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The MCP silencer of the *Drosophila Abd-B* gene requires both Pleiohomeotic and GAGA factor for the maintenance of repression

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SUMMARY

Silencing of homeotic gene expression requires the function of *cis*-regulatory elements known as Polycomb Response Elements (PREs). The MCP silencer element of the *Drosophila* homeotic gene *Abdominal-B* has been shown to behave as a PRE and to be required for silencing throughout development. Using deletion analysis and reporter gene assays, we defined a 138 bp sequence within the MCP silencer that is sufficient for silencing of a reporter gene in the imaginal discs. Within the MCP138 fragment, there are four binding sites for the Pleiohomeotic protein (PHO) and two binding sites for the GAGA factor (GAF), encoded by the *Trithorax-like* gene. PHO and the GAF proteins bind to these sites in vitro. Mutational analysis of PHO and GAF binding sequences indicate that these sites are necessary for silencing in vivo. Moreover, silencing by MCP138 depends on the function of the *Trithorax-like* gene, and on the function of the PcG genes, including *pleiohomeotic*. Deletion and mutational analyses show that, individually, either PHO or GAF binding sites retain only weak silencing activity. However, when both PHO and GAF binding sites are present, they achieve strong silencing. We present a model in which robust silencing is achieved by sequential and facilitated binding of PHO and GAF.

Key words: MCP silencer, *Abd-B* gene, Pleiohomeotic, GAF, PcG, trxG, *Drosophila melanogaster*

INTRODUCTION

Homeotic genes generate the morphological diversity of the body segments in *Drosophila* and other higher eukaryotes (Lewis, 1978; Duncan, 1987; McGinnis and Krumlauf, 1992). The expression of homeotic genes in specific groups of cells throughout development is required for normal morphogenesis. In *Drosophila*, domains of homeotic gene expression are regulated through both transcriptional activation and repression (Kennison, 1995; Paro and Harte, 1996). The domain of activation or repression must be precisely established and maintained during development, since misexpression of homeotic genes leads to severe homeotic transformations and lethality (Busturia and Morata, 1988; Gonzalez-Reyes and Morata, 1990; Lamka et al., 1992; Castelli-Gair et al., 1994).

Establishment of the activation and repression domains takes place early in embryonic development and depends on the function of the segmentation genes (Qian et al., 1991, 1993; Zhang et al.,1991). Maintenance throughout development involves transition to a different mechanism and a different set of proteins (Qian et al., 1991, 1993; Zhang et al., 1991; Müller and Bienz, 1991; Pirrotta et al., 1995; Kehle et al., 1998). The *trithorax* group of genes (*trx*G) is required for the maintenance of the active transcriptional state of the homeotic genes (Kennison, 1995). In contrast, the genes responsible for the maintenance of the repression, or silencing, are included in the *Polycomb* group (PcG) of genes. To date, thirteen PcG genes have been molecularly characterized. All the characterized PcG genes, with the exception of *pleiohomoetic* (*pho*), encode chromatin-associated proteins with motifs characteristic of chromatin-bound proteins or suggestive of protein-protein interactions (Paro and Harte, 1996). Indeed, some pairs of PcG proteins have been shown to interact in vitro (Peterson et al., 1997; Kyba and Brock, 1998) and, in vivo, they appear to be associated with large protein complexes (Franke et al., 1992; Shao et al., 1999; Tie et al., 2001). Two large PcG protein complexes have been described to be present in *Drosophila* embryonic extracts. One of these complexes, called PRC1, with an estimated molecular mass of greater than 2 MDa, contains at least four PcG proteins: PC, PH, PSC, and SCM (Shao et al., 1999). The other complex, with a molecular mass of approx. 600 kDa, includes at least two PcG proteins, ESC and E(Z) (Tie et al., 2001). The recent cloning of the *pho* gene has shown that it encodes a zinc finger protein related to the mammalian transcription factor YY1 and that it recognizes specific DNA sequences, making PHO the only PcG protein so far characterized that has specific DNA binding properties. This finding has led to the proposal that PHO protein binds to DNA and recruits the PcG silencing complexes to specific DNA sequences (Brown et al., 1998).

PcG proteins act on *cis*-regulatory elements of the homeotic genes to recognize target promoters and maintain the silenced

state. These silencing elements are known as Polycomb Response Elements, or PREs (Chan et al., 1994). Several PREs from the bithorax complex (Müller and Bienz, 1991; Busturia and Bienz, 1993; Chiang et al., 1995; Hagstrom et al., 1997) as well as from other genes regulated by the PcG proteins (Fauvarque and Dura, 1993; Kassis, 1994, Gindhart and Kaufman, 1995) have been characterized. They are DNA segments of several hundred base pairs that, when fused to a reporter, are able to silence target promoters in cis (Pirrotta, 1997a; Pirrotta, 1997b; Pirrotta, 1998; Pirrotta, 1999). Moreover, they can mediate pairing-sensitive silencing. Identification of key sequences bound by proteins within the characterized PREs is under way. Recently, specific sequences recognized by PHO have been shown to play an essential role in the silencing activity of three PREs (Brown et al., 1998; Fritsch et al., 1999; Shimell et al., 2000). The same studies showed, however, that the PHO recognition sequences by themselves are not sufficient to serve as a PRE, suggesting the existence of additional protein(s) that bind PREs.

Here we describe a detailed analysis of the MCP element from the *iab-5* regulatory region of the *Abd-B* gene. This fragment corresponds to the genomic region deleted in *Mcp* mutant alleles, which cause *Abd-B* to be expressed outside its normal domain (Lewis, 1978; Celniker et al., 1989; Sanchez-Herrero, 1991; Karch et al., 1994). Using reporter gene assays, we have previously shown that the 822 bp MCP behaves like a silencer during proliferation of the imaginal discs and that it is required throughout development (Busturia et al., 1997). Additional properties of the MCP822 fragment are: (1) it silences a reporter gene when placed either upstream or downstream of the associated enhancer (Busturia et al., 1997), (2) it functions in both orientations (Busturia et al., 1997), (3) it behaves as a PRE since PcG functions are required for its silencing activity (Busturia et al., 1997), and (4) it is capable of participating in long-distance trans silencing (Muller et al., 1999). In this paper, we define the MCP silencer as a 138 bp minimal element based upon its ability to maintain silencing during imaginal discs development. Within the minimal MCP element, there are four PHO binding sites and two GAGA factor (GAF) binding sites. We show by mutational analyses that both PHO and GAF binding sites are required for the silencing activity of the MCP element in vivo. We also show that silencing of the minimal element is dependent on the functions of *pleiohomeotic* and the GAF-encoding gene *Trithorax-like*.

MATERIALS AND METHODS

Fly strains, transformation and staining of discs

The *Trithorax-like* mutations used in this study were Trl^{R85} and Trl^{13C} (Farkas et al., 1994). The PcG mutations include Pc^{3} (Lewis, 1978), pho^{1}, pho^{b} (Girton and Jeon, 1994) and Psc^{h27} (Adler et al., 1991). *ciD-lacZ* (Orenic et al., 1990), *TM6B P[Ubi-GFP] Tb*^{1}, *TM3 P[Act-GFP] Ser*, and *CyO P[Ubi-GFP]* chromosomes were used to select homozygous and double heterozygous larvae. The majority of fly strains were obtained from the Bloominton Stock Center.

