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The *Drosophila pho-like* gene encodes a YY1-related DNA binding protein that is redundant with *pleiohomeotic* in homeotic gene silencing

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SUMMARY

Polycomb group proteins (PcG) repress homeotic genes in cells where these genes must remain inactive during *Drosophila* and vertebrate development. This repression depends on cis-acting silencer sequences, called Polycomb group response elements (PREs). Pleiohomeotic (Pho), the only known sequence-specific DNA-binding PcG protein, binds to PREs but *pho* mutants show only mild phenotypes compared with other PcG mutants. We characterize *pho-like*, a gene encoding a protein with high similarity to Pho. Pho-like binds to Pho-binding sites in vitro and *pho-like, pho* double mutants show more severe misexpression of homeotic genes than do the single mutants. These results suggest that Pho and Pho-like act redundantly to repress homeotic genes. We examined the distribution of five PcG proteins on polytene chromosomes from *pho-like, pho* double mutants. Pc, Psc, Scm, E(z) and Ph remain bound to polytene chromosomes at most sites in the absence of Pho and Pho-like. At a few chromosomal locations, however, some of the PcG proteins are no longer present in the absence of Pho and Pho-like, suggesting that Pho-like and Pho may anchor PcG protein complexes to only a subset of PREs. Alternatively, Pho-like and Pho may not participate in the anchoring of PcG complexes, but may be necessary for transcriptional repression mediated through PREs. In contrast to Pho and Pho-like, removal of Trithorax-like/GAGA factor or Zeste, two other DNA-binding proteins implicated in PRE function, does not cause misexpression of homeotic genes or reporter genes in imaginal disks.

Key words: Polycomb group genes, Gene silencing, *Drosophila*

INTRODUCTION

Polycomb-group (PcG) proteins are conserved transcriptional repressors with important roles during embryonic and post-embryonic development in *Drosophila* and vertebrates (for reviews, see Brock and van Lohuizen, 2001; Francis and Kingston, 2001; Mahmoudi and Verrijzer, 2001; Simon and Tamkun, 2002). Among the best-characterized PcG target genes are the homeotic genes in *Drosophila*. At least 15 different PcG genes are required to repress homeotic genes in cells in which these genes must remain inactive during development. These PcG proteins exist in at least two distinct multiprotein complexes that do not appear to bind to DNA directly but bind to chromatin (Shao et al., 1999; Ng et al., 2000; Tie et al., 2001; Saurin et al., 2001). Antibody staining of polytene chromosomes revealed that PcG proteins are associated with about 80-100 loci in the *Drosophila* genome (Zink and Paro, 1989; DeCamillis et al., 1992; Martin and Adler, 1993; Rastelli et al., 1993; Lonie et al., 1994; Carrington and Jones, 1996; Peterson et al., 1997). Subsequently, chromatin-immunoprecipitation experiments showed that several PcG proteins can be crosslinked with specific DNA sequences, called Polycomb group response elements (PREs), in PcG target genes (Strutt and Paro, 1997; Strutt et al., 1997; Orlando et al., 1998). Recent progress towards understanding how PcG protein complexes may be anchored at PREs has come from the dissection of PREs and the identification of proteins that directly bind to PRE DNA.

PREs were first identified in reporter gene assays as *cis*-regulatory elements in homeotic genes that render expression of a reporter gene construct sensitive to mutations in PcG genes (Simon et al., 1993; Chan et al., 1994; Christen and Bienz, 1994). Furthermore, addition of a PRE causes unusual silencing of a mini-*white* reporter gene in transgenic flies and creates a new PcG band on polytene chromosomes at the transgene insertion site (reviewed by Pirrotta, 1997a; Pirrotta, 1997b; Kassis, 2002). Characterization of a PRE from the *engrailed* gene led to the identification of the PcG protein Pleiohomeotic (Pho) as a PRE-binding factor (Brown et al., 1998). Pho is a DNA-binding protein related to the mammalian transcription factor YY1 (Brown et al., 1998). Pho-binding sites are found in many different PREs and are required for PRE function in many different reporter constructs (Brown et al., 1998; Mihaly et al., 1998; Fritsch et al., 1999; Shimell et

al., 2000; Mishra et al., 2001; Busturia et al., 2001). Despite this requirement for Pho-binding sites in reporter genes, *pho* mutants only weakly misexpress homeotic genes (Simon et al., 1992; Fritsch et al., 1999) and die as pharate adults with relatively weak homeotic transformations (Gehring, 1970). Thus, if Pho anchors PcG protein complexes on DNA, it most likely is not the only DNA-binding PcG protein that provides this function.

