A silencer is required for maintenance of transcriptional repression throughout *Drosophila* development

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SUMMARY

Transcriptional silencing by the Polycomb Group of genes maintains the position-specific repression of homeotic genes throughout *Drosophila* development. The Polycomb Group of genes characterized to date encode chromatin-associated proteins that have been suggested to form het-erochromatin-like structures. By studying the expression of reporter genes, we have identified a 725 bp fragment, called MCP725, in the homeotic gene *Abdominal-B*, that accurately maintains position-specific silencing during prolifer-ation of imaginal cells. Silencing by MCP725 requires the *Polycomb* and the *Polycomblike* genes, indicating that it contains a Polycomb response element. To investigate the mechanisms of transcriptional silencing by MCP725, we have studied its temporal requirements by removing MCP725 from the transgene at various times during devel-opment. We have discovered that excision of MCP725 during larval stages leads to loss of silencing. Our findings indicate that the silencer is required for the maintenance of the repressed state throughout cell proliferation. They also suggest that propagation of the silenced state does not occur merely by templating of a heterochromatin structure by virtue of protein-protein interactions. Rather, they suggest that silencers play an active role in the maintenance of the position-specific repression throughout development.

Key words: homeotic genes, imaginal disc silencers, transcriptional silencing, FLP-*FRT*, *Abdominal-B*, *Drosophila*, MCP, PRE, Polycomb Group genes

INTRODUCTION

Silencing, the clonal inheritance of a repressed transcriptional state (Brand et al., 1985; Laurenson and Rine, 1992; Loo and Rine, 1995), operates in the *Drosophila* homeotic genes to restrict their domains of expression during development. The repressed state is initiated early in development by the action of the transiently expressed gap segmentation proteins (Harding and Levine, 1988; Casares and Sánchez-Herrero, 1995). These proteins provide positional information by tran-scriptionally repressing the homeotic genes in specific paraseg-ments (ps) along the anterior-posterior axis of the embryo (Bienz, 1992). The maintenance, but not the initiation, of the repressed state throughout cell divisions depends on the Polycomb Group (PcG) of genes (Jürgens, 1985; Struhl and Akam, 1985; Simon et al., 1992). However, the mechanism by which the PcG proteins silence the homeotic genes in a position-specific manner is unclear, as the gap proteins are not detected after approximately 6 hours of embryonic develop-ment (Tautz, 1988; Gaul and Jäckle,1989), and the known PcG proteins are ubiquitously expressed and apparently do not bind specific DNA sequences (for reviews see Bienz, 1992; Bienz and Müller, 1995; Orlando and Paro, 1995; Simon, 1995; Pirrotta, 1997). It has been proposed that the early repressed state, initiated by the gap gene products, is recognized by the PcG proteins which promote the generation of multimeric protein silencing complexes at the silencer elements (Zhang and Bienz, 1992; Bienz, 1992). The PcG proteins are thought to induce the formation of stable heterochromatin-like struc-tures that are inherited, following DNA replication, through reassembly of their protein components (Locke et al., 1988; Orlando and Paro, 1995).

The proposition that the PcG proteins may induce hetero-chromatin-like structures, which stably and heritably inactivate transcription (Paro and Hogness, 1991; Eissenberg et al., 1995; Moehrle and Paro, 1994; Orlando and Paro, 1995), is based on indirect evidence. First, Polycomb protein shows sequence similarity within the 'chromo-domain' with heterochromatin-associated protein 1, HP1, encoded by the *Su(va)205* gene (Paro and Hogness, 1991). Mutations in *Su(va)205* suppress position effect variegation (PEV), a phenomenon thought to involve het-erochromatin (Eissenberg et al., 1990; Dernburg et al., 1996). Studies on PEV have shown that the distance which the hete-rochromatin extends in each cell is variable (producing a varie-gated phenotype), but once established, like homeotic gene silencing, it is stably and clonally inherited. Additional mecha-nistic similarities exist between PEV and silencing of homeotic genes. Reporter genes containing *cis*-regulatory Polycomb response elements of the homeotic genes can display variegated expression patterns in transformed flies (Pirrotta and Rastelli, 1994; Pirrotta, 1997). Furthermore, these reporter genes occa-sionally show spreading of the repressive effect on genes that flank the transgene insertion points (Chan et al., 1994; Pirrotta, 1997). Finally, in tissue culture cells, the inactive *Ultrabithorax* and *abdominal-A* homeotic genes are apparently coated with the

Polycomb protein while the active *Abdominal-B* (*Abd-B*) gene is relatively devoid of it (Orlando and Paro, 1993).

There are also results that argue against categorizing silenced chromatin as heterochromatin. Experiments in *Drosophila* embryos testing for highly compacted chromatin structure in the homeotic *Ultrabithorax* gene have been performed using heterologous DNA-binding proteins as probes for DNA accessibility (McCall and Bender, 1996). These experiments suggest that repression by the PcG proteins is not achieved by the exclusion of all DNA-binding proteins, as might be expected for highly compacted chromatin. Thus, the silenced chromatin does not have all the characteristics of heterochromatin. It has been suggested that PcG proteins may repress transcription not by inducing heterochromatin, but by interacting more directly with the transcription machinery (Gould et al., 1990; Jones and Gelbart, 1993; Bunker and Kingston, 1994; Pirrotta and Rastelli, 1994; Bienz and Müller, 1995). This could occur either by interaction of PcG repressor proteins directly with the promoter (Bienz and Müller, 1995) or by blocking looping interactions between enhancer and promoter (Pirrotta and Rastelli, 1994).

In this paper, we have investigated the mechanism of silencing by identifying a silencer element, MCP725, from the *Abd-B* gene, and testing if it is required throughout development to maintain a transcriptionally silenced state. We envisioned two possibilities. First, silencers might be dispensable for maintenance of silencing. This would suggest that the maintenance of homeotic gene silencing involves the formation of heterochromatin-like structures that, like heterochromatin in PEV, are able to self-propagate in the absence of specific silencer sequences. Second, silencers might be required throughout development to maintain the repressed state, suggesting an active role of the silencers in the reassembly or in the stability of the silencer complexes. Our results indicate that MCP725 is continuously required throughout development.