Transgenic flies were produced as previously described (Bienz et al., 1988). With all MCP constructs, including MCP822, the expression of the reporter gene in approximately one quarter of the transformant lines is completely silenced throughout the body axis, presumably due to position effects (see Busturia et al., 1997). The total number of transformant lines reported in Figs 1 and 3 excludes these lines. A line was classified as silenced if it showed background X-gal staining in less than 15% of the wing and haltere discs and if the derepressed patches were very small. Those classified as derepressed lines usually showed *lacZ* expression in nearly 100% of the wing and haltere discs examined. A line with less than complete penetrance of derepression was classified as derepressed if the penetrance was high and the derepressed patches were large. To assay for silencing of the reporter constructs in wing and haltere discs, larvae were grown continuously at 25°C in a humidity controlled chamber in uncrowded vials. X-gal staining of discs were done as previously described (Christen and Bienz, 1994).

FLP-induced excision of the MCP element in the germ line

To excise the MCP element with five mutated PHO sites (5MPHO), *cn*^{1}*; P[>5MPHO>, ry*$^{+}$*] ry*^{42} females (>=FRT) were crossed to w^{1118} *P[β2tub-FLP]; Sco P[β2tub-FLP]/S*^{2} *CyO cn bw* males (kindly provided by Kent Golic). F_1 *Sco P[β2tub-FLP]/cn; P[>5MPHO>, ry*$^{+}$*] ry*^{506}*/+* males were selected and crossed to *y; cn*^{1}*; ry*^{506} females. In the following generation, non-Sco, ry^{+} flies were isolated and independent lines were established from single flies by crossing them to *y; cn*^{1}*; ry*^{506} flies. To distinguish lines carrying the *P[Δ5MPHO, ry*$^{+}$*]* chromosome, wing and haltere discs were assayed for *lacZ* expression. To identify lines from which 5MPHO had been excised, genomic DNA was prepared from each line and used to amplify a diagnostic fragment using a primer in the *Ubx* promoter and a reverse primer in the PBX enhancer fragment.

Transgene construction, in vitro mutagenesis and deletion analysis

The PBX-MCP-*Ubx* promoter-*lacZ* reporter construct has been described previously (Busturia et al., 1997). This reporter is built upon the *ry+ Ubxpp-lacZ* transformation vector described by Müller and Bienz (Müller and Bienz, 1991). It carries 3.1 kb of the *Ubx* promoter sequence upstream of the start site (Saari and Bienz, 1987) and includes the *Ubx* transcribed sequence to the first 7 amino acids of the ORF, where it is fused to the *lacZ* sequence. The PBX element is the 5.2 kb PBXO1 fragment, also described previously (Castelli-Gair et al., 1992; Christen and Bienz, 1994; Busturia et al., 1997). In all constructs, the MCP822 element or its mutated or deleted versions are inserted between the PBX and the *Ubx* promoter fragments (Fig. 1A). The 822 bp MCP element is the *Sal*I-*Xba*I fragment located about 50 kb downstream of the *Abd-B* transcriptional unit (Zavortink and Sakonju, 1989), and corresponds to residues 1153 to 1976 of the sequence shown in Fig. 4A in the paper by Karch et al. (Karch et al., 1994). The MCP fragment used in our study lacks the *Xba*I site at residue 1878 and is also missing two bases present in the Karch sequence. The length of the *Sal*I-*Xba*I fragment is therefore 822 bp long, even though it is designated in our previous publication as MCP725 (Busturia et al., 1997). MCP fragments in many of our constructs are flanked by 34 bp FRT elements (Qian and Cox, 1995).

To alter consensus sequences for PHO or GAF binding sites, oligonucleotides carrying desired mutated sequences were used to amplify PCR products. Oligonucleotide sequences and the protocol used to construct mutant MCP reporters are available upon request. The mutated sequences are shown in Fig. 5. MCP deletion constructs were generated by amplifying desired segments using appropriate primers. Every mutant construct was sequenced to verify that the intended mutant sequences had been introduced and other PCR-induced mutations did not exist.

Electrophoretic mobility shift assays

PHO protein was synthesized in vitro in TNT coupled transcription/translation reticulocyte lysate (Promega) from a T7 promoter-PHO cDNA template ($PHO_{2\text{-}520}$ pT7 of Fritsch et al., 1999; kindly provided by J. Müller). In the binding assay, 25 fmole of a radioactively labeled oligonucleotide pair was mixed with 2 μl of the

in vitro translation lysate in 20 μl of 20 mM Hepes (pH 7.6), 4% Ficoll 400, 5 mM $MgCl_2$, 40 mM NaCl, 0.1 mM EDTA, 0.5 mM DTT, 50 μM $ZnCl_2$ and 200 μg/ml poly dI/poly dC (Pharmacia). After 30 minutes on ice, the binding reaction was immediately loaded on a prerun 4% polyacrylamide gel (1×TBE running buffer) and electrophoresed for 2.5 hours at 12 V/cm at 4°C. X-ray film exposures were typically for 4 to 16 hours. In competition experiments, a 100× molar excess of non-radioactive competitor DNA was mixed with the radioactive DNA fragment before addition of translation lysate. As the non-specific competitor control, an oligonucleotide containing the ADF1 transcription factor binding site (GCATCCGTCGACGT-CGACTGCACTCGCCCC) was used. To demonstrate that mobility shift is due to PHO binding to the PHO consensus sequence, ATGGC on the tested oligos was changed to CGTGC (see Fig. 7), and the mutated oligos were used in the binding reaction as unlabeled excess competitor (e.g. see Fig. 1C).

For the purpose of synthesizing GAF in vitro, the GAF ORF encoding a 519 amino acid polypeptide was amplified from the GAF cDNA cloned in pCK vector (Soeller et al., 1993; kindly provided by T. Kornberg) and blunt-end ligated into Bluescript. GAF protein was synthesized in vitro from the T7 promoter using the coupled transcription/translation reticulocyte lysate as described above. The binding assay and competition experiments were similar to that described above except that it was done in 10 mM Tris pH 7.5, 50 mM KCl, 1 mM DTT, 10 μg/μl BSA, 5% glycerol, 100 μg/ml poly dI:dC (Pharmacia).

To prepare embryonic nuclear extract, Canton-S embryos were collected overnight (0-20 hours) and processed according to the protocol described by Franke et al. (Franke et al., 1992). Total protein concentration in nuclear extracts was 8.5-9.5 mg/ml. For EMSAs, 1-3 μl of nuclear extract was used in the binding reaction that was identical to that described above for PHO binding assays, except that $ZnCl_2$ was not added.

Effects of PcG mutations and testing genetic interactions

When examining the effect of a single mutation, the mutant chromosome usually came from females and the reporter-carrying chromosome from males. In all experiments, balancer chromosomes were marked with larval markers (GFP, *Tb*, or *Ci-lacZ*) so that appropriate genotypes could be identified unambiguously. A chromosome carrying both Trl^{R85} and the *P[MCP822, T8]* reporter was used for genetic interaction studies. In these tests, Trl^{R85} *P[T8]/TM6, GFP Tb* females were crossed to PcG mutant males (pho^{b}*/Ci-lacZ* or Psc^{h27}*/CyO, GFP*). For each experiment, wing and haltere discs from wild-type siblings were isolated and stained as controls. The numbers reported in Table 1 are compilations from at least two separate experiments for each genotype. The number of wing discs examined ranged from 61 to 590, but for most genotypes 100 to 300 discs were examined.

To study the genetic interactions between PcG genes and *Trl* using adult homeotic mutant phenotypes (Table 2), Trl^{R85}*/TM3, Ser* females were crossed to Pc^{3}*/TM3, Sb* or pho^{1}*/CiD* males and the percentage of F_1 double heterozygous males showing extra sex combs in either the second or third leg, or double heterozygous females with antenna to leg transformation, was determined. The number of flies examined ranged from 50 to 187. Homeotic transformations observed in these interaction studies appear to be sensitive to crowding conditions, and therefore care was taken to avoid this.