Because of the weak homeotic gene misexpression in *pho* mutants, we searched the *Drosophila* genome for *pho*-related sequences and identified a gene that we call *pho-like* (*phol*). The Phol protein binds to the same DNA sequence as Pho. The strong PcG phenotype of *phol, pho* double mutants shows that Phol is another DNA-binding protein required for PcG repression. We examined the distribution of five different PcG proteins on polytene chromosomes in *phol, pho* double mutants. Our data show that binding of PcG proteins to a few chromosomal bands requires *pho* and *phol*, but that at most chromosomal locations PcG proteins remain bound in the absence of Pho and Phol.

Finally, we analysed the requirement for two other DNA-binding proteins that have recently been implicated in PcG repression: GAGA factor, which is encoded by the gene *Trithorax-like* (*Trl*); and Zeste (Horard et al., 2000; Mishra et al., 2001; Hodgson et al., 2001; Busturia et al., 2001; Hur et al., 2002). We report experiments aimed at testing the role of these two proteins in PcG repression in imaginal disks. Our results provide no evidence for a requirement of either *Trl* or *zeste* in PcG repression in imaginal disks.

MATERIALS AND METHODS

Cloning of the Pho-like zinc finger domain into the T7-link expression construct

PCR was performed on the on the EST clone, LD42095 using the rphoBam (5′GCTGGTAATGCCATGGGATCCGCTGGCGCGG-CCGGC3′) and rphoXhoR (5′CGGTGTCTTGTTGTTCACGAGTT-ACGGTGCGCGCGGCCA3′) primers. rphoBam introduces an in-frame *Bam*HI site upstream of the first zinc finger (at amino acid 490) of Phol. rphoXhoR introduces an in-frame stop codon immediately downstream of the fourth zinc finger of Phol (amino acid 624) followed by a new *Xho*I site. The PCR product was subcloned downstream of the β-globin 5′ UTR and ATG in *pT7link* (provided by R. Treisman) to generate Pho-like$_{490\text{-}624}$T7. The integrity of the inserted PCR product was confirmed by sequencing.

Gel mobility shift assay

^{35}S-labeled full-length Pho protein was translated in vitro from the Pho$_{2\text{-}520p}$T7 construct (Fritsch et al., 1999) and Phol from the Pho-like$_{490\text{-}624}$T7 construct using the TNT reticulocyte lysate system (Promega). Gel mobility shift assays were performed as described previously (Americo et al., 2002) using 3 μl of the in vitro translation reaction.

Isolation of *phol* mutants

Virgin females from the stock EP0559 were crossed to males containing the immobilized transposase insertion, P[*ry*$^{+}$(Δ2, 3)]99B (Robertson et al., 1988) to generate deletions of the P-element and flanking DNA. Males of the genotype *w*; EP0559/*ry*^{506} *Sb* P[*ry*$^{+}$(Δ2, 3)]99B were crossed to *w; TM3, Ser/Sb* males. Individual *w*$^{-}$, *Ser* male flies resulting from loss of the *w*$^{+}$ marker present in the EP were crossed to *w; TM3, Ser/Sb* virgins and stocks were established that were EP0559(*w*$^{-}$)/*TM3*. DNA was made from homozygous EP0559(*w*$^{-}$) flies from 120 lines and checked for loss of the EP element using primers to the 5′ and 3′ ends of the P-element and flanking sequences. Lines that appeared to be missing *phol* DNA were subjected to further molecular analyses.

Identification of *phol, pho* double mutant larvae

phol homozygous (*Tb*$^{+}$) male larvae were collected from a stock that was *pho*^{1}/*ci*^{D}; *phol*^{81A}/*TM6B, Tb*. After removal of the salivary glands, DNA was prepared from the remaining carcass. The *pho*^{1} mutation is associated with a DOC element insertion at codon 272 upstream of the zinc finger domain of the Pho protein (Brown et al., 1998). To identify homozygous *pho*^{1} mutant larvae, we used primers 5′TTTGGCATTGATGGCTTCACG3′ and 5′GCATTGCAGATGAA-TCTCTGA3′ in a Long PCR reaction (Brown et al., 1998) with the DNA from individual larvae. The *ci*^{D} chromosome gives a 618 bp PCR product, while the *pho*^{1} chromosome (which carries the DOC element insertion) generates a fragment in excess of 5 kb. *pho*^{1} homozygotes produce only the larger PCR product. The *pho-like* mutation was confirmed by the absence of a PCR product using the rpho14 (5′CGGTAGCCTCATCATCCTC3′) and rpho23 (5′AGGGTTGCATTGTGG3′) primers.