MATERIALS AND METHODS

Fly strains and transformant lines

Host flies for P-element transformation were *cn¹*; *ry⁴²*. *ciᴰ-lacZ* (Orenic et al., 1990) and *en-lacZ* (Hama et al., 1990) were used as parasegmental markers. *Pc³* is a null mutation for the *Polycomb* gene (Lewis, 1978). The *FRT²ᴿ⁻ᴳ¹³* and *FRT²ᴿ⁻ᴳ¹³ Pcl¹* (a null *Pcl* mutation; Duncan, 1982) transgenic flies were kindly provided by Martha Soto (Soto et al., 1995). The X chromosome *FLP* recombinase stock used was *w¹¹¹⁸P[hsp70FLP]3F*, constructed by Robert Petersen (Golic et al., 1997) and kindly provided by Kent Golic. In our experiments, these transgenic flies constitutively expressed FLP at low levels at 25°C, accounting for 3-20% of the clones observed in ps5 of the haltere disc (Table 2). 50% of the transformant lines carrying silencing constructs (constructs 1, 2, 3 and 4; Table 1) show spatially restricted repression. The repression exhibited for each line in ps5 of the haltere ranges from 86% to 100% (Table 2). There is some variablity in the penetrance and expressivity of repression in the wing disc; a low percentage of discs showed small patches of β-gal expression, usually located in ps5. We do not fully understand the complete repression that we and others (Chan et al., 1994; Christen and Bienz, 1994) have observed in some lines carrying silencing constructs (25% of the lines; Table 1). However, the complete repression is also dependent on the presence of the MCP725 element in the construct throughout larval development (data not shown).

Transgenes

The *ry⁺ Ubxpp lacZ* transformation construct has been previously described (Müller and Bienz, 1991). The PBX element used in our experiments is the 5.2 kb PBX01 fragment, also previously described (Castelli-Gair et al., 1992; Christen and Bienz, 1994). The MCP725 element is a *Sal*I-*Xba*I fragment obtained from the 4.3 kb MCP fragment (Fig. 1) (Busturia and Bienz, 1993). The 200 bp *FRT*s were obtained from pP[RS3] (Golic and Golic, 1996). All plasmids containing *FRT*s were sequenced to verify their orientation. The PBX, MCP725 and *FRT* fragments were placed in the transformation construct using standard cloning procedures. Detailed maps of the plasmids used are available upon request.

Dependence on PcG genes and generation of clones lacking *Polycomblike* function

To study the heterozygous effect of *Polycomb* (*Pc*) and *Polycomblike* (*Pcl*), we used the null mutations *Pc³* and *Pcl¹*, respectively, and the transformant line *P[T8]* which is a 3rd chromosome, homozygous viable transformant line carrying the silencing construct 1 (Table 1). *P[T8]* showed background X-gal staining; small patches of β-gal expression were found in 4% of the homozygous or heterozygous wing imaginal discs (9 out of 194 wing discs studied). *P[T8]/P[T8]* females were crossed to *Pc³/TM6B* males. *Pc³/P[T8]* larvae were selected by the absence of the dominant larval marker, *Tb*, associated with TM6B, and the wing imaginal discs were dissected and stained with X-gal. 116 out of 200 wing imaginal discs studied showed expression of β-gal. Similarly, 101 wing imaginal discs were studied from larvae of the *FRT²ᴿᴳ¹³Pcl¹/CyO; P[T8]/P[T8]* stock and 32 showed β-gal expression. When the background staining of the *P[T8]* line (4%) is taken into account, derepression of β-gal in the wing discs due to the lack of one dose of the *Pc* and the *Pcl* genes becomes 54% and 28% respectively.

Homozygous mutant clones for *Pcl* were generated by FLP-mediated recombination (Xu and Rubin, 1993), using the following transgenic flies: *FRT²ᴿ⁻ᴳ¹³*, which carries an *FRT* at the base of chromosome arm 2R; *FRT²ᴿ⁻ᴳ¹³ Pcl¹* containing the *FRT* in *cis* with the *Pcl¹* mutation (Soto et al., 1995); the X chromosome *FLP* recombinase stock *w¹¹¹⁸P[hsp70FLP]3F*; and the *P[T8]* stock. Males of the genotype *P[hsp70FLP]3F/Y; FRT²ᴿ⁻ᴳ¹³/CyO* were crossed to *FRT²ᴿ⁻ᴳ¹³Pcl¹/CyO; P[T8]/P[T8]* females. The progeny of this cross were heat shocked at 38°C for 1 hour at 24-48 hours after egg laying (AEL) as described by Golic (1993). Late 3rd instar larvae were dissected and stained with X-gal, and β-gal expression was examined in the wing disc. Out of 335 female wing imaginal discs studied, 180 showed expression of β-gal. The rationale for the calculation of the percentage of imaginal discs showing derepression of β-gal due to complete lack of *Pcl* function is as follows. The 335 female discs are divided into three groups of 111, as the different viable genotypes resulting from the cross cannot be morphologically distinguished. It is assumed that all three genotypes hatch with the same frequency. Out of 111 imaginal discs of the genotype *P[hsp70FLP]3F/X; FRT²ᴿ⁻ᴳ¹³/CyO*, 4 discs (4%) are expected to show β-gal derepression due to the background staining of the *P[T8]* line. Similarly, out of 111 imaginal discs of the genotype *P[hsp70FLP]3F/X; FRT²ᴿ⁻ᴳ¹³Pcl¹/CyO; P[T8]/+*, 4 discs (4%) should show β-gal derepression due to the background staining of the *P[T8]* line, and 31 (28%) discs show derepression due to the heterozygous effect of *Pcl¹* on silencing. Finally, in the experimental genotype, *P[hsp70FLP]3F/X; FRT²ᴿ⁻ᴳ¹³/ FRT²ᴿ⁻ᴳ¹³Pcl¹;P[T8]/+*, 4 discs (4%) are expected to show β-gal derepression due to the background staining of the *P[T8]* line, and 31 (28%) discs due to the heterozygous effect of *Pcl¹* on silencing. Therefore, the total number of wing imaginal discs showing derepression of β-gal not due to complete lack of *Pcl* function is 74. Substracting 74 from the 180 total discs showing derepression of β-gal results in 106. Therefore, nearly 100% (106/111) of the experimental discs show derepression of β-gal due to the lack of *Pcl* function.