To examine the expression of the endogenous *Ubx* gene in the wing discs, Pc^{3}*/TM6 Tb* and Trl^{R85}*/TM3,GFP* flies were selected from the corresponding stocks. For the double heterozygote analysis, Pc^{3}*/*Trl^{R85} larvae were selected from the cross between Pc^{3}*/TM6 Tb* males and Trl^{R85}*/TM3,GFP* females. Imaginal discs were dissected, stained with the FP.3.38 monoclonal anti-UBX antibody (kindly provided by Rob White; White and Wilcox, 1984), and analyzed with a Biorad Radiance 2000 confocal microscope.

RESULTS

Pleiohomeotic binding is necessary but not sufficient for MCP silencing activity

PHO binding sites have been shown to be required for silencing activity of three previously characterized PREs (Brown et al., 1998; Fritsch et al., 1999; Shimell et al., 2000). Within the 822 bp MCP (Busturia et al., 1997), there are five PHO consensus binding sequences (ATGGC; Fritsch et al., 1999). One is

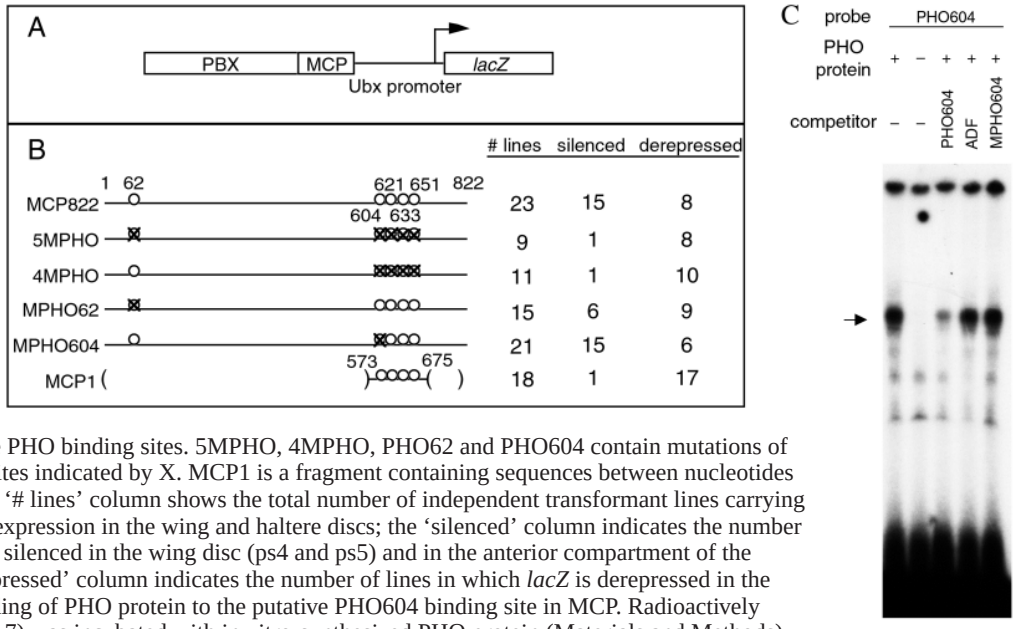


Fig. 1. Pleiohomeotic binding sites are required but not sufficient for MCP silencing function. (A) The basic structure of the reporter construct used to assay the silencing activity of MCP fragments (not drawn to scale). The MCP element was replaced with mutant or deletion fragments discussed in the text. (B) Diagram of the MCP fragments. The top line shows the wild-type MCP822 element which is a *Sal*I-*Xba*I fragment. The numbers represent nucleotide residues corresponding to those indicated in Fig. 7. Open circles denote locations of the putative PHO binding sites. 5MPHO, 4MPHO, PHO62 and PHO604 contain mutations of PHO binding consensus sequence at sites indicated by X. MCP1 is a fragment containing sequences between nucleotides 573 and 675. In the adjacent table, the '# lines' column shows the total number of independent transformant lines carrying the constructs and examined for *lacZ* expression in the wing and haltere discs; the 'silenced' column indicates the number of lines in which expression of *lacZ* is silenced in the wing disc (ps4 and ps5) and in the anterior compartment of the haltere disc (ps5) (Fig. 2A); the 'derepressed' column indicates the number of lines in which *lacZ* is derepressed in the wing or haltere discs. (C) In vitro binding of PHO protein to the putative PHO604 binding site in MCP. Radioactively labeled oligonucleotide PHO604 (Fig. 7) was incubated with in vitro-synthesized PHO protein (Materials and Methods). Arrow indicates the PHO-oligo complexes. Unlabeled competitors were PHO604, ADF (non-specific sequence control) and MPHO604 (PHO604 site mutated; see Fig. 7).

located at residue 62 while four other sites are clustered between residues 604 and 651 (Fig. 1 and see Fig. 7). To determine if these consensus sites represent bona fide PHO binding sites, we synthesized five oligonucleotides containing the potential binding sites (PHO62, 604, 621, 633, and 651; see Fig. 7) and tested them in electrophoretic mobility shift assays (EMSAs) using in vitro-synthesized PHO protein (see Materials and Methods). Fig. 1C shows an example of such assays using oligo PHO604. The shifted band (arrow) is competed by unlabeled PHO604 but not by nonspecific oligo (ADF), nor by MPHO604 in which the PHO binding site has been mutated from ATGGC to CGTGC. Similar results were obtained with oligos PHO62, PHO621 and PHO651, but not with oligo PHO633 (data not shown). These in vitro results indicate that four of the five ATGGC sites within MCP are bona fide binding sites for PHO protein.

To test if the PHO binding sites are required for silencing activity in vivo, we mutated the PHO consensus sequences from ATGGC to CGTGC, which eliminates binding as seen above, and tested the mutated silencers in an in vivo reporter assay (Müller and Bienz, 1991; Busturia et al., 1997). This assay utilizes a transgene comprising the PBX enhancer and the proximal *Ubx* promoter fused to the *lacZ* reporter gene (Fig. 1A, see Materials and Methods). In embryos, the PBX element activates the *Ubx* promoter in parasegment (ps) 6 and more posterior segments, while repressing the *lacZ* expression in ps5 and more anterior segments. However, this repression is not maintained during larval development, leading to derepression of the *Ubx* promoter in wing discs (ps4 and 5) and the anterior compartment of haltere discs (ps5). In contrast, when the MCP silencer is fused to the PBX-*Ubx* promoter-*lacZ* construct, repression in ps5 and more anterior segments is maintained throughout development (Busturia et al., 1997). Thus, this reporter transgene provides a system to assay the ability of silencers to maintain silencing established early in a spatially specific manner.

Figs 1 and 2 show the results of the experiments in which the activity of the mutated MCP fragments were assayed in vivo. Mutating four or five PHO binding sites (4MPHO or 5MPHO constructs) resulted in derepression of the *Ubx* promoter in the wing discs of almost all transformed lines. Within each derepressed line, penetrance of derepression was nearly 100%, with only a few lines showing a mixture of silenced and derepressed *lacZ* expression in the wing discs. The degree of derepression, or expressivity, varied from line to line. Some lines showed only small patches of derepression, while in others almost entire wing discs expressed *lacZ* (e.g. compare Fig. 2B and D). We presume some of this variability results from position effects. However, the presence of weakly derepressed lines suggest that PHO binding site mutations do not remove the entire silencing activity of MCP822. We conclude that PHO binding sites are required for full silencing activity by MCP, but silencing could be mediated by sequences other than PHO binding sites (see below).

