nucleotides without leaving the promoter region (Rougvie and Lis, 1988; Rasmussen and Lis, 1995). When the polymerase is engaged, thymidines located within the single-stranded transcription bubble are more reactive to permanganate. As shown in Figure 4, permanganate reactivity is observed at positions +25, +38, and +39 in the nonrepressed ΔP M15 promoter, showing that the polymerase is transcriptionally engaged. This reactivity is nearly undetectable in the repressed parent line FPF M15, and no new bands appear in the initiation region except for a faint band at position +10. Therefore, although TBP and RNA polymerase are bound, PcG repression apparently interferes with some early step in transcription initiation.

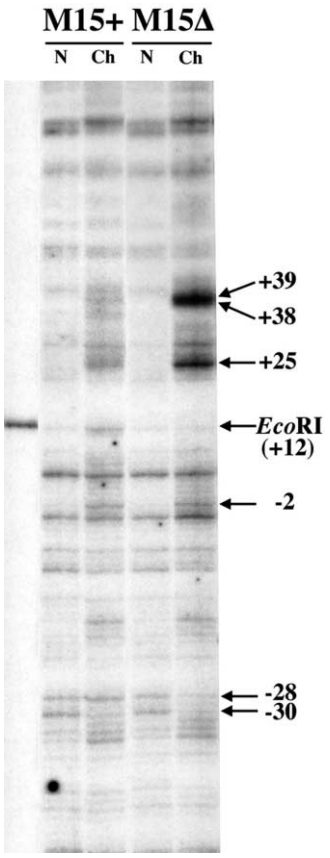

Figure 4. $KMnO_4$ Analysis of the *hsp26* Transgene

Chromatin of the FPF M15 line or its ΔP derivative was treated with $KMnO_4$ before (Ch) or after phenol extraction to produce the naked DNA control sample (N). The products were analyzed by LM-PCR using primers specific for the transgenic *hsp26* promoter region. Sites sensitive to permanganate, indicative of strand opening and the presence of an engaged RNA polymerase, are visible in the downstream region of the ΔP line but not in the M15 line. The TATA box region (position −30) shows the protection of one (M15) or two Ts (ΔP) due to the bound TBP. The lane on the left shows DNA cleavage with EcoRI at position +12.

Holstege et al. (1997) have identified distinct steps in promoter opening by pol II. A functional preinitiation complex requires ATP to open the strands in the −9 to +2 region. We do not see evidence of this strand opening in the repressed promoter. Initiation of synthesis extends the open bubble to +8, but closure of the trailing edge of the bubble occurs only when synthesis reaches +11. The fact that we only see a faint permanganate band at +10 suggests that most of the pol II bound

at the promoter in the M15 line has failed to open the strands and initiate transcription. In addition, the TBP may be seated imperfectly on the DNA. When TBP binds to the TATA box it protects the two Ts in the TATA sequence against $KMnO_4$ (Giardina et al., 1992). This protection is clearly visible in the ΔP M15 line (nucleotides −28 and −30 in Figure 4). In the repressed state, only one of the two Ts is still protected, suggesting that although TBP is present it is improperly seated on the TATA box sequence. Recent work shows that the TBP-DNA complex at the TATA box undergoes a slow transition from an unbent to a bent DNA complex, which is mediated by TFIIB (Zhao and Herr, 2002). That this transition might be inhibited by PcG silencing is suggested by the report that PC protein coimmunoprecipitates with TFIIB from *Drosophila* cells (Breiling et al., 2001).

Conclusions

Our results imply that, at least in the configuration studied here, PcG silencing does not involve coating the chromatin, condensing it, and preventing the access of *trans*-acting factors. The predominant effect of the PRE on the *hsp26* promoter is not so much to prevent access of promoter factors to the DNA as to interfere with the subsequent events necessary for opening the promoter and producing the initiation complex. The decrease in XbaI cleavage is consistent with the modest decrease in RNA polymerase binding at repressed promoters observed in the ChIP experiments. A possible explanation for both effects is that when polymerase does not form the initiation complex, its binding is less stable. Dissociation of the polymerase would in turn destabilize the preset chromatin state and decrease the accessibility to the XbaI sites. The findings reported here emphasize the importance of events at the primary chromatin fiber, rather than the involvement of "higher-order structures," to achieve regulation by the PcG system.

Experimental Procedures

Transposon Constructs

The YGPhsW and YGFPFhsW constructs were assembled in the C4 Yellow P element vector, which contains the *yellow* gene as a marker for transformation (Sigrist and Pirrotta, 1997), separated from the rest of the construct by the 436 bp Su(Hw) insulator element. The PRE, a 661 bp PstI-NdeI fragment containing the core region of the *bxd* PRE, was placed immediately in front of a *hsp26* promoter fragment containing 351 bp of the upstream region, including two sets of HSEs and two sets of GAGA factor binding sites, as well as 641 bp of the *hsp26* transcription unit, fused to the *lacZ* coding region. Finally, the *miniwhite* gene was included as a visible silencing indicator. In the FPF lines, the PRE was flanked by FRT sites to allow excision.

