Polycomb Complexes and the Propagation of the Methylation Mark at the Drosophila Ubx Gene

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Polycomb group proteins are transcriptional repressors that control many developmental genes. The Polycomb group protein Enhancer of Zeste has been shown in vitro to methylate specifically lysine 27 and lysine 9 of histone H3 but the role of this modification in Polycomb silencing is unknown. We show that H3 trimethylated at lysine 27 is found on the entire Ubx gene silenced by Polycomb. However, Enhancer of Zeste and other Polycomb group proteins stay primarily localized at their response elements, which appear to be the least methylated parts of the silenced gene. Our results suggest that, contrary to the prevailing view, the Polycomb group proteins and methyltransferase complexes are recruited to the Polycomb response elements independently of histone methylation and then loop over to scan the entire region, methylating all accessible nucleosomes. We propose that the Polycomb chromodomain is required for the looping mechanism that spreads methylation over a broad domain, which in turn is required for the stability of the Polycomb group protein complex. Both the spread of methylation from the Polycomb response elements, and the silencing effect can be blocked by the gypsy insulator.
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Proteins are present at the PRE and promoter regions, H3 K27 methylation is localized over the PRE but K9 methylation is widely distributed. We find that PcG proteins are localized primarily to the PREs while me3K27 is spread over the entire gene. Surprisingly, the PREs are the least methylated parts of the Ubx gene, probably because of the fact that these regions are depleted of nucleosomes. We show that the spread of H3 methylation is blocked by a gypsy Su(Hw) insulator, consistent with the observation that the insulator prevents silencing of a gene when interposed between PRE and promoter. Our results suggest that trimethylation of H3 at K27 is unlikely to serve as a recruiter of PcG proteins to their response elements but may be important for stabilizing the complexes and may participate more directly in transcriptional repression.

EXPERIMENTAL PROCEDURES

Fly Lines—The YGPhsW transposon construct in lines F22 and M7 is described in Ref. 21. The PGM62 line is described in Ref. 6, the site of transposon insertion in this line was determined by inverse-PCR using the following primers: Plac1 (CACCAAGGCTCTGCTCCCAAAAT) and Plac4 (5′-ACTGTGCGTATGTTCTGTTATCATT-3′), Plac1 and Pwh1 (5′-GTAACCGTAAATCCTCCGCAGGTCACA-3′), Pry1 (5′-CTTTAGCATGTCCGTGGGGTTTGAAT-3′), and Pry2 (5′-CTTGCGGACGGACCCACCTATTATT-3′).

Nuclease Sensitivity Assay—Nuclei from Schneider L2 cells at a concentration of 1 mg of DNA/ml were incubated for 2 min at 24 °C with 0–80 units/ml DNase I. The purified DNA products were then cut with suitable restriction enzymes, analyzed by gel electrophoresis, and Southern blot hybridization with corresponding probes. To produce nucleosome ladders, the nuclei were digested with MNase (1–5 units/ml) for 1 min at 24 °C, and the Southern blot was then successively hybridized with probes ES, PA, and AK from the bxd PRE region (see Fig. 3).

Antibodies—Unless specified, all antibodies were rabbit polyclonal. Anti-E(Z) antibodies were raised against a peptide containing amino acids 8–190 fused to GST and affinity-purified as described in Ref. 22. Anti-PC and anti-PSC antibodies were described in Ref. 22. The anti-me3K9 was described in Ref. 23; anti-me2K7 and anti-me3K7 were described in Ref. 24 and generously supplied by T. Jenuwein. Antibodies against me3K27 (ab6002) and the C-terminal part of histones H3 (ab1791) were purchased from Abcam. Anti-H2B (07-371) antibodies were from Upstate. Anti-TBP antibodies were a gift from J. Kadonaga and mouse monoclonal anti-RNA polymerase (8WG16) antibodies were purchased from Covance.

Chromatin Cross-linking and Immunoprecipitation—Cells of a derivative of the Schneider L2 cultured cell line, Sg4, were grown and cross-linked as described (25), the embryos were collected overnight and cross-linked as described in Ref. 21. Fixed cells or embryos were resuspended in TEN140 buffer (140 mM NaCl, 10 mM Tris-HCl pH 8.0, 1 mM EDTA) to a concentration of ~1 × 10⁸ cells/ml or 100 mg/ml, respectively. 5 ml of suspension were subjected to sonication with a Branson 250 sonifier equipped with microtip under conditions described in Ref. 25. The resulted lysate was further adjusted to radioimmunoprecipitation assay 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 phenylmethylsulfonyl fluoride) and subjected to 5 min of centrifugation at 16,000 × g. For ChIP, 500 μl of supernatant were precleared by incubation with protein A-Sepharose (Sigma) and further incubated with appropriate antibody overnight at 4 °C. 5–6 μg of anti-PC, PSC, me3K27, me2K7, H2B, 1–3 μg of anti-H3, E(Z), Trithorax (TRX), GAGA, 1 μl of anti-TBP, and 5 μl of anti-me3K9 and anti-RNA polymerase (8WG16) antibodies were used per one reaction. The antibody complexes were precipitated, and DNA recovered as described (25). Immunoprecipitated DNA was dissolved in 150 μl of water (V₁).