To test if all five PHO binding sites are required for silencing

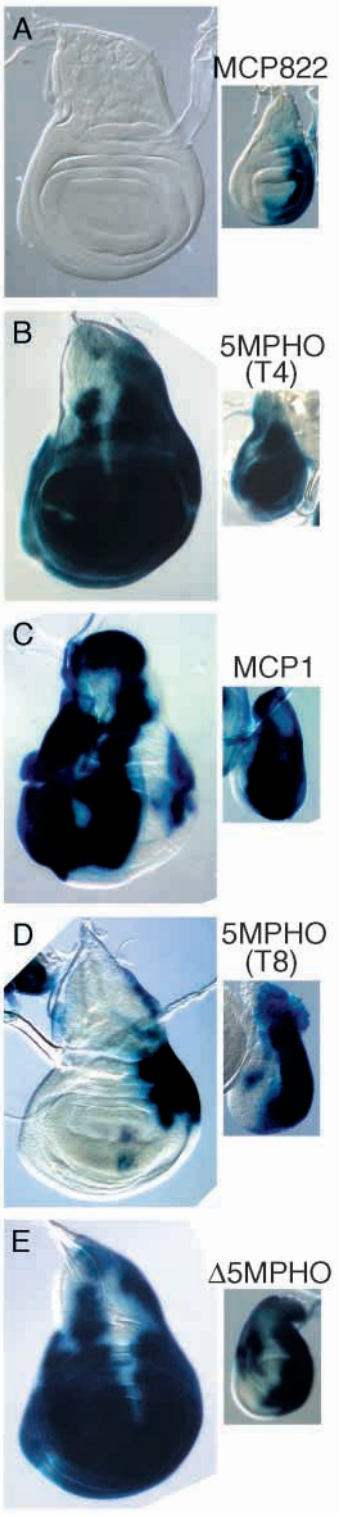
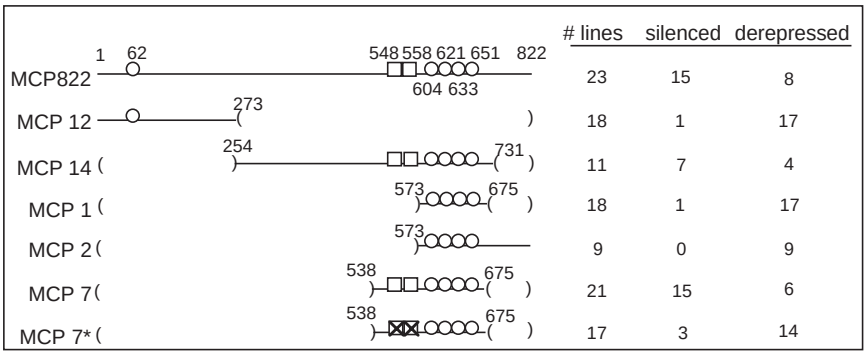


Fig. 2. Reporter gene expression patterns in the wing and haltere imaginal discs. (A) MCP822 construct containing the wild-type element. *lacZ* expression is correctly maintained: it is expressed in the posterior compartment of the haltere disc (ps6) but is silenced in the wing disc (ps4 and ps5) and in the anterior compartment of the haltere disc (ps5). (B) 5MPHO construct in which all the putative PHO binding sites are mutated. The expression of transformant line T4 is shown. *lacZ* is strongly derepressed in the wing and haltere discs**.** (C) MCP1 construct carrying the fragment from residues 573 to 675. *lacZ* is derepressed in the wing and haltere discs. (D) 5MPHO line T8, in which *lacZ* is weakly derepressed. (E) Δ5MPHO: a derivative line of 5MPHO line T8 from which 5MPHO had been excised. Expression is strongly derepressed in wing and haltere discs.



	# lines	silenced	derepressed
MCP822	23	15	8
MCP 12	18	1	17
MCP 14	11	7	4
MCP 1	18	1	17
MCP 2	9	0	9
MCP 7	21	15	6
MCP 7*	17	3	14

Fig. 3. Deletion analysis of MCP. Top line shows a diagram of the wild-type MCP element, with the location of the five PHO consensus sites (circles) and the two putative GAF binding sites (squares) indicated. The deletion constructs MCP12, MCP14, MCP1, MCP2 and MCP7 are shown. The deleted portions are denoted by parentheses and the extent of the deletions are indicated by the nucleotide numbers. MCP7* has mutations in the two putative GAF binding sites, indicated by Xs. In the adjacent table, three columns are shown: '# lines' are the total number of independent transformant lines examined for *lacZ* expression; 'silenced' is the number of lines in which silencing is correctly maintained in the wing disc and the anterior compartment of the haltere disc; 'derepressed' is the number of lines in which *lacZ* is derepressed in the wing or haltere discs.

activity we made two constructs, one with the PHO site at residue 62 mutated (PHO62) and another with one of the clustered PHO binding sites mutated (PHO604). These single mutation constructs were capable of maintaining silencing, although in one case (PHO62) with a reduced efficiency (Fig. 1B). It appears therefore that four PHO binding sites are sufficient in the context of the full 822 bp MCP fragment. We then tested the 103 bp fragment between residues 573 and 675, which contains four PHO binding sites, for its ability to maintain silencing. We find that this fragment (MCP1) is not sufficient to maintain silencing (Figs 1B and 2C). The observed expressivity of derepression indicates that little residual silencing activity remains in the MCP1 fragment. Therefore, the PHO binding sites are necessary but the presence of four PHO binding sites is not sufficient for the MCP silencer function.

Sequences other than PHO binding sites can independently maintain silencing

The presence of low expressivity lines among transformants carrying constructs with PHO binding sites mutated suggested that sequences other than PHO binding sites may contribute to silencing. To distinguish between residual silencing activity in 5MPHO and contributions from position effects, we compared the silencing activity of a 5MPHO insertion line with that of its derivative line (Δ5MPHO) from which 5MPHO had been excised. If the 5MPHO line contained silencing activity, then it should silence more effectively than its derivative Δ5MPHO line. Since both are inserted at the same genomic location, the difference can be attributed to the MCP sequences outside of the PHO consensus binding sites. To construct Δ5MPHO lines, we started with relatively weakly derepressed 5MPHO lines. Since the 5MPHO sequences were flanked with FRTs, we excised them by passaging through male germ cells that expressed FLP recombinase (see Materials and Methods). Fig. 2 shows the results from one of the lines studied. Compared to its parental 5MPHO line (Fig. 2D), the derivative Δ5MPHO line (Fig. 2E) shows a greater degree of derepression in the wing discs. Δ5MPHO lines derived from two other 5MPHO lines also showed an increased derepression of the reporter in the wing discs (data not shown). These results indicate that there are sequences distinct from PHO binding sites in MCP that can mediate silencing.