Polytene chromosome staining

Squashes were performed as described elsewhere (Zink et al., 1991), except incubation in solution 1 was for 30 seconds and in solution 2 was for 4.0-4.5 minutes. Slides were washed in PBS 10 minutes, incubated in blocking buffer (PBS, 5% BSA, 5% dried milk, 0.4% Tween 20) for 30 minutes, and incubated overnight at 4°C with primary antibody (αPc, 1:100; αScm, 1:50; αPsc, 1:50; αE(z), 1:25; αPh, 1:500; αMsl3, 1:200; and αPho, 1:100) in blocking buffer. Slides were rinsed 30 minutes in PBS (adjusted to 300 mM NaCl with 0.4% Tween 20) then incubated with secondary antibody for 1 hour at room temperature. Secondary antibodies were FITC-, Cy2- or Cy3-labelled affinity-purified F(ab′)$_2$ fragments (Jackson ImmunoResearch Labs). The slides were washed twice for 30 minutes in PBS (plus 0.1% Tween 20), stained with DAPI for 5 minutes, rinsed with PBS and mounted in 1 mg/ml phenylenediamine/70% glycerol in PBS. In some experiments, antibodies against Msl3, a male-specific protein that coats the male X (reviewed by Kelley and Kuroda, 2000), were used to identify unambiguously the X-chromosome.

Pho antibody

Rabbit polyclonal antibodies were raised against a gel-purified HIS-tag/Pho full-length fusion protein. The crude polyclonal antisera specifically super-shifted a Pho/DNA complex in gel shift assays (data not shown). In western blots the antibody detects a strong band corresponding to Pho and a very weak band corresponding to Phol in 0-12 hour nuclear embryonic extracts (data not shown).

Drosophila strains

The following strains were used in this study (*FRT2A* is an abbreviation for *P{w*$^{+mW.hs}$*=FRT(w*^{hs}*)}2A. FRT82B* is an abbreviation for *P{ry*$^{+t7.2}$*=neoFRT}82B*):

pho^{1}/*ci*^{D}
w; phol^{81A} *FRT 2A/TM6B*
w; phol^{81A}*/TM6B; pho*^{1}/*ci*^{D}
w; Trl^{R85}*/TM6B*
z^{v77h}
z^{v77h}*; pho*^{1}/*ci*^{D}
w; FRT82B trx^{E2}*/TM6C*
w; FRT82B trx^{B11}*/TM6C*
yw hs-flp; M(3)^{i55} *hs-nGFP FRT2A*
ywhs-flp; FRT82B hs-nGFP

For analysis of *phol*^{81A} or *Trl*^{R85} mutant clones in *pho*^{1} homozygotes, *yw hs-flp/+; M(3)*^{i55} *hs-nGFP FRT2A/TM6B; pho*^{1}/+ virgins were crossed to *w; phol*^{81A} *FRT2A/TM6B*; *pho*^{1}/+ males or *w; Trl*^{R85} *FRT2A/TM6B*; *pho*^{1}/+ males, respectively. In both cases,