Analysis of ChIP Products—The primers used for real-time PCR analysis of the immunoprecipitated chromatin are listed in supplementary Table S1. PCR reactions were assembled by mixing 5 μl (V₂) of immunoprecipitated DNA solution with 10 μl of 2×SYBR Green PCR Master Mix, 0.1 μM of corresponding primers and pure water to 20 μl. The starting amount of the template DNA relative to the total amount of the same DNA fragment that had been present in the corresponding ChIP reaction (the X value expressed in percent) was derived from the comparison of the reaction threshold cycle value (Ct) to the appropriate 5-point standard curve. Standard curves were made separately for each combination of cross-linked lysate and primer pair by amplification of serial dilutions of the input DNA isolated from an aliquot of lysate that did not undergo immunoprecipitation. The yield of ChIP reaction (N) was then calculated using Equation 1.

\[ N = X \times \frac{V_1}{V_2} \]  

(Eq. 1)

The hybridization of ChIP materials with GeneChip Drosophila Forward Tiling Array (Affymetrix; P/N: 511262) is described in details elsewhere (26). The hybridization data are deposited to ArrayExpress data base with accession number: E-MEXP-535.

RESULTS

PREs Are the Major Binding Sites of PcG Proteins—In Drosophila Schneider L2 (SL2) tissue culture cells, the Ubx gene is silenced by PcG mechanisms (27). The PREs are known to recruit PcG proteins but we were interested in determining if in a repressed gene other sites, the promoter in particular, are associated with characteristic components of PcG complexes. We performed chromatin immunoprecipitations using cross-linked chromatin and antibodies against PC, PSC, and E(Z) proteins. The localization of these proteins was determined by sampling sequences uniformly scattered along the Ubx gene (Fig. 1A) and amplifying them by quantitative real-time PCR. The absolute amount of DNA immunoprecipitated was calculated from the threshold cycle value and a five-point standardization curve made by serial dilution of DNA isolated from chromatin immunoprecipitation input material. This is essential to obtain meaningful data because different sequences and different primers have very different amplification efficiencies, which cannot be taken into account by simple end-point PCR analysis. The precipitation profiles for these three proteins look qualitatively similar (Fig. 1C). Two distinct peaks of precipita-
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The two PREs of the Ubx gene (black rectangles), are situated one in the upstream regulatory region and the other in the third intron of the transcription unit. The Ubx transcription start site is marked by an arrow, and exons are indicated as white rectangles. The positions of DNA fragments amplified for quantification of ChIP results are shown below the Ubx map relative to the DNA sequence coordinates of chromosome 3R (sequence release 4). B, precipitation of DNA fragments with anti-H3 me3K27 (purple) and anti-H3total (yellow) antibodies was quantified by real-time PCR and shown after normalizing to the value for the Ubx promoter fragment. The results of ChIP with anti-PC (blue), anti-PSC (green), and anti-E(Z) (red) antibodies are shown below (C). In this case, the precipitation values are normalized to those of the bxd-PRE (BP). This internal reference enables comparison between different experiments. D, genomic tiling arrays were hybridized with probes made from ChIP material obtained using the indicated antibodies and corresponding chromatin inputs. To compensate for sequence-based variation in amplification efficiency and hybridization properties of individual array features, the data were computed in terms of ChIP/Input ratios. The values were further smoothed by taking a trimmed mean over a sliding 675 bp window to reduce the impact of isolated, aberrant probes. The gap in the profiles seen next to the bxd-PRE is caused by the lack of unique oligonucleotides in the corresponding DNA sequence.