Deletion analysis of the MCP element to localize sequences required for silencing

To identify sequences other than PHO binding sites that contribute to silencing activity of the MCP element, we generated a number of deletion constructs and tested their silencing activity in the reporter assay. The constructs we generated and the results of in vivo functional tests are shown in Fig. 3. Deletions of MCP from the proximal (left) end up to residue 538 (constructs MCP14 and MCP7), did not significantly alter silencing activity. However, deleting MCP to residue 573 (constructs MCP1 and MCP2) eliminated its silencing activity (see Fig. 2C for *lacZ* expression in the imaginal discs). MCP2 retains the MCP sequence between residues 675 and 822 that is absent from MCP1. Since neither construct retains silencing activity, a key sequence for silencing must be located proximal to the cluster of PHO binding sites. This loss of silencing is not simply due to lack of the PHO site at residue 62 since a number of constructs that lack this PHO site (PHO62, MCP14, MCP7) were able to silence effectively. Rather, comparison of MCP7 and MCP1 indicates that the sequence required for silencing is located between residues 538 and 573. From the distal end, MCP can be deleted to residue 675 without significantly reducing the silencing activity (MCP14, MCP7); however, as expected, deleting to residue 273 (MCP12) eliminated its silencing activity. This deletion analysis indicates that the minimal MCP construct that retains robust silencing activity is the 138 bp (MCP7) fragment that spans from residues 538 to 675.

To test whether the minimal MCP construct is functioning as a PRE, we examined two MCP7 transformant lines, P[T3]

Table 1. Silencing activity of MCP in PcG mutants

	Derepression of *lacZ* in wing discs (%)		
Genotype	MCP822 P[T8]	MCP7 P[T3]	MCP7 P[T9]
+/+	13	5	10
Pc^3/+	100	85	88
Trl^{R85}/+	17	37	46
pho^1/+	60	64	58
pho^b/+	50	–	–
Trl^{R85}/+;pho^b/+	46	–	–
Psc^{h27}/+	30	–	–
Psc^{h27}/+;Trl^{R85}/+	35	–	–

–, not determined.

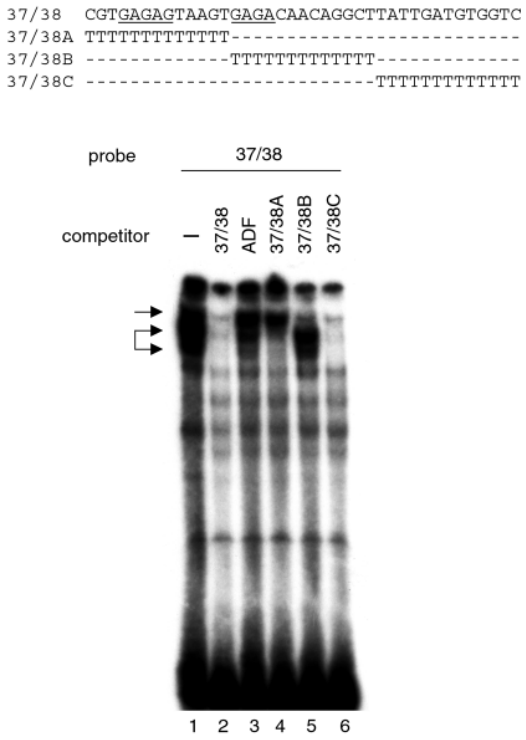


Fig. 4. Electrophoretic mobility shift assays. Top panel shows the sequences of the oligonucleotides 37/38 and the competitors 37/38 A, B and C used in the assay. The oligo 37/38 contains sequences between residues 543 and 581 including the two putative GAGA binding sites (underlined) at positions 548 and 558 (Fig. 7). The competitor sequences are identical to oligo 37/38 except for the residues replaced with Ts. The lower panel shows the results from EMSA when radioactively labeled 37/38 oligonucleotide was used as probe and incubated with embryonic nuclear extracts in the absence or in the presence of unlabeled competitors (Materials and Methods). Lane 1: oligo 37/38 forms three shifted bands (arrows) in the absence of competitors. Lane 2: 37/38 competitor. The three bands are competed. Lane 3: ADF competitor (non-specific sequence). No competition is observed. Lane 4: oligo 37/38 A as a competitor. The bottom two bands (connected arrows) are competed. Lane 5: 37/38 B competitor. The top band (arrow) is competed. Lane 6: 37/38 C competitor. All three bands are competed. Summary: the top band is competed by sequences included in the left third of the 37/38 oligo and the bottom two bands are competed by sequences in the middle third of the 37/38 oligo. No protein binds to the right third of the oligo 37/38. The results are schematically shown in Fig. 7.

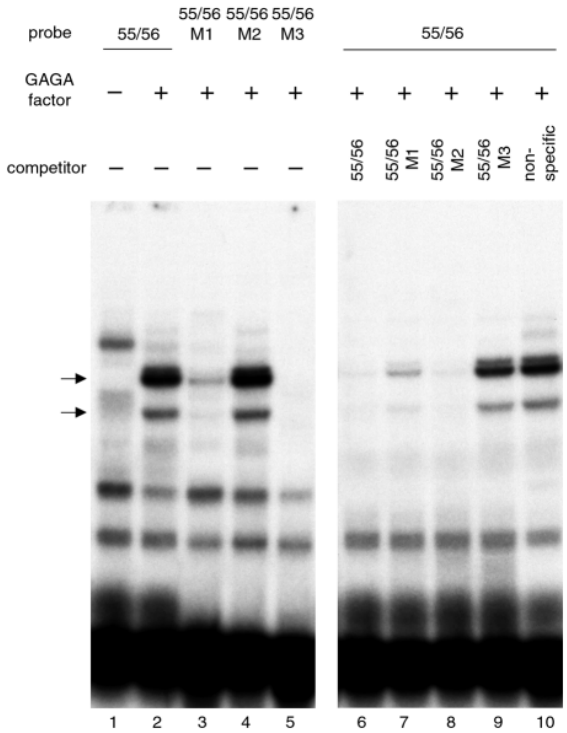


Fig. 5. Binding of in vitro synthesized GAF to two putative GAGA binding sites at MCP residues 548 and 558. Lanes 1-5: EMSA performed in the absence of competitor. Oligonucleotide 55/56 contains the sequence between residues 533 and 572 that includes the putative GAGAG548 and GAGA558 binding sites (Fig. 7). Oligo 55/56M1 has the GAGA548 site mutated, oligo 55/56M2 has the GAGA558 site mutated, and oligo 55/56M3 has mutations in both sites. Radioactively labeled oligonucleotide 55/56 (lane 2) or one of the mutated versions (lanes 3-5) was incubated with in vitro-synthesized GAF factor and run on a 5% acrylamide gel (Materials and Methods). Two shifted bands (arrows) are present when incubated with GAF (lane 2) but not without (lane 1). The two shifted bands presumably correspond to protein-DNA complexes formed by the full-length and a shorter GAF polypeptides synthesized in our in vitro translation system (data not shown). Lanes 6-10: EMSA in the presence of competitors. The binding is competed by excess, unlabeled oligo 55/56 (lane 6) but not by non-specific competitor oligo (lane 10). Lanes 7-9 are competition experiments in which oligo 55/56 was labeled and the mutated oligos were used as excess, unlabeled competitors. The shifted band using 55/56M1 (lane 3) is faint. However, we believe this to be a true shift rather than due to spillover from adjacent lanes, as lane 7 shows shows that oligo 55/56M1 can compete for GAF binding.

and P[T9], in animals heterozygous for PcG mutations. These inserts show background derepression of the *lacZ* reporter in 5% and 10%, respectively, of the wing discs from wild-type larvae. In contrast, P[T3] and P[T9] reporters in heterozygous Pc^3 larvae were derepressed in 85% and 88%, respectively, of the wing discs (Table 1). Similarly, P[T3] and P[T9] reporters in pho^1 heterozygous larvae showed *lacZ* derepression in 64% and 58%, respectively, of the wing discs (Table 1). These results confirm that the minimal MCP138 element contains sequences that recruit PcG silencing complexes.