Fly Strains and Transgenic Fly Lines

The host fly stock used for germ line transformation was *Df(1)w*^{67c23} *y*$^{-}$*w*$^{-}$. Mutations used were *Pc*^{3}, *Su(var)2-5*^{01}, *trx*^{E2}, *brm*^{2}, *ISWI*^{2}. Eye colors were photographed using 2-day-old flies raised at 22°C. To excise the PRE, the FPF line was crossed with flies carrying a heat shock-inducible FRT transposase (Golic, 1994). The progeny were heat shocked for 1 hr at 37°C on 2 successive days during larval growth. In the following generation, flies were selected for a change in eye color, and PRE excision was verified by genomic Southern blots. To induce the *hsp26-lacZ* gene, third instar larvae were heated at 37°C for 30 min in a waterbath. After recovery for 1 hr at room temperature, the larvae were dissected, fixed with glutaraldehyde, and stained with X-gal to reveal the β-galactosidase activity (Poux et al., 1996). β-galactosidase activity was assayed in extracts prepared 1 hr after the heat shock, according to Simon and Lis (1987) with some modifications, using chlorophenol red-β-D-galactopyranoside (CPRG, Roche) as substrate and measuring the OD_{595} during the linear part of the reaction.

Chromatin Immunoprecipitation

Overnight embryo collections were heat shocked at 37°C for 30 min and immediately frozen in liquid nitrogen in parallel with control non-heat-shocked embryos. The frozen embryos were dechorionated and fixed with 1.8% formaldehyde for 20 min at room temperature (Cavalli et al., 1999). Fixed embryos (0.5 g) were resuspended in 5 ml RIPA buffer (140 mM NaCl, 10 mM Tris-HCl [pH 8.0], 1 mM EDTA, 1% Triton X-100, 0.1% SDS, 0.1% sodium deoxycholate, 1 mM PMSF) and lysed by sonication in the presence of 2 ml of glass beads (150–212 μ, acid-washed, Sigma). The lysate was cleared by centrifugation for 5 min at 20000 g, divided in 500 μl aliquots and used immediately or stored at −80°C.

For immunoprecipitations, 500 μl of lysate was precleared by incubation with Protein A Sepharose beads (Sigma). Clear lysate was incubated with appropriate antibodies overnight at 4°C. Antibodies used were rabbit polyclonal anti-PC (3 μl) (Poux et al., 2001), rabbit polyclonal anti-TBP (1 μl) (obtained from J. Kadonaga), mouse monoclonal anti-RNA polymerase 8WG16 (5 μl) (Covance), rabbit polyclonal anti-HSF (2 μl) (obtained from J. Lis and C. Wu). The antibody complexes were bound to Protein A Sepharose beads (Sigma), washed five times with 1 ml RIPA, once with 1 ml LiCl buffer (250 mM LiCl, 10 mM Tris-HCl [pH 8.0], 1 mM EDTA, 0.5% NP-40, 0.5% sodium deoxycholate), and twice with 1 ml TE (10 mM Tris-HCl [pH 8.0], 1 mM EDTA). The DNA was recovered as described by Cavalli et al. (1999) and dissolved in 60 μl water. Control mock immunoprecipitations were done in parallel with no added antibodies.

Real-Time PCR Analysis

DNA from 2 μl of each sample was amplified in 20 μl reaction mixtures in the presence of 10 μl 2xSYBR Green PCR Master Mix (Bio-Rad) and 0.5 μM of corresponding primers. Primers used to amplify the transgenic *hsp26* promoter were PRE hs2, 5′-AGTTCTA GAGCGGCCGAATTGG-3′, and P(3′)hsp26, 5′-AGTTGCTTTGAGTT GTTCAC-3′. Primers used for the analysis of the endogenous *hsp26* promoter were P1 hsp26 5′-CTTTTGCGCTCTTTCTA-3′ and P(3′)hsp26. To amplify the endogenous *bxd* PRE, we used primers BP3 5′-GCCATAACGGCAGAACCAAAG-3′ and BP4 5′-ATGAGGCCATCT CAGTCGC-3′. All primers were annealed at 56°C. Real-time PCR was performed in 96-well plates with the iCycler Real-Time PCR Detection System controlled by iCycler iQ software v3.0A (Bio-Rad).

The fraction of input DNA immunoprecipitated in the ChIP reaction (expressed in % input) was calculated from the reaction threshold cycle value using the appropriate 5-point standard curve (for details see the Supplemental Data at http://www.molecule.org/cgi/content/full/13/6/887/DC1). Standard curves were made separately for each combination of crosslinked lysate and primer pair by amplification of serial dilutions of the input DNA isolated from an aliquot of lysate. All experiments were repeated several times, and mean values and standard deviations were calculated.

Potassium Permanganate Genomic Footprinting

Genomic footprinting with $KMnO_4$ was performed as described by Weber et al. (1997). The products were analyzed by LM-PCR to determine the reactivity of the transcribed strand of the *hsp26-lacZ* transgene using primer PREhs1, 5′-AGTTCTAGAGCGGCCG-3′ for elongation, primer PREhs2 and linker primer 5′-GTGACCCGGGA GATCTGAATTG-3′ for amplification, and primer P(5′)hsp26, 5′-GTT TATCAAACGATACAAAGCTATAATTCAT-3′ for labeled extension.

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