FIGURE 1. Distribution of histone H3 and PcG proteins in the Ubx region. A, Ubx locus has a broad regulatory region containing a number of embryonic and imaginal disc enhancers (white and gray circles, respectively). The two PREs of the Ubx gene (black rectangles), are situated one in the upstream regulatory region and the other in the third intron of the transcription unit. The Ubx transcription start site is marked by an arrow, and exons are indicated as white rectangles. The positions of DNA fragments amplified for quantification of ChIP results are shown below the Ubx map relative to the DNA sequence coordinates of chromosome 3R (sequence release 4). B, precipitation of DNA fragments with anti-H3 me3K27 (purple) and anti-H3total (yellow) antibodies was quantified by real-time PCR and shown after normalizing to the value for the Ubx promoter fragment. The results of ChIP with anti-PC (blue), anti-PSC (green), and anti-E(Z) (red) antibodies are shown below (C). In this case, the precipitation values are normalized to those of the bxd-PRE (BP). This internal reference enables comparison between different experiments. D, genomic tiling arrays were hybridized with probes made from ChIP material obtained using the indicated antibodies and corresponding chromatin inputs. To compensate for sequence-based variation in amplification efficiency and hybridization properties of individual array features, the data were computed in terms of ChIP/Input ratios. The values were further smoothed by taking a trimmed mean over a sliding 675 bp window to reduce the impact of isolated, aberrant probes. The gap in the profiles seen next to the bxd-PRE is caused by the lack of unique oligonucleotides in the corresponding DNA sequence.
Chromatin of the Repressed Ubx Gene Is Enriched in Methylated Histone H3—To determine the relationship of histone H3 methylation to the distribution of PcG proteins, we performed chromatin immunoprecipitations using antibodies against histone H3 trimethylated at lysine 27 (me3K27). Fig. 1B shows that most fragments from the Ubx region, comprising 40-kb upstream of the transcription start and the entire 75 kb of the transcribed region, are precipitated well above the background level. A similar distribution profile was obtained using anti-me3K9. As background control, we took the degree of precipitation of DNA from the coding region of the white gene, which is not transcribed in these cells.

Surprisingly, while all parts of the silenced Ubx gene appear to be associated with me3K27, little methylated histone H3 could be detected at fragment BP (Fig. 1B), which corresponds to the core of the bxd-PRE, the major PRE of the Ubx gene (28). To a lesser extent, this is also true for the BX1 fragment, which corresponds to the weaker BX-PRE (5, 2).

When chromatin immunoprecipitation results were analyzed by hybridization to the Affymetrix genomic tiling microarrays, the resulting profile confirms the results obtained by real-time PCR. The level of me3K27 is high over the upstream regulatory region and entire Ubx gene, tapering off around the 3'-end of the transcription unit. Instead of a level plateau, methylation shows a reproducible series of rapid oscillations with a period of 2 kb. It is possible that these fluctuations reflect a higher order structure of the Ubx chromatin or the process by which methylation spreads from the PRE site (see “Discussion”). We also note that the methylation profile drops sharply over the two PRE sites, in agreement with the real-time PCR data. This undermethylation of presumptive PREs is found frequently but not universally at other PcG target genes (26).

These results are in contradiction with several recent publications that reported finding H3 K27 methylation localized over the bxd PRE, not elsewhere on the Ubx gene (12, 19, 20). This led us to devote considerable effort to ascertain the validity of our results and the reasons for the discrepancy. We first confirmed that our results were not antibody-dependent. We used two different anti-me3K27 antibodies with essentially similar results. We also tested different degrees of chromatin cross-linking and found that, except at the extremes of under- or over-cross-linking, the results were not qualitatively altered over a 10-fold range of formaldehyde concentration. We asked if the methylation profile reflected that of total histone H3. To determine this, we performed chromatin IP using antibodies against the C-terminal part of H3 (anti-H3total). This region of H3 is not subject to known modifications hence anti-H3total should recognize all forms of this histone. As shown in Fig. 1B, DNA from the coding region of the white gene as well as most DNA fragments from the Ubx locus were precipitated with anti-H3total roughly to the same extent. In contrast, there is indeed much less precipitation of the BP fragment. Some decrease in precipitation compared with the surrounding regions is also visible for the BX1 fragment. In contrast with specific binding proteins or histone modifications, antibody against H3total is expected to precipitate nearly the entire genomic DNA complement. In this case clearly, the antibody added was only a very small fraction of the amount necessary to bind all the H3 present, probably explaining the lower signal to noise ratio obtained. We have also carried out chromatin immunoprecipitation with anti-histone H2B with similar results (not shown) indicating a local depletion of histone at the PRE. These results suggest the possibility that the H3 K27 undermethylation might be because of the absence of nucleosomes at the PRE core.

Detailed Analysis of the PRE Region—The bxd PRE, the major PRE of the Ubx gene, is a complex region encompassing more than one 1.5 kb of DNA whose core sequences, rich in binding sites for PHO and GAGA factors have the strongest silencing activity (28). The core is flanked by sequences that, on their own, have weaker silencing activity. Both core and flanking sequences can bind in vitro PcG complexes from embryonic nuclear extracts, implying that they contain binding sites for PcG-associated recruiting proteins (28). We examined in greater detail the PRE region by ChIP assays for PC, PSC, E(Z), GAGA factor, TRX as well as H3 me3K27, H3 me2K27 and total histone H3.