Assaying DNA binding activities within the minimal MCP silencer element

To identify the essential sequences located outside the PHO cluster region, we synthesized two pairs of oligonucleotides, 37/38 and 39/40, covering the sequences between residues 543 and 607 and tested them in EMSAs using *Drosophila* embryonic extracts (see Fig. 7 for location of oligos). With oligo 37/38, three shifted bands were observed (Fig. 4, lane 1, arrows). To localize the region of the oligo responsible for the shift, we replaced three subregions of the oligo with runs of T residues (Fig. 4 top) and used them as unlabeled competitors in the bandshift assay. The top shifted band (arrow) is competed away by oligos 37/38B and 37/38C (Fig. 4, lanes 5 and 6). This indicates that the recognition sequence for the protein(s) bound in the top band is in the left third of the oligo. The lower pair of bands (connected arrows) are competed away

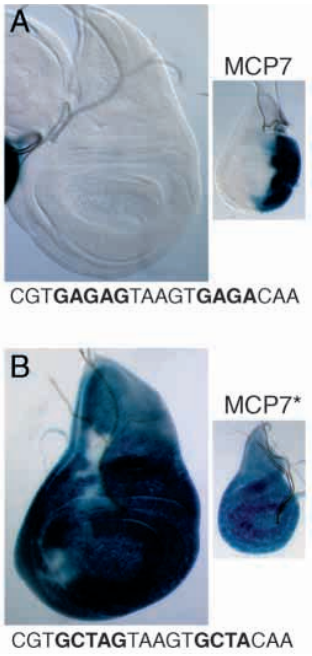


Fig. 6. GAGA binding sites are required for the maintenance of silencing. (A) Pattern of *lacZ* expression in the wing and haltere imaginal discs from larvae containing the MCP7 construct (138 bp MCP minimal sequence that includes the GAGA binding sites, Fig. 3). *lacZ* is silenced in the wing disc (ps4 and ps5) and in the anterior compartment of the haltere disc (ps5), but it is expressed in the posterior compartment of the haltere disc (ps6). (B) MCP7* construct (138 bp MCP minimal sequence with GAGA binding sites mutated as shown). Both the wing and haltere discs show strong derepression of *lacZ*.

by oligos 37/38A and 37/38C (Fig. 4, lanes 4 and 6). This indicates that the middle third of the oligo contains the recognition sequence for the protein(s) bound in the lower two bands. Taken together, this analysis suggests that a protein(s) is bound to the left third, and a different protein is bound to the middle third of the oligo, while no protein is bound to the right third. Using a similar logic with overlapping oligos, we determined that a protein binds to a sequence around residues 582-592. Oligo 39/40 band-shifts in EMSA using an embryonic extract that lacks PHO activity, but the overlapping oligo PHO604 does not (data not shown). These analyses suggest that, in addition to PHO proteins, at least three more proteins bind to the 138 bp minimal MCP element. The proposed protein interaction regions are indicated in Fig. 7.

Table 2. Interaction of *Trl* with PcG genes

Genotype	Expression of Ubx in wing discs (%)	Extra sex combs (%)	Antenna to leg (%)
+/+	0	0	0
Pc^3/+	97	7	8
Trl^{R85}/+	0	0	0
pho^1/+	0	0	0
Trl^{R85}/Pc^3	95	45	25
Trl^{R85}/+;pho^1/+	0	11	0

GAGA binding sites are required for MCP silencing activity

Within the two subregions of oligo pair 37/38 exhibiting protein binding activity there are two potential recognition sequences for GAGA factor (GAF), GAGAG and GAGA at residues 548 and 558, respectively (Fig. 7). Most GAF binding sites are composed of longer GA repeats, but as little as three bases (GAG) has been recognized by GAF in vitro (Wilkins and Lis, 1998). To determine if putative GAGA binding sites at residue 548 and 558 are bona fide GAGA binding sites, we tested the ability of in vitro-synthesized GAF to bind to the oligonucleotide 55/56 containing sequence between residues 533 and 572 (Fig. 7). Oligo 55/56 is shifted when incubated with in vitro-synthesized GAF (Fig. 5, lane 2). The two shifted bands (arrows) are likely to correspond to complexes of a full-length GAF and a partial GAF that are synthesized in our in vitro translation system (data not shown). The shifted bands are competed by excess unlabelled competitors with the same sequence as the labeled oligo (lane 6) but not by an unrelated

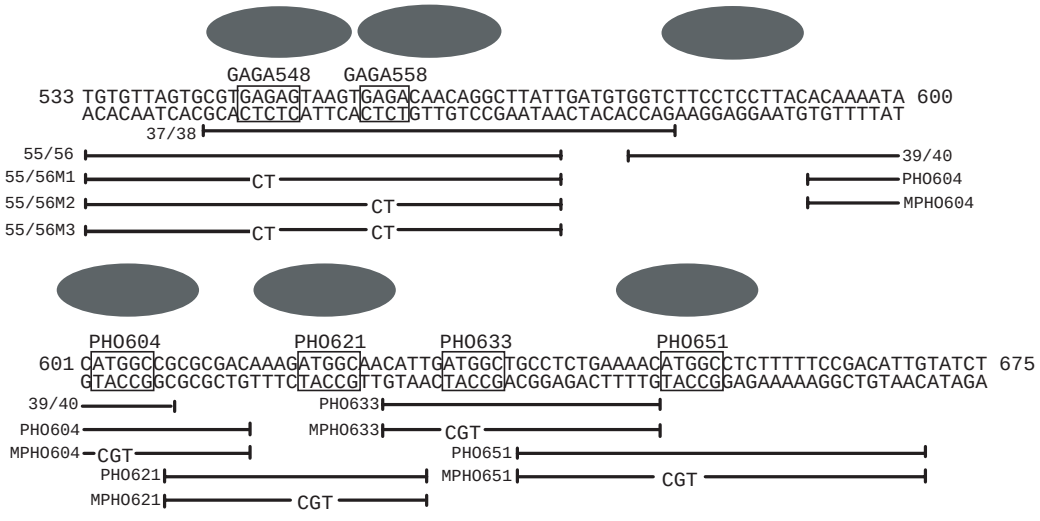


Fig. 7. The minimal MCP silencer element. The sequence of the 138 bp minimal MCP silencer element is shown. Putative GAGA and PHO binding sites are boxed. Above the sequence, grey ovals show the approximate locations of protein binding sites deduced from DNA binding studies using embryonic nuclear extracts and in vitro-synthesized proteins. The protein in nuclear extracts that interacts with sequences near GAGA558 is unlikely to be GAF since the binding is not competed by the strong GAGA binding sequence at GAGA548 (see text and Fig. 4). Below the sequence, the extent of the oligonucleotides and the mutated residues are indicated.

sequence oligo (lane 10). To determine if both the GAGAG548 and GAGA558 sites can bind GAF, we synthesized oligos with mutations in the putative GAF binding sequence at either 548 or 558 (55/56M1 or M2 in Fig. 7), and tested them in the EMSA using in vitro-synthesized GAF. A small amount of labeled oligo 55/56M1, carrying a mutation at GAGAG548, still shifts (Fig. 5, lane 3), suggesting that GAGA558 weakly binds GAF. When GAGA558 is mutated (55/56 M2), mobility shift is robust (Fig. 5, lane 4), suggesting a preferential binding of GAF to the GAGAG548 site. An oligonucleotide with both GAGAG548 and GAGA558 mutated (55/56 M3) does not bind GAF (lane 5). Results consistent with this were obtained in separate experiments in which the mutated oligos were used as unlabelled competitors. The 55/56M1 oligo moderately competes (lane 7) while the 55/56M2 oligo does so strongly (lane 8), but the 55/56M3 oligo with both GAGA sites mutated competes as poorly as the nonspecific competitor (lane 9). These results indicate that both of the potential GAGA binding sites can be recognized by GAF, but that the GAGAG548 site does so much more strongly than the GAGA558 site.