FLP-induced excision and inversion of the MCP725 element

For the excision experiment we used the transformant lines T3 and T12 which are homozygous viable and carry the silencing construct 3 (Table 1) on the 2nd and 3rd chromosome, respectively. The transformant lines T2 and T5 carrying the silencing construct 4 (Table 1) on the 3rd and 2nd chromosome, respectively, were used for the inversion experiment. Both the excision and the inversion of the MCP725 element was induced by heat treating (38ºC for 1 hour) the progeny of the w^{1118} *P[hsp70FLP]*3F/Y×*P[TX]/P[TX]* cross, where *TX* is a given transformant line, at 24-48, 48-72 or 72-96 hours AEL. Late 3rd instar wing and haltere discs were dissected and stained with X-gal to study β-gal expression. The results of the excision experiment are shown in Table 2. We observed that the frequency of clones decreased with later heat treatments. Two explanations might be possible. First, in a larval population of 72-96 hours AEL, a significant portion of the haltere discs have almost completed cell division (Ashburner, 1989). Second, the decrease may result from the need for more that one cell division to achieve loss of silencing. Our experimental assay cannot distinguish between these two possibilities.

Detection of inversion of the MCP725 element

To ascertain that MCP725 had indeed inverted in the control construct where the *FRT*s flanking MCP725 were in opposing orientations (construct 4, Table 1), the expected PCR products before and after inversion were amplified. To detect uninverted DNA, PCR products were amplified between a nested pair of primers in the PBX sequence (primers AB5 and AB6) and another nested pair in MCP725 (primers AB7 and AB8). Amplification between these sets results in a 415 bp PCR product, which includes the *FRT* element located between PBX and MCP725. Inverted DNA was amplified by the same pair of primers in PBX and a different pair of nested primers in MCP725 (primers AB11 and AB12), oriented in the opposite direction from primers AB7 and AB8. When MCP725 is inverted, amplification between primers AB5/AB6 and AB11/AB12 produces a 676 bp PCR product. Genomic DNA from line T2, with or without hs-FLP (*P[hsp70FLP]3F*) which had been heat shocked (Table 2) was isolated from whole animals and used as templates in two rounds of PCR, 30 cycles each, in the presence of primers which amplify inverted or noninverted MCP725. The PCR products were separated in an agarose gel, blotted onto Duralon-UV membrane (Stratagene) and probed with radioactively labelled MCP725. From genomic DNA of T2 without FLP, only the 415 bp product was detected. From genomic DNA of T2 with FLP, both 415 bp and 676 bp products were detected, indicating MCP725 had indeed been inverted. Sequences of primers are available upon request.

X-gal staining of the imaginal discs

Larvae were dissected in PBS and the staining and mounting of the discs was done as described by Christen and Bienz (1994).

RESULTS

Identification of the MCP725 imaginal disc silencer

The *Abd-B* gene of the bithorax complex contains enhancers and silencers that control its expression (Sánchez-Herrero and Akam, 1989; Busturia and Bienz, 1993). The *Mcp¹* mutation, a 3.5 kb deletion in the *cis* regulatory region (Fig. 1) (Karch et al., 1985) results in derepression of *Abd-B* (Celniker et al., 1990; Sánchez-Herrero, 1991), suggesting that the deletion removes a silencer element. Moreover, the 4.3 kb MCP element, completely covering *Mcp¹* (Fig. 1), when fused to a *lacZ* reporter minigene, functions as a silencer throughout embryonic development (Busturia and Bienz, 1993). We
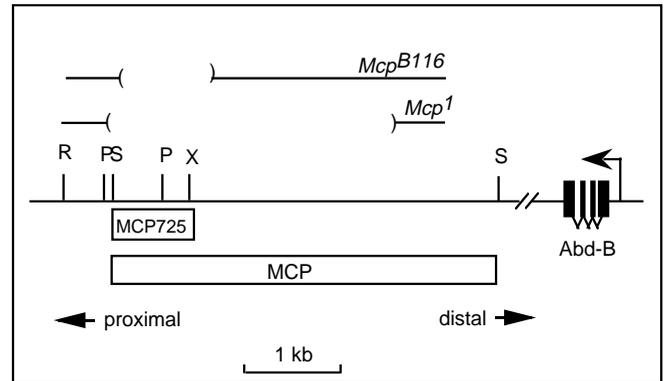

Fig. 1. Genomic location of the MCP725 silencer. MCP725 is located (Karch et al., 1985; Busturia and Bienz, 1993) about 50 kb downstream of the *Abd-B* transcriptional unit (Zavortink and Sakonju, 1989). Black boxes indicate exons and the arrow indicates start and direction of transcription. MCP725 is entirely contained within the MCP fragment (Busturia and Bienz, 1993). Deletion mutants *Mcp¹* (3.5 kb, completely covering MCP725) and *Mcp^B116* (about 900 bp, partially covering MCP725) result in a similar transformation of the 4th abdominal segment to the 5th (Karch et al., 1985, 1994). Proximal and distal indicate position with respect to the centromere on the 3rd chromosome. R, *Eco*RI; P, *Pst*I; S, *Sal*I; X, *Xba*I.

looked for a fragment within the MCP element that acts as a silencer during proliferation of the imaginal discs, where, compared to embryonic development, many more cell divisions occur (Ashburner, 1989). We have investigated silencing in the imaginal cells using the PBX enhancer of the homeotic *Ultrabithorax* (*Ubx*) gene (Müller and Bienz, 1991). In embryos carrying a transgene composed of the PBX element, the proximal *Ubx* promoter (*Ubx*pp), and the *lacZ* gene, expression of β-gal is repressed anterior to ps6 (Müller and Bienz, 1991). However, this repression is not maintained through larval development, as shown by the ubiquitous presence of β-gal in the wing and haltere imaginal discs (corresponding to ps4, ps5 and ps6; Fig. 2A) (Castelli-Gair et al., 1992; Christen and Bienz, 1994). The ability to initiate silencing, but not to maintain it, makes the PBX element ideal for identifying sequences required for silencing during proliferation of imaginal cells.