The results, shown in Fig. 2, confirm that the PRE core is most highly enriched for PC and, at the same time, contains the least H3 me3K27 or total histone H3. Immediately flanking fragments contain progressively less PC and increasing amounts of me3K27 and core histone. We suppose that these fragments are probably still capable of intrinsically recruiting PC complexes through DNA binding recruiting proteins. Interestingly, none of the PRE fragments showed strong precipitation with antibodies against H3 dimethylated at lysine 27 (me2K27). The distribution of E(Z) and PSC, is more sharply localized at the PRE core than that of PC, much as indicated by the microarray profiles. A similar very sharp localization is obtained for TRX and for GAGA factor. The latter, a DNA-binding protein binds cooperatively to clustered consensus sequences GAGAG and should be confined to DNA containing these motifs. In fact, the binding observed reflects remarkably well the number of clustered GAGA consensuses. We note also that these results imply that the PcG complexes constitute no hindrance to antibody access to GAGA factor bound to DNA. Remarkably TRX, which is thought to be antagonistic to PcG binds exactly the same DNA fragments.

Chromatin State of the PRE Core—The ChIP experiments reveal that the bxd PRE is the binding site of a large number of proteins but, curiously, not histone H3 whether methylated or not. To account for this absence, we considered three possible explanations: 1) The binding of large PcG complexes at the PRE might prevent the access of the antibody to the histone H3. This would be surprising, as accessibility problems with chromatin immunoprecipitation have not been reported previously. There appears to be no interference with the access of antibody to other PRE proteins, including GAGA factor, which lies closest to the DNA and is thought to form a multimeric complex that binds to clustered DNA consensus sequences. Another possible explanation is that the me3K27 epitope is pre-empted by interactions with the chromodomain of PC or other proteins. The fact that both me3K27 and total H3 appear similarly depleted at the PRE argues against the idea that the me3K27 epitope might be masked by the PC chromodomain. As a further control, we
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A

PRE core

1kb

B' AS F A FFAA RF F A B PF F SFF A K F F FPFF H

12592873

FM1 FM2 FM3 FM4 BP FM5 FM6 FM7 FM8

12581421

B

PC

C

E(z)

D

PSC

E

GAGA

F

TRX

G

me2K27

H

me3K27

I

H3

0.26 0.36 0.33 0.69 1.00 0.86 0.27 0.32 0.28 0.10 0.02

0.17 0.15 0.19 0.12 0.16 0.11 0.18 0.16 0.22 0.27 1.00

0.04 0.04 0.10 0.30 1.00 0.83 0.11 0.05 0.03 0.03 0.02

0.04 0.07 0.07 0.26 1.30 0.13 0.07 0.06 0.48 0.06

0.89 1.03 1.00 0.58 0.27 0.41 0.95 0.95 1.11 1.00 0.99

0.01 0.02 0.02 0.14 1.00 0.52 0.05 0.01 0.01 0.02 0.01

0.17 0.15 0.19 0.12 0.16 0.11 0.18 0.16 0.22 0.27 1.00
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The Gypsy Insulator Blocks the Spread of Histone H3 Methylation—The data presented above are not consistent with the idea that methylation of H3 is involved in the recruitment of PcG complexes to the PREs but suggest that it may serve a different, subsequent function that may contribute to the stability or be more directly related to the silencing mechanism itself. If the PC complex and the E(Z) complex are both recruited to and localized at the PRE, how does the entire \textit{Ubx} gene become methylated? Experiments in which the PRE is excised in vivo have shown that its continued presence is required for the maintenance of silencing (35). Furthermore, the silencing process proceeds from the PRE and, like enhancer action, is blocked by the insertion of a chromatin insulator between the PRE and the promoter (6, 36). If so, would the spreading of H3 methylation from the PRE to the chromatin of a target gene be prevented when its silencing action is blocked by an insulator element?

To test this, we used a transposon construct in which a core 650-bp fragment of the \textit{bxd} PRE is placed upstream of the \textit{white} gene and separated from it by the gypsy \textit{Su(Hw)} insulator (Fig. 4A). In line PGM62 containing this transposon, the silencing effect of the PRE is blocked by the insulator and the \textit{white} gene is active, resulting in strong eye pigmentation (6). In a \textit{su(Hw)} mutant background, the insulation is lifted and the PRE silences the \textit{white} gene completely, resulting in white eyes.

ChIP analysis of PGM62 embryos was done to map the localization of PC, PSC, E(Z), total histone H3, and me3K27 H3. The PC protein, as expected, is localized over the PRE region (Fig. 4B) and the same is true for PSC and E(Z) proteins (not shown). The distribution of these PcG proteins is not greatly changed in a \textit{su(Hw)} mutant background. When we looked at the distribution of me3K27, we found again that it is very low at the PRE itself but high in the sequences flanking the transposon 3′-end (Fig. 4C). The \textit{white} gene on the other side of the insulator is not appreciably methylated in wild type embryos but becomes strongly methylated in \textit{su(Hw)} mutant embryos. The distribution of bulk histone H3 was the same in the wild type and \textit{su(Hw)}− background (Fig. 4D). These results suggest that
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spreading of H3 methylation from the PRE to the chromatin of the white gene is blocked by the gypsy insulator element. Interestingly, the chromatin flanking the 5′ end of the transposon is not methylated in either the wild type or su(Hw) mutant. Sequencing of the regions flanking the transposon insertion site, obtained by inverse PCR, showed that the insertion has occurred one nucleotide downstream of the transcription start site of the CG8776-RA transcript, one of the transcripts encoded by the putative carbon monoxide oxygenase gene (CG8776, Berkeley Drosophila Genome Project, (37)). The promoter of this transcript is immediately to the right of the transposon 5′-end in Fig. 4A. Neither the promoter nor the downstream region contain me3K27 in flies lacking the transposon insertion. It is possible that CG8776 promoter activity prevents the spreading of methylation beyond the white gene.