To test the requirement for GAF binding sites in the minimal MCP element (MCP7) in vivo, we mutated both GAGA binding sequences (mutated sequences are shown in Fig. 6) and tested the ability of the mutant construct (MCP7*) to maintain silencing. We find that, unlike the parental MCP7 construct, this mutant construct MCP7* is unable to maintain silencing in most transformed lines (Fig. 3). In these derepressed lines, the *lacZ* reporter was expressed in nearly the entire wing and haltere discs (Fig. 6). Therefore, in the context of MCP7, GAF binding sites appear to be absolutely required for silencing. Stated another way, this result indicates that PHO binding sites by themselves possess little silencing activity. The same conclusion can be extended to the two other proteins that bind to the MCP7 (Fig. 7). We have not directly tested their participation in silencing, but we can at least conclude that they do not have any silencing function on their own.

Genetic evidence that GAF is required for silencing by MCP

To determine whether GAF is involved in silencing *in vivo*, we examined MCP-mediated silencing in flies mutated for the GAF encoding gene, *Trithorax-like* (Farkas et al., 1994). Animals homozygous for the putative null allele Trl^{R85} (Farka et al., 1994) die before wing imaginal discs develop. Therefore, we examined the expression of MCP reporter constructs in heterozygous animals. We examined silencing of three reporter constructs in *Trl* mutant animals: the 822 bp MCP P[T8] line and two independent lines carrying the 138 bp MCP7 element, P[T3] and P[T9]. When compared to their wild-type sibling controls, Trl^{R85} heterozygous animals carrying MCP822 showed a modest but reproducible increase in the percentage of wing discs expressing *lacZ* (Table 1). The effect of Trl^{R85} mutation is more pronounced in MCP7 lines. In Trl^{R85} heterozygous larvae, the P[T3] and P[9] lines showed derepression in 37% and 46%, respectively (Table 1). The greater effects of the Trl^{R85} mutation on MCP7 inserts presumably reflect a greater dependence of the minimal element on GAF. These results indicate that *Trl* function is required for silencing by the MCP element. Combined with the result that GAGA consensus sequence is required in vivo, the genetic results strongly suggest that GAF binds to the MCP element and contributes to the maintenance of silencing.

Interactions of *Trl* and PcG genes

Trl is usually considered to be a member of the trxG genes (Paro and Harte 1996). If GAF is required for silencing, however, *Trl* mutations might be expected to enhance the phenotypes caused by PcG mutations. Strutt et al. (Strutt et al., 1997) have noted that male flies doubly heterozygous for *Pc* and *Trl* mutant alleles show an increased appearance of extra sex combs. In contrast, Kehle et al. (Kehle et al., 1998) did not detect genetic interaction between *Pc* and *Trl* mutations. To determine if *Pc* and *Trl* genetically interact, we measured the penetrance of homeotic transformations in doubly heterozygous animals (Table 2). In Pc^3/Trl^{R85} double heterozygotes, 45% of males show the extra sex combs phenotype (on either the second, third or both legs). Moreover, 25% of the females show the antenna-to-leg transformation phenotype associated with the PcG mutations. These frequencies are significant increases in penetrance since only 7% of $Pc^3/+$ and no $Trl^{R85}/+$ males show the extra sex combs phenotype, and 8% of $Pc^3/+$ and no $Trl^{R85}/+$ females show the antenna-to-leg transformation. Similar results were obtained with another mutant allele of *Trl*, Trl^{13C} (data not shown). We have also examined genetic interactions between *pho* and *Trl* alleles (Table 2). In these experiments, 11% of $Trl^{R85}/+$; $pho^1/+$ males show the extra sex comb phenotype. This is a modest but still significant interaction as neither $pho^1/+$ nor $Trl^{R85}/+$ males show extra sex combs. These results indicate *Trl* and PcG genes interact to silence homeotic genes. It is not clear why Kehle et al. (Kehle et al., 1998) did not observe genetic interactions. We note, however, that Kehle et al. used different alleles from those used by us or by Strutt et al. and measured expressivity, rather than penetrance, of the extra sex comb phenotype.

We have also tested if *Trl* and PcG genes interact to silence the endogenous *Ubx* gene and MCP reporter constructs in double heterozygous animals. Compared to that seen for the extra sex comb phenotype, however, genetic interaction between *Trl* and PcG genes for the *Ubx* gene or the reporters was not detectable. The endogenous *Ubx* gene was not derepressed in the third instar wing imaginal discs of Pc^3/Trl^{R85} larvae any more frequently than in the $Pc^3/+$ wing imaginal discs (Table 2). Moreover, there was no difference in the size of UBX-expressing clones in Pc^3/Trl^{R85} and $Pc^3/+$ animals (data not shown). We also examined the effect of *Trl* and two PcG mutations, *Psc* and *pho*, on silencing of the MCP822 P[T8] reporter. The combination of Psc^{h27} and Trl^{R85} mutations does not induce derepression any more frequently than the combined effect of Psc^{h27} and Trl^{R85} individual mutations (Table 1). Similarly, the combination of Trl^{R85} and pho^b did not show any increase over $pho^b/+$ animals. This apparent lack of synergy between *Trl* and PcG mutations, when looking at the expression of UBX and the reporter genes in the wing discs, may simply mean that these heterozygous conditions do not sensitize animals sufficiently to reveal genetic interactions. It may also reflect the silencing mechanism at this promoter. A possible explanation is presented in the Discussion.

DISCUSSION

PREs that are capable of maintaining silencing have been identified from several homeotic genes (reviewed by Pirrotta, 1999). However, the complexity of DNA binding activity has not been fully determined. Here we have shown that a 138 bp MCP fragment (MCP7 construct) from the *Abd-B* gene of the bithorax complex behaves as a PRE and is sufficient for maintaining silencing at least through the larval stages. Within this fragment, there are four PHO protein binding sites and one strong and one weak GAF binding site. In addition, we have detected two other DNA binding activities in embryonic nuclear extracts. Our results show that both PHO and GAF binding sites are required for the maintenance of silencing by the 138 bp MCP fragment.