By assaying the ability of MCP fragments to silence PBX-driven expression, we have identified a 725 bp fragment (MCP725; Fig. 1) that is able to maintain the ps6-restricted expression of the PBX element throughout proliferation of the imaginal discs. A construct was made carrying the PBX element, the MCP725 fragment, the *Ubx*pp and the *lacZ* gene (construct 1; Table 1). The expression of this transgene in the imaginal discs shows spatially restricted repression (Table 1): complete repression in the wing disc (ps4 and ps5), repression in the anterior compartment (ps5) of the haltere disc and strong expression in the posterior compartment of the haltere disc (ps6) (Fig. 2B). Therefore, the MCP725 element is a silencer that functions throughout proliferation of the imaginal discs.

Transcriptional characteristics of MCP725

The orientation of the MCP725 element within the transgene does not affect its silencing ability (constructs 1 and 2; Table

Table 1. Transformant lines and patterns of expression

Construct*	Lines†	Spatially restricted repression‡	Complete repression§	No repression¶
1-PBX MCP725 Ubxpp	11	6	2	3
2-PBX 527PCM Ubxpp	3	1	2	0
3-PBX <MCP725 <Ubxpp	9	5	1	3
4-PBX <MCP725 >Ubxpp	7	3	2	2
Total (%)	30 (100)	15 (50)	7 (25)	8 (25)

*Description of silencing constructs. In construct 2, 527PCM indicates the presence of MCP725 in opposite orientation to that of construct 1. Construct 3 and 4 are the excision and the inversion minigenes respectively (> and < indicate FRT and its orientation). †Total number of independent transformant lines carrying the constructs. ‡Number of lines showing repression in ps4 and ps5, but expression in ps6. §Number of lines showing repression in ps4, ps5 and ps6. ¶Number of lines showing expression in ps4, ps5 and ps6. The total number of lines and its relative percentage in each repression category is shown.

1). In addition, MCP725 functions independently of its position, as spatially restricted repression is observed in the imaginal discs when MCP725 is either downstream (Table 1) or upstream of the PBX element. Two independent lines carrying the construct with the MCP725 element upstream of the PBX element (MCP725 PBX *Ubx*pp *lacZ* construct) were obtained, and both of them show spatially restricted repression anterior to ps6 (data not shown). Finally, MCP725 by itself does not activate transcription. Five independent transgenic lines carrying a MCP725 *Ubx*pp *lacZ* construct, which lacks the PBX element, did not express β-gal either in the embryo or in the imaginal discs.

Silencing by MCP725 is dependent on the PcG genes

To investigate whether MCP725-mediated silencing is dependent on the PcG genes, we studied the β-gal expression in the wing imaginal discs of the *P[T8]* transgenic line (construct 1; Table 1; see Methods) in backgrounds mutant for two members of the PcG genes, *Polycomb* (*Pc*) (Lewis, 1978) and *Polycomblike* (*Pcl*) (Duncan, 1982). Because null mutations for the PcG genes are homozygous embryonic lethal, we first studied whether the lack of one dose of *Pc* or *Pcl* has an effect on silencing by MCP725. The results show that β-gal is derepressed in 54% of imaginal discs from *P[T8]/Pc³* larvae and 28% of imaginal discs from *Pcl¹/+*; *P[T8]/P[T8]* larvae, indicating a heterozygous effect of both *Pc³* and *Pcl¹* on silencing mediated by the MCP725 element (see Methods).

Next, we analyzed whether the percentage of discs showing derepression of β-gal increases in *Pcl* homozygous mutant conditions. We generated *Pcl* homozygous mutant clones using the *FLP-FRT* recombination system (*FLP*: recombinase, *FRT*: FLP Recombination Target) (Golic and Lindquist, 1989; Xu and Rubin, 1993). Male flies with the genotype *P[hsp70FLP]3F/Y*; *FRT²ᴿ⁻ᴳ¹³/CyO* were crossed to *FRT²ᴿᴳ¹³Pcl¹/CyO*; *P[T8]/P[T8]* female flies (see Methods). The progeny were subjected to a heat-shock at 24-48 hour AEL, and β-gal expression was analyzed in the wing disc. The female progeny carry the FLP transgene, and therefore contain *FRT*-mediated *Pcl¹* homozygous clones. The male progeny, without the FLP transgene, serve as controls for the effects of heat treatment. Out of 335 female wing discs, 180 showed expression of β-gal. Out of 194 male wing discs, 50 expressed β-gal, due to the heterozygous effect of *Pcl¹*. After accounting for the background of the *P[T8]* line and the heterozygous effect of *Pcl¹*, and considering that only one third of the female

progeny are of the appropriate experimental genotype (*P[hsp70FLP]3F/X*; *FRT²ᴿ⁻ᴳ¹³/FRT²ᴿ⁻ᴳ¹³Pcl¹*; *T8/+*), the results indicate that β-gal is derepressed in nearly 100% of the experimental wing discs due to the complete lack of *Pcl* function (see Methods).

These results indicate that MCP725-mediated silencing requires the Pc and Pcl proteins. As Pc and Pcl act in concert with other PcG proteins (Duncan, 1982; Lonie et al., 1994), it is likely that other members of the PcG also interact with the MCP725 silencer.

Excision of the MCP725 silencer at any time during larval development results in loss of silencing

The ability of MCP725 to maintain spatially restricted expression throughout proliferation of imaginal cells allowed us to investigate when the MCP725 silencer is required during development. We studied the temporal requirements of the MCP725 silencer using the *FLP-FRT* system (Golic and Lindquist, 1989; Golic, 1993). We made a construct where *FRT*s flanking the MCP725 element are oriented as direct repeats (construct 3; Table 1). This allows the in vivo excision of MCP725 when FLP is provided (Golic, 1993). For this experiment, we focused our quantitative analysis on the haltere disc for two reasons. First, as shown in Fig. 2B, the haltere disc

Table 2. Percentage of haltere discs showing β-gal expression in ps5 after excision or inversion of the MCP725 silencer

	No hs*	hs at hours AEL†		
		24-48	48-72	72-96
Excision				
T3 – FLP	14	10	nd	nd
T3 + FLP	17	100	80	41
T12 – FLP	0	0	nd	nd
T12 + FLP	20	92	65	20
Inversion				
T2 – FLP	9	11	nd	nd
T2 + FLP	9	14	12	8
T5 – FLP	0	0	nd	nd
T5 + FLP	0	0	0	0