H3 K27 Methylation and Levels of Repression—We confirmed the effect of the Su(Hw) insulator on the spreading of histone H3 methylation using another transposon construct of a type earlier described as YGPhsW (21). In this construct the bxd PRE is placed in front of the hsp26-lacZ gene followed by the miniwhite gene. On the other side of the PRE, the Su(Hw) insulator protects the yellow gene from silencing (Fig. 5B). We used two lines carrying this YGPhsW transposon, one (F22) displaying strong silencing of both the miniwhite gene and the hsp26-lacZ gene, the other (M7) displaying very little silencing, strong uniform eye pigmentation in most flies and strong inducible expression of hsp26-lacZ. We examined these lines in a wild type or in a su(Hw) mutant background. Interestingly, silencing is distinctly enhanced in the mutant background. In the F22 line, the eye color becomes virtually white instead of strongly variegated and even in line M7 the eye is visibly lighter although it remains uniformly pigmented (Fig. 5A). Both positive and negative effects of a neighboring Su(Hw) insulator on PcG silencing have been previously reported and attributed to trans-interactions (6). We then carried out a ChIP analysis to determine the distribution of PC protein, of H3 K27 methylation and of total histone H3 in the presence or absence of Su(Hw) protein. We were interested in comparing the effect of the insulator and the degree of histone methylation in the strongly versus the weakly repressed lines.

The ChIP results in Fig. 5, C–E confirm the essential block created by the Su(Hw) insulator to the spread of H3 methylation from the PRE. In the su(Hw) mutant background the methylation of the yellow gene region, which was very low in the wild-type background, increases to the level seen at the hsp26 promoter. Equally interesting, the PC signal also increases systematically over the yellow gene region when the insulator block is relieved. The levels reached in the mutant embryos are not high but the increase is consistent in the F22 and M7 lines and a similar effect can be detected over the white gene in the experiment shown in Fig. 4A. We interpret these results as showing that, in the absence of Su(Hw) protein, the region beyond the insulator becomes accessible to transient interactions with the PcG complex residing at the PRE with the result that histone methylation now takes place in this region. Note that the distance between the insulator and the yellow promoter here is 2.3 kb, making it highly unlikely that the insulator

FIGURE 3. The PRE region is nucleosome-free. A, map of the 6.7-kb HindIII fragment containing the bxd PRE indicating selected restriction sites, the position of the PRE core, and the position of the probes used in the nuclease sensitivity experiments. The asterisks indicate the DNase I hypersensitive sites. B, nucleosome ladder was produced by digestion of chromatin (1 mg of DNA/ml) with MNase (1–5 units/ml) for 2 min at 24 °C, and the Southern blot was then successively hybridized with probes ES, PA, and AK from the bxd PRE region. Probe PA shows that the nucleosome array is disrupted over the PRE core region. C, chromatin from S2 tissue culture cells (1 mg of DNA/ml) was digested with DNase I (0–80 units/ml) for 2 min at 24 °C. After phenol extraction, the Southern blot was then successively hybridized with probes ES, PA, and AK from the bxd PRE region. Probe ES indicates selected restriction sites, the position of the PRE core, and the position of the probes used in the nuclease sensitivity experiments. The asterisks indicate the DNase I hypersensitive sites.

TABLE 1
Quantitative results of ChIP with α-TBP and α-Pol II antibodies

The precipitation of each DNA fragment was quantified by real-time PCR. The resulting values were normalized to the amount of hsp26 promoter fragment precipitated. The values given are the averages of two independent experiments and the standard deviation.

| Ab         | hsp26 promoter Mean | White Mean | White +/- Mean | Ubx promoter Mean | Ubx +/- Mean |
|------------|---------------------|------------|----------------|-------------------|-------------|
| α-TBP      | 1.000               | 0.032      | 0.015          | 0.030             | 0.015       |
| α-Pol II   | 1.000               | 0.030      | 0.015          | 0.036             | 0.018       |
complex interferes directly with the local nucleosome organization in the promoter region.

We note also that, while consistent, the increased PC interaction detected in the yellow region is not directly proportional to the amount of methylation. When the insulation block is lifted, we find a significant level of PC in both the upstream and coding region of yellow but H3 K27 methylation, while very high in the coding region, is absent in the yellow upstream region. A loose correlation is also seen between the level of repression and the amount of both PC and me3K27 precipitation in the two YGPhsW lines used. The more repressed line F22 has higher levels of both than the less repressed M7 line. In the su(Hw) mutant background, we find that the level of repression of the white gene increases in both lines and we also see a concomitant rising trend in both methylation and PC binding over the hsp26-lacZ and mini-white region.