Requirement of PHO for maintenance of silencing

We have shown that PHO binding sites within MCP are necessary for maintenance of silencing. We identified five sites within the 822 bp MCP showing homology to the PHO binding consensus sequence (Brown et al., 1998; Mihaly et al., 1998). Four of the five sites bound to in vitro-synthesized PHO protein. Our mutational analysis of these sites indicates that they are required for silencing. However, not all five sites are needed. In the context of the 822 bp MCP sequence, mutated fragments with four functional PHO binding sites were capable of maintaining silencing. That these PHO consensus sites are indeed recognized by PHO in vivo is supported by the effect of *pho* mutations on the transgene expression: the PBX-MCP-*Ubx*p-*lacZ* transgene is partially derepressed in animals heterozygous for pho^1 or pho^b mutations. At present, the molecular mechanism of how PHO contributes to silencing is unknown. PHO exhibits partial sequence similarity to the mammalian transcription factor YY1 and binds to DNA in a sequence-specific manner (Brown et al., 1998). Based on this, it was suggested that PHO acts as a recruiter of the PcG silencing complexes to DNA (Brown et al., 1998). However, it has been shown recently that a LexA-PHO fusion protein, when bound to LexA binding sites, is unable to silence the expression of a reporter gene, while a LexA-Polycomb fusion protein is able to do so in the same system (Poux et al., 2001). Furthermore, PHO is unable to recruit PcG proteins, as observed in immunoprecipitation experiments. These results suggest that PHO cannot by itself recruit silencing complexes. Our finding that MCP constructs with four PHO binding sites (MCP1 and MCP7*) show little silencing activity supports their conclusion that PHO protein cannot recruit silencing complexes by itself. How then does PHO contribute to silencing? As will be discussed more fully below, the contribution of PHO to silencing may require an additional factor. Only in the presence of binding sites for this additional factor can PHO's contribution be revealed. Under this condition, it is still possible that PHO may recruit PcG complexes. The inability of Lex-PHO protein to recruit PcG complexes may also be explained by the absence of a required adaptor protein in the extracts. YY1 has been shown to interact with a newly identified zinc-finger protein called RYBP, which also interacts with the mammalian PcG proteins with ring-finger domains (Garcia et al., 1999). RYBP also interacts with *Drosophila* PHO protein in vitro (Garcia et al., 1999). There is at least one candidate for a RYBP homolog in the *Drosophila* genome (our unpublished observation). The *Drosophila* counterpart of RYBP may also serve as an adaptor protein between PHO and PcG ring-finger domain proteins such as PSC and SU(Z)2.

Silencing function of GAGA binding sites in vivo

We have demonstrated that GAF binding sites are required for efficient silencing by MCP in vivo. We do not know if both sites are required in vivo. In vitro binding of GAF to the GAGAG548 sequence is much stronger than to the GAGA558 sequence (Fig. 5). In addition, the oligonucleotide containing only the GAGA558 sequence does not detectably compete for the nuclear extract factor (presumed GAF) that binds to the oligonucleotide containing GAGAG548 (Fig. 4). Therefore, occupancy of the stronger site may be sufficient to contribute to the maintenance of silencing.

We have not directly demonstrated that it is GAF that binds to these sites in vivo. It remains possible that another GAGA binding protein recognizes these sites and contributes to silencing. However, several lines of evidence suggest the involvement of GAF in silencing. First, PC protein and GAF were found to be co-localized on polytene chromosomes in salivary glands (Strutt et al., 1997). Second, the combination of *Pc* and *Trl* mutant alleles enhances the extra sexcomb phenotype and antenna-to-leg transformation caused by *Pc* mutations (Strutt et al., 1997; our results, Table 2). Third, *Trl* mutations can compromise silencing mediated by a Fab-7 PRE fragment (Hagstrom et al., 1997). Finally, the in vitro formation of complexes between PcG proteins and the bxd PRE is dependent on consensus recognition sequences for GAF, and GAF in embryonic extracts co-immunoprecipitates with PC protein (Horard et al., 2000). These results make GAF a good candidate for the protein that binds to the GAGA sites within MCP.

Contribution of GAF and PHO to the maintenance of silencing

How does GAF or perhaps another GAGA binding protein contribute to the silencing by MCP, and what is its relationship to the PHO protein function? We suggest two models to explain their relationship that leads to strong silencing. These models are based on the following observations. First, PHO binding sites by themselves show little silencing activity (MCP1 and MCP7* constructs). Second, GAF or some other protein that binds to MCP can weakly recruit silencing complexes in the absence of PHO binding (5MPHO construct). Third, when present together, GAF and PHO binding sites exhibit robust silencing activity (MCP7 construct). In the first model, GAF and PHO bind to the MCP silencer in a sequential order. One version would be that GAF binding is absolutely required for binding or activity of PHO. GAF may open up chromatin at MCP, allowing binding of PHO. Upon binding, PHO may recruit PcG silencing complexes, although there is still little evidence that this happens. GAF has been shown to induce DNase I hypersensitive sites, or nucleosome-free regions (Lu et al., 1993), and this may create a prerequisite condition for PHO to bind to its recognition sites. There is indeed a DNase hypersensitive region associated with MCP that includes the location of the GAF binding site (Karch et al., 1994).

In a second version of the model, PHO acts as a facilitator of GAF binding by creating some pre-condition, perhaps by

bending DNA as YY1 does (Natesan and Gilman 1993). Since PHO binding sites are not absolutely required for MCP silencing activity, GAF presumably can bind weakly to MCP in the absence of PHO. Enhanced binding of GAF leads to increased recruitment of silencing complexes. GAF bound to MCP may recruit PcG silencing complexes by directly interacting with PC or other members of PcG complexes (Horard et al., 2000). Alternatively, GAF could first recruit SIN3 histone deacetylation complexes through its interaction with SAP18 (Espinas et al., 2000), which then might generate a chromatin state favorable for PcG complex binding. Whichever version of the model is correct, the important feature of the model is the sequential recruitment of DNA binding proteins, GAF and PHO, to MCP. Binding of one protein creates a condition favorable to the binding of a second protein, eventually leading to the recruitment of PcG complexes. Note that the requirement of GAF and PHO proteins applies to MCP silencing, but not necessarily to all PREs. Other PREs may use other combinations of proteins. Our model is analogous to Swi5 protein binding to the yeast HO promoter and recruiting the chromatin remodeling complex Swi/Snf. Swi/Snf in turn recruits the histone acetylase complex SAGA, eventually leading to the binding of the transcription factor SBF to the HO promoter (Cosma et al., 1999). In such a sequential recruitment model, compromising one step in the sequence may become rate limitating so that combining two mutations that disable two different steps may not necessarily lead to synergistic effects. This may explain why we did not observe synergistic effects in our assay system when Trl and PcG mutations were combined.

In the second model, GAF and PHO bind to MCP independently of each other. Each protein may induce a unique chromatin modification that, together, can have a positive synergistic effect on the recruitment of PcG silencing complexes. Multi-layered recruitment of silencing complexes may also explain why recruiting LexA-PHO or LexA-GAF fusion protein individually to a LexA binding site failed to assemble PcG silencing complexes (Poux et al., 2000).

A dual role of Trl in regulating homeotic gene expression

Trl is thought to be required for the maintenance of the activation of homeotic genes. Consistent with this, *Trl* mutations show phenotypes indicative of the loss of activity of homeotic genes and enhance hypomorphic loss-of-function phenotypes (Farkas et al., 1994). Nevertheless, previous studies (Strutt et al., 1997; Hagstrom et al., 1997; Horard et al., 2000) and our work presented here clearly implicate *Trl* in the repression of homeotic gene transcription. *Trl* therefore should be classified as belonging to both the trxG and PcG groups. *Trl* would not be the first gene to be classified as belonging to both positive and negative regulatory groups. LaJeunesse and Shearn (LaJeunesse and Shearn, 1995) found that a mutant allele of *Enhancer of zeste* (*E(z)*) caused both derepression and loss of expression of homeotic genes. A recent screen for second site non-complementers of an *ash1* mutation, a trxG gene mutation, found that mutations in six PcG genes – *E(Pc)*, *Psc*, *Su(z)2*, *Asx*, *E(z)* and *Scm* – enhanced, rather than suppressed, the *ash1* mutation (Gildea et al., 2000). Thus, there appeared to be a group of genes that act in both activation and silencing of homeotic genes. Gildea et al. (Gildea et al., 2000) have called these genes the ETP (enhancer of trithorax and Polycomb mutations) group. Based on genetic and molecular evidence, we suggest that *Trl* should be included in the ETP group. The mechanism as to how the ETP proteins contribute to both activation and silencing is unclear. Perhaps ETP proteins are core chromatin proteins needed to form both activation and repression complexes, and depending on the cellular transcriptional state, either one or the other is formed.

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