*Non-heat-shocked haltere discs without FLP (–FLP) indicating the penetrance of the repression, and with FLP (+FLP) showing the FLP constitutive activity at 25°C. †Heat-shocked haltere discs (1 hour, 38°C) at different times (in hours) AEL. At least 150 discs were studied for each time point.
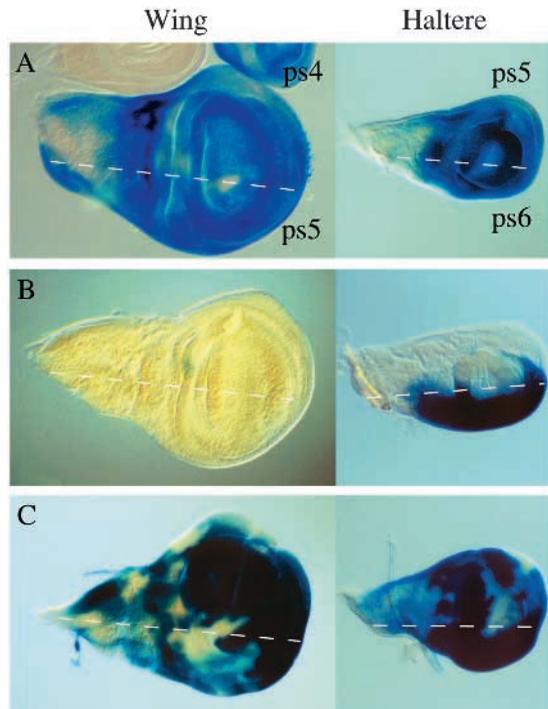
Fig. 2. β-gal expression patterns in the wing and haltere imaginal discs. (A) PBX *Ubxpp lacZ* construct: The PBX element drives expression in ps4 and ps5 in the wing disc, and ps5 and ps6 in the haltere disc (Castelli-Gair et al., 1992; Christen and Bienz, 1994). The approximate locations of the parasegmental boundaries are indicated by dashed lines. (B) PBX MCP725 *Ubxpp lacZ* construct: The presence of the MCP725 silencer restricts the PBX expression to ps6 in the haltere disc. (C) PBX<MCP725<*Ubxpp lacZ* subjected to a heat-shock to induce FLP-mediated recombination: removal of MCP725 at any time during larval development results in loss of the repression, indicated by the appearance of clones of β-gal expression in ps4 and ps5 in the wing disc and ps5 in the haltere disc. The 3rd instar wing and haltere discs shown here were heat-shocked at 24-48 hours AEL.

allows us to observe both expressed and repressed domains, and second, the penetrance and expressivity of the repression is almost 100% in the haltere disc (see Methods). Two independent lines, T3 and T12, carrying construct 3 (Table 1), were chosen for a detailed analysis (Table 2). In the absence of FLP, 14% of the haltere discs from line T3 and 0% of the haltere discs from line T12 show small patches of β-gal expression in ps5 (Table 2). Male flies carrying the FLP transgene, $w^{1118}P[hsp70FLP]$3F/Y, were crossed to female flies homozygous for the different silencing lines, and eggs were collected for 24 hours. Excision of the MCP725 silencer was induced by heat treating larvae at the 1st, 2nd or 3rd instar, and the pattern of β-gal expression was assayed in late 3rd instar imaginal discs. Female larvae, which carry the FLP transgene, were selected and at least 150 discs were studied for each time point (Table 2). Male larvae, without the FLP transgene, were also selected as a control for the heat-shock effect. In the control males, none of the discs showed β-gal expression due to the heat treatment. In contrast, when MCP725 was removed from the silencing construct at any time during larval development (Table 2), clones of β-gal expression appeared in ps5 of the

haltere disc (Table 2; Fig. 2C) and in ps4 and ps5 of the wing disc (Fig. 2C; see Methods). Therefore, maintenance of the silenced transcriptional state throughout development requires the continous presence of the silencer.

Inversion of the MCP725 silencer during proliferation does not have an effect on silencing

It could be argued that the recombination event itself, rather than loss of the silencer, disrupted the silenced state and thus caused derepression of β-gal expression in the imaginal discs. To address this possibility, we made a construct where the *FRT*s flanking MCP725 were in opposing orientations (construct 4; Table 1). This arrangement of the *FRT*s results in inversion (Golic, 1993), rather than excision, of the intervening MCP725 fragment. Thus, this construct provides a control for the effect of recombination on silencing. Two lines were chosen for the experiment which was performed as described above (see Methods). Inversion of MCP725 did not induce derepression of β-gal at any time during larval development (Table 2). We confirmed molecularly that inversion of the MCP725 element did take place in our experimental conditions. PCR primers were designed based on the MCP725 and the PBX sequences. When used in PCR amplification, these primers produced specific fragments differing in size depending upon the orientation of the MCP725 element (data not shown, see Methods). These results indicate that the recombination process is not sufficient to disrupt the silenced state.

DISCUSSION

The MCP725 element

We have described the identification of MCP725, an imaginal disc silencer in the *Abd-B* gene. The MCP725 silencer has the following characteristics: it does not activate transcription either in the embryo or in the imaginal discs; it acts on different promoters (*Abd-B*, its in vivo target, and *Ubx*) and independently of orientation; it contains a nuclease hypersensitive region (Karch et al., 1994) and mutations removing this silencer (e.g. Mcp^1, Mcp^{B116}, Fig. 1) (Karch et al., 1994) produce phenotypes consistent with derepression of *Abd-B* (Celniker et al., 1990; Sánchez-Herrero, 1991; Karch et al., 1994). Furthermore, as silencing provided by MCP725 is dependent on the PcG genes, this silencer can be included in the class of elements known as *Polycomb* response elements (Simon et al., 1993). Two imaginal disc silencers have been identified in the *Ubx* gene (Chan et al., 1994; Christen and Bienz, 1994). However, it has not been shown if these silencers need to be present throughout development.

The MCP725 element contains sequences that have been proposed to act as a chromatin boundary influencing *Abd-B* gene expression (Karch et al., 1994; Schedl and Grosveld, 1995). This boundary has been suggested to insulate a *cis* regulatory domain from the transcriptional influences of adjacent *cis* regulatory domains. Data presented here suggest that MCP725 does not act as a boundary element. Our results indicate that MCP725 functions independently of its position relative to the PBX element and the promoter. Even though the MCP725 element is the smallest silencer so far described in the bithorax complex genes, it may still be quite complex, and additional assays (Hargstrom et al., 1996; Zhou et al., 1996) are required to rule

out the presence of a boundary element within the MCP725 fragment.