FIGURE 4. The spreading of histone methylation is blocked by the gypsy Su(Hw) insulator. A, schematic representation of the transposon insertion site in the PGM62 line. Genomic DNA (thin line), P element ends (black arrowheads), bx-d-PRE (dashed box), gypsy insulator (black oval), and miniwhite fragments are indicated. The PCR probes used in the flanking regions are designated according to their distance from the transposon insertion site. The results of chromatin immunoprecipitations with antibodies specific for PC (B), me3K27 (C), and H3total (D) for each position probed are shown by gray bars for wild-type chromatin and white bars for su(Hw)- chromatin.

FIGURE 5. Correlation between H3 K27 methylation and the level of repression. A, expression of the white gene in the YGPhsW transgenic lines. The eye color of the strongly repressed F22 line and the weakly repressed M7 line are shown in wild type and su(Hw) backgrounds. B, YGFPPhsW construct is shown schematically with P element ends (arrows), bx-d-PRE, gypsy insulator (black oval), yellow, hsp26-lacZ fusion and miniwhite fragments indicated. Transcription start sites for all genes are shown by broken arrows. The positions of DNA fragments amplified for ChIP quantification are indicated below. The distribution of PC (C), H3 me3K27 (D), and H3total (E), along the transposon in embryos of F22 su(Hw)- (gray columns), F22 su(Hw)+ (white columns), M7 su(Hw)- (black columns), and M7 su(Hw)+ (shaded columns) is shown. Note that to see the transposon sequences rather than the endogenous sequences, the 5' hsp probe chosen cannot be either in the PRE or in the hsp26 promoter but utilize the linker between the two. As a consequence this probe detects both PRE and promoter.

DISCUSSION

Chromatin immunoprecipitation is the technique of choice to map the distribution of regulatory factors along their target genes. Yet the need to quantitate ChIP results to interpret them correctly remains poorly appreciated. It is common practice to declare a protein to be specifically bound to a given sequence if, under certain conditions, the sequence is PCR-amplified from a fraction of immunoprecipitated DNA with a better yield than from an equal fraction of DNA precipitated without antibody.
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(“mock IP”). Or, a sequence is considered to represent a protein binding site if it is amplified better then control non-binding sequences when PCR conditions are adjusted to produce no visible product with a template generated in mock IP. Such analyses are usually displayed as pictures of agarose gels showing the end products of PCR. We argue that these approaches are not properly controlled and are often highly misleading.

In our experience, the mock IP product contains generally at least 10-fold less background genomic DNA than ChIP with any but highly diluted antibody. Hence, for any given sequence, more end product will be obtained when material precipitated in the presence of antibody rather then mock IP is used as a template for PCR. Secondly, it is known that different amplifiers can have very different amplification efficiencies. It is important to keep in mind that because of the exponential nature of the process even small differences in amplification efficiencies will result in a dramatic difference in the amounts of end product after 30 or more PCR cycles (38). The efficiency of PCR amplification is largely determined by the properties of the oligonucleotide primers used, but the properties of the DNA template are also a major contributing factor. As we have noticed earlier different regions of cross-linked chromatin may have very diverse sensitivity to ultrasound shearing, which is employed to produce soluble chromatin used as an input for ChIP. In fact, the bxd-PRE, which is a subject of the present study, was found to be sonication hypersensitive (25). The difference in the size of template DNA becomes of even greater concern if the ChIP material is amplified by linker-mediated (LM) or random PCR and then hybridized to Southern blots or to genomic microarrays. We believe that the only reliable way to interpret ChIP results correctly is to compare quantitatively the amount of a precipitated of fragment to its initial amount in the chromatin IP. This can be done by relating the quantitative real-time PCR to a calibration curve made with serial dilutions of DNA isolated from the chromatin IP. Alternatively, probes prepared in the same manner from ChIP and input material can be hybridized in parallel to Southern blot or genomic microarray and the ratios of the two signals determined. Relative precipitation values have then to be compared with those of positive and negative control regions to decide if the fragment under study shows significant binding to the protein of interest.

Our point is nicely illustrated by two attempts to map components of transcription machinery at the promoter of the silenced Ubx gene. In an earlier study Breiling et al. (27) reported the results of ChIP experiment analyzed as described in the first paragraph of this section and concluded that general transcription factors are still bound to the promoters of repressed genes. In the present study, although we do see more Ubx promoter DNA precipitating in the presence of anti-TBP or anti-Pol II antibodies than in the mock IP reaction, a careful quantitation and comparison reveal that these TBP and Pol II levels at the Ubx promoter are not higher than background levels. The absence of promoter complexes has similarly been observed in repressed Hox genes in mouse using real-time quantitative PCR analysis of ChIP (39).

Histone H3 K27 Trimethylation Forms a Broad Domain—The experiments described here show clearly that all three PcG proteins tested are preferentially located at the PREs. This specificity is clearest and most sharply delineated in the case of PSC and E(Z). In the case of PC, the peaks centered at the PREs are much broader, including secondary peaks, and although the binding detected at other Ubx regions decreases to low values, it never reaches the level seen at control sites such as the white gene that possess no PRE. The second basic conclusion from these experiments is that, in contrast to the localization of PcG proteins, the H3 me3K27 profile forms a broad domain that includes the entire Ubx transcription unit and upstream regulatory region. The third important observation is that, contrary to previously published accounts, the PREs themselves appear to contain the lowest levels of me3K27 of the entire domain. We will consider this surprising result first. The lack of apparent methylation at the PREs does not depend on the antibody used or on the level of cross-linking. We obtained comparable results with two different anti-me3K27 antibodies and with anti-me3K9. Furthermore, the fact that a similar result was obtained with antibody against total histone H3 or histone H2B (not shown) suggests that nucleosomes are underrepresented at the bxd PRE core. The result is not because of lack of accessibility to the histone or to the epitope: GAGA factor bound to the PRE appears as easily accessible as GAGA factor bound to the Ubx promoter. Salt extraction of the PcG complexes before cross-linking does not qualitatively change the me3K27 binding profile.

We think that the reasons for the difference of these results from those previously reported are several but the most important come from the methods used to evaluate the products of chromatin immunoprecipitation. A peak of H3K27 methylation over the bxd-PRE was repeatedly reported by the same group (12, 20), who based their conclusion on the high amounts of end product obtained after amplification of PRE DNA under PCR conditions adjusted to produce no visible product with a template generated in mock IP. As we have discussed above this approach cannot be properly controlled and thus may lead to misinterpretation. It is curious to note that in the work of Wang et al. (20) antibodies directed against trimethyl K27 were used to detect H3K27 methylation over bxd-PRE while in the earlier work Cao et al. (12) have reported essentially the same results using antibodies specifically against H3-dimethylated K27. In fact, ChIP with anti-me2K27 reveals that, not surprisingly, me2K27 is depleted in regions containing me3K27, relative to non-genic regions (our results, not shown). The same work also reported H3 dimethyl K9 to be uniformly distributed in the Ubx gene. Neither we nor others were able to detect appreciable levels of me2K9 in this region or any connection between this histone modification and PcG silencing (13, 19, 40). In another study Ringrose et al. (19) reported a distinct peak of me3K27 over the bxd-PRE. In this case the immunoprecipitated chromatin was amplified by LM-PCR, labeled and hybridized to a set of 1-kb fragments covering part of the Bithorax-Complex. It is important to note that the chromatin used for this ChIP experiment was purified by centrifugation in CsCl density gradient. As we have shown earlier bxd-PRE is quantitatively depleted during this procedure (25). The authors did recognize this problem and attempted to mathematically correct the data to compensate for the loss of PRE DNA. However the correction procedure itself is cause of concern. As stated in the article the
correction factor was derived from the comparison between hybridization signals produced by non-amplified total genomic DNA and DNA recovered from bulk chromatin fractions of CsCl gradient (19). This factor does not take into account the fact that the actual ChIP product is amplified by LM-PCR before hybridization analysis. Because of its sonication hypersensitivity, the bxd PRE region yields shorter DNA fragments during preparation of cross-linked chromatin and thus would be expected to amplify better then less sonication sensitive neighboring DNA (41). In that case post-experimental correction of hybridization data is likely to exaggerate bxd-PRE precipitation. The correction coefficient for bxd-PRE used by Ringrose et al. (19) is not reported, but can be deduced to be around 6 from comparison of Fig. 4A in Ref. 19 and Fig. 4A in Ref. 42, in which no correction was applied. Using this value the original data could be readily restored and would show essentially uniform distribution of me3K27 along the Ubx gene (see Ref. 19). Note, that the absence of me3K27 from the bxd-PRE core would be partly smoothed out by hybridization to 1-kb fragments because of the low resolution of the analysis.

In sum, we believe, that careful quantitative analysis of ChIP indicates that while PcG proteins are principally localized at the PRE, the histone H3 methylation they produce is distributed over the entire Ubx gene. Similar results have been reached by Papp and Müller (43). It is evident from this and from the undermethylation of the PRE core that that K27 methylation does not, by itself, recruit PcG complexes. This does not preclude an important role for methylation in PcG binding and silencing but suggests that the relationship between the two requires a more dynamic model.