Recently, an improved method for analysing the distribution of proteins on target loci has been applied to the MCP element (Strutt et al., 1997). Surprisingly, despite the strong silencing activity that MCP725 shows in our functional assay, the MCP725 fragment is only slightly enriched above background in their Pc protein binding assay. However, this method has also failed to detect Pc binding to the FAB silencer (Busturia and Bienz, 1993), which suggests that the method may have also missed the functional PREs within the MCP725 element. Perhaps, high levels of Pc binding activity are separable from silencing activity.

The role of silencers in the maintenance of repression

We have shown that the MCP725 silencer is needed for inheritance of the repressed state throughout larval development. Our results eliminate one model of the silencer function, namely that it is required only for the initial establishment of silenced chromatin. Furthermore, our results argue against a model by which propagation of silenced chromatin is mediated by templating of a heterochromatin-like structure, which would occur independently of specific DNA sequences and solely by virtue of protein-protein interactions among the PcG proteins.

The repressed state of the homeotic genes is initiated early in development by the binding of the repressor gap proteins, like Hunchback, on specific *cis* regulatory sequences of the homeotic gene promoters (Zhang et al., 1991; Bienz, 1992). This repression is position-specific and marks the domain where the homeotic genes should be continuously repressed throughout development. The gap proteins are only briefly expressed during embryogenesis (Tautz, 1988; Gaul and Jäckle, 1989), and, therefore, other mechanisms or other proteins must take over their role as position-specific repressors. *Cis* regulatory elements, like MCP725, have been shown to be involved in the maintenance of the position-specific repression, which is dependent on the PcG genes (Chan et al., 1994; Christen and Bienz, 1994). However, the Pc proteins so far identified are both ubiquitously expressed and unable to bind to specific DNA sequences. Thus, the process of maintenance of the position-specific repression remains unknown.

Our findings that the MCP725 element is continuously required during development to maintain the repressed state provides insight into the mechanism by which position-specific repression is maintained. We can envision two different models. First, silencers may function to 'lock-in' the initial repressor complex that contains the information as to where in the embryo the homeotic gene should be repressed. The stable propagation of the repressor complex could be accomplished if the repressor complex remains bound to the silencer during DNA replication. Indeed, Raff et al. (1994) have found that a transcriptional regulator can associate with specific DNA sequences at all stages of the cell cycle. If this were the case, the initial position-specific repression could be maintained by a mechanism that does not require additional positional cues. Second, silencers, like MCP725 might serve as DNA binding sequences for proteins that functionally replace the position-specific repressor role of the gap proteins at later stages of development. These proteins, if they exist, would likely have DNA binding specificity and be expressed in a spatially restricted pattern throughout development. Candidates for these include either the remaining uncharacterized members of the PcG genes or the homeotic proteins, which themselves might replace the role of the gap proteins as repressors.

Several characteristics of homeotic proteins make this latter suggestion attractive: (1) they are known transcriptional repressors, as well as activators (Krasnow et al., 1989; Scott et al., 1989; Gehring et al., 1994); (2) they are known to bind the promoters of other homeotic gene family members (Krasnow et al., 1989; Winslow et al., 1989; Appel and Sakonju, 1993); (3) they are expressed and functionally required throughout development (Sánchez-Herrero et al., 1985; Duncan, 1987); (4) they show highly complementary expression patterns with each other; for example, the limit of *Ubx* repression domain coincides with that of *Antennapedia* expression domain (Akam, 1987). According to this model, the repression domain of *Ubx* would first be established by the function of the gap Hunchback (Hb) protein (Bienz, 1992). After early stages of embryogenesis when Hb is no longer expressed (Tautz, 1988; Gaul and Jackle, 1989), its function to repress *Ubx* would be replaced by the Antp protein. In this way, PcG proteins would recognize the repressed state of the homeotic genes by interacting with the homeotic proteins. Then, PcG proteins will stabilize the multimeric repressor complexes. As homeotic proteins are present throughout development, the silencing complexes could be reformed and position-specific repression maintained after every round of DNA replication.

Gene silencing is a phenomenon also observed in organisms other than *Drosophila*. The human X-inactivation center (XIC) has been shown to be required for the establishment of repression but not for its maintenance (Brown and Willard, 1994). Therefore, the XIC appears to function differently from the *Drosophila* silencer we characterized in this study. The silencer from the mating type locus in the yeast *S. cerevisiae* is required for inheritance of the repressed state through cell division but not for its maintenance during the cell cycle (Holmes and Broach, 1996). In *Drosophila*, previous studies have indicated that the PcG genes are required even in the absence of cell division, at least in embryonic cells (Gould et al., 1990). Our assay was not designed to determine whether the silencer is continuously required in post-mitotic cells. However, this will be an important issue to investigate as it will shed light on the molecular mechanism of the silencer function. The comparison of the role of silencers in a variety of organisms should clarify whether similar mechanisms underlie similar phenomena.

REFERENCES

Akam, M. E. (1987). The molecular basis for metameric pattern in the *Drosophila* embryo. *Development* **101**, 1-22.
Appel, B. and Sakonju, S. (1993). Cell-type-specific mechanisms of transcriptional repression by the homeotic gene products Ubx and abd-A in *Drosophila* embryos. *EMBO J.* **12**, 1099-1109.
Ashburner, M. (1989). *Drosophila*: *A Laboratory Handbook*. Cold Spring Harbor: Cold Spring Harbor Laboratory Press.

Bienz, M. (1992). Molecular mechanisms of determination in *Drosophila*. *Curr. Opin . Cell Biol*. **4**, 955-961.

Bienz, M. and Müller, J. (1995). Transcriptional silencing of homeotic genes in *Drosophila*. *BioEssays* **17**, 775-784.

Brand, A. H., Breeden, L., Abraham, J., Sternglanz, R. and Nasmyth, K. (1985). Characterization of a 'silencer' in yeast: A DNA sequence with properties opposite to those of a transcriptional enhancer. *Cell* **41**, 41-48.

Brown, C. and Willard, H. (1994). The human X-inactivation center is not required for maintenance of X-chromosome inactivation. *Nature* **368**, 154-156.

Bunker, C. and Kingston, R. (1994). Transcriptional repression by *Drosophila* and mammalian Polycomb group proteins in transfected mmammalian cells. *Mol. Cell Biol*. **14**,1721-1723.

Busturia, A. and Bienz, M. (1993). Silencers in *Abdominal-B*, a homeotic *Drosophila* gene. *EMBO J.* **12**, 1415-1425.

Casares, F. and Sánchez-Herrero, E. (1995). Regulation of the *infraabdominal* regions of the bithorax complex of *Drosophila* by gap genes. *Development* **121**, 1855-1866.

Castelli-Gair, J., Müller, J. and Bienz, M. (1992). Function of an *Ultrabithorax* minigene in imaginal cells. *Development* **114**, 877-886.

Celniker, S. E., Sharma, S., Keelan, D. J. and Lewis, E. B. (1990). The molecular genetics of the Bithorax complex of *Drosophila*: cis-regulation in the *Abdominal-B* domain. *EMBO J.* **9**, 4277-4286.

Chan, C.-S., Rastelli, L. and Pirrotta, V. (1994). A *Polycomb* response element in the *Ubx* gene that determines an epigenetically inherited state of repression. *EMBO J.* **13**, 2553-2564.

Christen, B. and Bienz, M. (1994). Imaginal disc silencers from *Ultrabithorax*: evidence for Polycomb response elements. *Mech. Dev.* **48**, 255-266.

Dernburg. A.F., Broman, K. W., Fung, J.C., Marshall, W.F., Philips, J., Agard, D.A. and Sedat, J.W. (1996). Perturbation of nuclear architecture by long-distance chromosome interactions. *Cell* **85**, 745-759.

Duncan, I. (1982). *Polycomblike*: A gene that appears to be required for the normal expression of the bithorax and Antennapedia gene complexes of *Drosophila melanogaster*. *Genetics* **102**, 49-70.

Duncan, I. (1987). The bithorax complex. *Ann. Rev. Genet.* **21**, 285-319.

Eissenberg, J.C., James, T.C., Foster-Hartnett, D.M., Martnett, T., Ngan, V. and Elgin, S. (1990). Mutation in a heterochromatin-specific chromosomal protein is associated with suppression of position effect variegation in *Drosophila melanogaster*. *Proc. Natl. Acad. Sci. USA* **87**, 9923-9927.

Eissenberg, J. C., Elgin, S. C. R. and Paro, R. (1995). Epigenetic regulation in *Drosophila*: a conspiracy of silence. In *Chromatin Structure and Gene Expression* (ed. S. C. R. Elgin), pp. 147-171. Oxford: IRL Press.

Gaul, U. and Jäckle, H. (1989). Analysis of maternal effect mutant combinations elucidates regulation and function of the overlap of *hunchback* and *Krüppel* gene expression in the *Drosophila* blastoderm embryo. *Development* **107**, 651-662.

Gehring, W. J., Qian, Y. Q., Billeter, M., Furukubo-Tokunaga, K., Schier, A. F., Resendez-Perez, D., Affolter, M., Otting, G. and Wuthrich, K. (1994). Homeodomain-DNA recognition. *Cell* **78**, 211-223.

Golic, K. and Lindquist, S. L. (1989). The FLP recombinase of yeast catalyzes site-specific recombination in the *Drosophila* genome. *Cell* **59**, 499-509.

Golic, K. (1993). Generating mosaics by site specific recombination. *Cellular Interactions in Development. A Practical Approach.* (ed. D. A. Hartley), pp. 1-31. Oxford University Press.

Golic, M. M. and Golic, K. G. (1996). A quantitative measure of the mitotic pairing of alleles in *Drosophila melanogaster* and the influence of structural heterozygosity. *Genetics* **143**, 385-400.

Golic, M. M., Rong, Y., Petersen, R., Lindquist, S. L., and Golic, K. (1997). FLP-mediated DNA mobilization to specific target sites in Drosophila chromosomes. *Nucl. Acids Res.* (in press).

Gould, A.P., Lai, R.Y.K., Green, M.J. and White, R.A.H. (1990). Blocking cell division does not remove the requirement for Polycomb function in *Drosophila* embryogenesis. *Development* **110**, 1319-1325.

Hama, C., Ali, Z. and Kornberg, T. (1990). Region specific recombination and expression are directed by portions of the *Drosophila* engrailed promoter. *Genes Dev.* **4**, 1079-1093.

Harding, K. and Levine, M. (1988). Gap genes define the limits of Antennapedia and Bithorax gene expression during early development in *Drosophila*. *EMBO J.* **7**, 205-214.

Hargstrom, K., Muller, M. and Schedl, P. (1996). Fab-7 functions as a chromatin domain boundary to ensure proper segment specification by the *Drosophila* bithorax complex. *Genes Dev*.**10**, 3202-3215.

Holmes, S. G. and Broach, J. R. (1996). Silencers are required for inheritance of the repressed state in yeast. *Genes Dev*.**10**, 1021-1032.

Jones, R. S, and Gelbart, W. M. (1993). Genetics analysis of the *Enhancer of zeste* and its role in gene regulation in *Drosophila melanogaster*. *Genetics* **126**, 185-199

Jürgens, G. (1985). A group of genes controlling the spatial expression of the bithorax complex in *Drosophila*. *Nature* **316**, 153-155.

Karch, F., Galloni, M., Spios, L., Gausz, J., Gyurkovics, H. and Schedl, P. (1994). *Mcp* and *Fab-7*: molecular analysis of putative boundaries of *cis*-regulatory domains in the bithorax complex of *Drosophila melanogaster*. *Nucl. Acids Res.* **22**, 3138-3146.

Karch, F., Weiffenbach, B., Peifer, M., Bender, W., Duncan, I., Celniker, S., Crosby, M. and Lewis, E. B. (1985). The abdominal region of the Bithorax complex. *Cell* **43**, 81-96.

Krasnow, M. A., Saffman, E. E., Kornfeld, K. and Hogness, D. S. (1989). Transcriptional activation and repression by Ultrabithorax proteins in cultured *Drosophila* cells. *Cell* **57**, 1031-1043.

Laurenson, P. and Rine, J. (1992). Silencers, silencing and heritable transcriptional states. *Microbiol. Rev.* **56**, 543-563.

Lewis, E. B. (1978). A gene complex controlling segmentation in *Drosophila*. *Nature* **276**, 566-570.