**Relationship between PcG proteins and H3 K27 Methylation—** PREs have been shown to recruit PcG complexes and to produce new binding foci detectable in polytene chromosomes. It is not surprising therefore to find the three PcG proteins tested to be associated with the two Ubx PREs. A much smaller peak in microarray profiles for all three proteins can be discerned the vicinity of the Ubx 3′-exon but its significance is unknown. More surprising to us was the striking difference between the distributions of PSC and E(Z) and that of PC. E(Z) and PSC belong to two different complexes that do not co-precipitate (except in the very early embryo) but are both recruited to the PRE. PC and PSC are core components of the PRCA-type of PcG complex yet, while PSC is detected almost exclusively at the PREs, PC has a much broader distribution peaking at the PREs but tailing over considerable distances along the Ubx gene and regulatory regions. The simplest interpretation of this is that a second type of complex containing PC but not PSC is recruited by a different mechanism to the rest of the Ubx sequences. Alternatively, the same complex, containing both proteins is involved in both cases but the nature of the chromatin contact is different, such that in case both proteins are well cross-linked to the chromatin but in the second case only PC is efficiently cross-linked.

Just as striking is the fact that, although the E(Z) complex is responsible for the H3 K27 methylation spread over the entire Ubx gene, the E(Z) protein is found localized at the PREs. We conclude that the E(Z) complex methylates the Ubx domain by a hit-and-run type of mechanism. Because the methylation is stable, the E(Z) complex needs only visit each nucleosome once on the average every cell cycle. We note that E(Z)-dependent histone H3 K27 dimethylation is highly abundant and widely distributed in the genome (40) but we do not detect E(Z) complexes associated with it. Where then does the E(Z) that methylates PcG target genes come from? While more complicated scenarios may be imagined, the simplest one involves the E(Z) complex bound at the PRE.

**Spreading of Histone Methylation from the PREs—** We suppose that the PcG complexes are recruited to PREs by DNA-binding proteins independently of histone methylation. To methylate the entire Ubx domain, the E(Z)/ESC histone MTase complex might then detach from the PRE and slide along the chromatin from one nucleosome to the next to survey the entire domain. However, we think it more likely that both the PC and the E(Z) complexes assembled at the PRE remain associated with the PRE sequences, where they are detected, but that the whole PRE assembly loops over to scan the entire region, methylating all accessible nucleosomes (Fig. 6). Such looping models were originally proposed to be mediated by sites of weak PcG complex formation (see Refs. 44 and 45). In a modern version of this type of model, the looping activity would be mediated by the distinct affinity of the PC protein for histone H3, which is greatly increased by K27 methylation (18). These affinities would mediate transient interactions of the complexes bound at the PRE with the surrounding chromatin and allow continuous scanning and methylation of unmethylated or hemimethylated nucleosomes.

In such a model, ChIP experiments would always detect a strong PcG presence at the PRE but PcG interactions with the rest of the repressed gene would be distributed over a region, which is very large in the case of the Ubx gene, smaller in the case of the YGPhsW transposon, hence the signal detected at any one site would be weaker in proportion to the extent of the methylated domain. In addition, the contacts between the PcG complex and the rest of the silenced gene would be much more transient than contacts with the PRE. Together, these considerations would explain why our ChIP assay gives such low values for PcG proteins over the rest of the methylated domain.

The looping mechanism proposed for the PRE-bound complex strongly resembles that suggested for the interaction between the Locus Control Region and β-globin genes (46) or for enhancer-promoter interactions (reviewed in Ref. 47). Like these interactions, the silencing of a promoter by the PRE is blocked by insulator elements (6, 36). In our transposon constructs, the insertion of a gypsy Su(Hw) insulator between PRE and promoter blocks the spread of methylation. At present, the mechanism of insulator action is not clear and we cannot tell how the block to methylation is achieved. It is possible that the insulator element produces topological constraints that prevent the PRE-bound complexes from looping beyond the insulator. This would be consistent with the observation that a significant level of Pc presence becomes detectable over the yellow gene in YGPhsW when the insulator block is lifted (Fig. 5).

**The Role of Histone Methylation in PcG Repression—** Although our data argue against a principal role of histone methylation in the recruitment of Polycomb proteins to their
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response elements, it seems to be important for both transcriptional repression and stable association of PcG proteins with chromatin. Loss of catalytic E(Z) function eventually results in derepression of HOX genes (15) and dissociation of PcG proteins from polytene chromosomes (13, 48). We speculate that once the me3K27 domain is established, modified nucleosomes will pave the way for looping interactions of the PRE-bound PcG proteins with the parts of the silenced gene including promoter or enhancer regions. Silencing might then result from hit-and-run interactions with either or both, possibly even resulting in methylation of the associated factors. Alternatively or in addition, trimethylation of K27 and possibly K9 may directly interfere with the signaling cascade of consecutive histone modifications that guide the multistep process of transcription initiation and elongation (49, 50). As histone methylation is thought to persist through cell division its immediate presence at the very beginning of the subsequent interphase might win the time necessary for the full assembly of PcG complexes on the PREs before competing transcription has taken over.

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