Locke, J., Kotarski, M. and Tartof, K. (1988). Dosage-dependent modifiers of position effect variegation in *Drosophila* and a mass action model that explains their effect. *Genetics* **120**, 181-198.

Lonie, A., D'Andrea, R., Paro, R. and Saint, R. (1994). Molecular characterization of the *Polycomblike* gene of *Drosophila melanogaster*, a trans-acting negative regulator of homeotic gene expression. *Development* **120**, 2629-2636.

Loo, S. and Rine, J. (1995). Silencing and heritable domains of gene expression. *Ann. Rev. Cell Biol*.**11**, 519-48.

McCall, K. and Bender, W. (1996). Probes for chromatin accessibility in the *Drosophila* bithorax complex respond differently to *Polycomb* mediated repression. *EMBO J*., **15**, 569-580

Moehrle, A. and Paro, R. (1994). Spreading the silence: epigenetic transcriptional regulation during *Drosophila* development. *Dev. Genet.* **15**, 478-484.

Müller, J. and Bienz, M. (1991). Long range repression conferring boundaries of *Ultrabithorax* expression in the *Drosophila* embryo. *EMBO J.* **10**, 1415-1425.

Orenic, T., Slusarski, D., Kroll, K. and Holmgren, R. (1990). Cloning and characterization of the segment polarity gene *cubitus interruptus Dominant* of *Drosophila*. *Genes Dev*. **4**, 1053-1067.

Orlando, V. and Paro, R. (1993). Mapping Polycomb-repressed domains in the bithorax complex using in vivo formaldehyde cross-linked chromatin. *Cell* **75**, 1187-1198

Orlando, V. and Paro, R. (1995). Chromatin multiprotein complexes involved in the maintenance of transcription patterns. *Curr. Opin. Genet. Dev.* **5**, 174-179.

Paro, R. and Hogness, D. (1991). The Polycomb protein shares a homologous domain with heterochromatin-associated protein in *Drosophila*. *Proc. Natl. Acad. Sci. USA* **88**, 263-267.

Pirrotta, V. (1997). PcG complexes and chromatin silencing. *Curr. Opin. Genet. Dev.* **7**, 249-258.

Pirrotta,V. and Rastelli, L. (1994). *white* gene expression, repressive chromatin domains and homeotic gene silencing in *Drosophila*. *BioEssays* **16**,549-555

Raff, J. W., Kellum, R. and Alberts, B. (1994). The *Drosophila* GAGA transcription factor is associated with specific regions of heterochromatin throughout cell cycle. *EMBO J*. **13**, 5977-5983.

Sánchez-Herrero, E. (1991). Control of the expression of the bithorax complex genes *abdominal-A* and *Abdominal-B* by *cis*-regulatory regions in *Drosophila* embryos. *Development* **111**, 437-449.

Sánchez-Herrero, E. and Akam, M. (1989). Spatially ordered transcription of regulatory DNA in the bithorax complex of *Drosophila*. *Development* **107**, 321-329.

Sánchez-Herrero, E., Vernos, I., Marco, R. and Morata, G. (1985). Genetic organization of *Drosophila* bithorax complex. *Nature* **313**, 108-113.

Schedl, P. and Grosveld, F. (1995). Domains and boundaries. In *Chromatin Structure and Gene Expression* (ed. S. C. R. Elgin), pp. 172-196. Oxford: IRL Press.

Scott, M. P., Tamkun, J. W. and Hartzell, G. W. (1989). The structure and function of the homeodomain. *Biochem. Biophys. Acta* **989**, 25-48.

Simon, J. (1995). Locking in stable states of gene expression: transcriptional control during *Drosophila* development. *Curr. Opin. Cell Biol.* **7**, 376-385.

Simon, J., Chiang, A. and Bender, W. (1992). Ten different *Polycomb* group genes are required for spatial control of the abd-A and Abd-B homeotic products. *Development* **114**, 493-505.

Simon, J., Chiang, A., Bender, W., Shimell, M. J. and O'Connor, M. (1993). Elements of the *Drosophila* Bithorax complex that mediate repression by Polycomb group products. *Dev. Biol.* **158**, 131-144.

Soto, M., Chou, T. and Bender, W. (1995). Comparison of germline mosaics of genes in the *Polycomb* Group of *Drosophila melanogaster*. *Genetics* **140**, 231-243.

Struhl, G. and Akam, M. (1985). Altered distributions of *Ultrabithorax* transcripts in *extra sex combs* mutant embryos of *Drosophila*. *EMBO J.* **4**, 3259-3264.

Strutt, H., Cavalli, G., and Paro, R. (1997). Co-localization of Polycomb protein and GAGA factor on regulatory elements responsible for the maintenance of homeotic gene expression. *EMBO J.* **16**, 3621-3623

Tautz, D. (1988). Regulation of the *Drosophila* segmentation gene *hunchback* by two maternal morphogenetic centres. *Nature* **332**, 281-284.

Winslow, G. M., Hayashi, S., Krasnow, M., Hogness, D. S. and Scott, M. P. (1989). Transcriptional activation by the Antennapedia and fushi tarazu proteins in cultured *Drosophila* cells. *Cell* **57**, 1017-1030.

Xu, T. and Rubin, G. (1993). Analysis of genetic mosaics in developing and adult *Drosophila* tissues. *Development* **117**, 1223-1237.

Zavortink, M. and Sakonju, S. (1989). The morphogenetic and regulatory functions of the *Drosophila Abdominal-B* gene are encoded in overlapping RNAs transcribed from separate promoters. *Genes Dev.* **3**, 1969-1981.

Zhang, C. and Bienz, M. (1992). Segmental determination in *Drosophila* conferred by hunchback (hb), a repressor of the homeotic gene *Ultrabithorax*. *Proc. Natl. Acad. Sci. USA* **89**, 7511-7515.

Zhang, C., Müller, J., Hoch, M., Jackle, H. and Bienz, M. (1991). Target sequences for hunchback in a control region conferring *Ultabithorax* expression boundaries. *Development* **113**, 1171-1179.

Zhou, J., Barolo, S., Szymanski, P. and Levine, M. (1996). The Fab-7 element of the bithorax complex attenuates enhancer-promoter interactions in the *Drosophila* embryo. *Genes Dev*. **10**, 3195-3201.