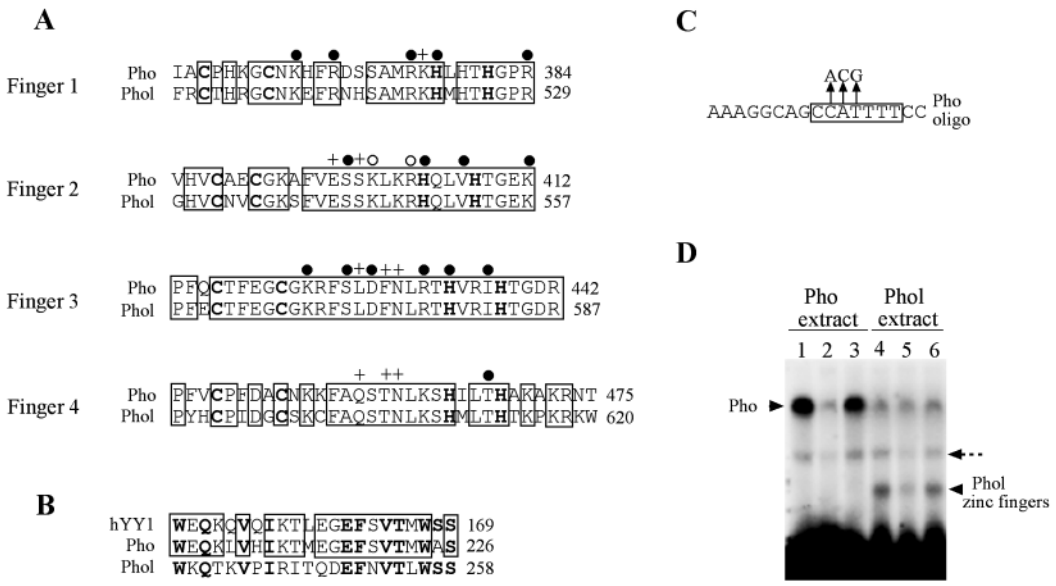

Fig. 1. Comparison of amino acid conservation and DNA-binding properties of Pho and Pho-like. (A) Amino acid identity (boxed) between Pho and Phol over the four zinc fingers. Amino acids from human YY1 that have been shown by X-ray crystallography to interact with DNA are marked (Houbaviy et al., 1996). Black circles represent amino acids that contact the DNA backbone. (+) represents positions that contact the DNA bases. White circles represent amino acids that contact both the DNA backbone and the bases. Cys and His residues of the zinc fingers are in bold. (B) Amino acid conservation within the spacer region between Pho, Phol and human YY1 (hYY1). Bold type indicates amino acids that are conserved between all three proteins. (C) The DNA sequence of the Pho and mutated Pho-binding site oligonucleotides used to test the DNA-binding specificity of the Phol zinc fingers. The mutated bases are denoted by the arrows. The Pho binding site is boxed. (D) Autoradiogram of a gel mobility shift assay showing binding of full-length Pho (lanes1-3) and Phol zinc-finger protein (lanes 4-6) to the Pho-binding site. Lanes 1 and 4, no competitor DNA; lanes 2 and 5, 100× unlabeled Pho-binding site; lanes 3 and 6, 100× unlabeled mutated Pho-binding site. The specific Pho and Phol complexes are indicated by arrowheads. The broken arrow denotes a faint gel shift due to endogenous YY1 protein in the reticulocyte lysate.

clones are marked by the absence of GFP and *pho*^{1} homozygotes were identified by their misexpression of Ubx protein.

Clonal analysis

Mitotic clones were generated by crossing the appropriate fly strains listed above and heat-shocking the F_1 larvae. Heat shock treatment was for 1 hour in a 37°C water bath; larvae were then allowed to develop for 96 hours at 25°C. Prior to dissection, larvae were subjected to another 1 hour heat shock, followed by a 1 hour recovery period, to induce expression of the GFP marker protein.

Staining procedures

Inverted larval carcasses were fixed and labelled with antibodies against Ubx or Abd-B or β-gal and, in the case of clonal analyses, double labelled with GFP antibody, followed by incubation with fluorescently labelled secondary antibodies as described (Beuchle et al., 2001). X-Gal staining was carried out as described (Christen and Bienz, 1994). Embryos were stained with β-gal antibodies and biotinylated secondary antibodies, followed by DAB staining using standard protocols.

Reporter gene constructs

BP01 females (Müller and Bienz, 1991) were crossed to *pho*^{1}*/ci*^{D} males and *BP01/+; pho*^{1}*/+* flies were inbred. *pho*^{1} homozygotes were identified by the misexpression of the *BP01* reporter gene in a quarter of the embryos. *Mcp725-P[T8]*, an insertion on the third chromosome (Busturia et al., 1997), was recombined onto a *Trl*^{R85} *FRT2A* chromosome; recombinants were selected and tested for the presence of *Mcp725-P[T8]*, *Trl*^{R85} and *FRT2A*. Males from a *Trl*^{R85} *FRT2A Mcp725-P[T8]/TM6B* stock were crossed with *yw flp122; M(3)i5 5hs-nGFP FRT2A* virgins and clones in their progeny were analysed for misexpression of the *Mcp725-P[T8]* transgene using antibodies against β-gal and GFP.

The *PRE*$_{DGAGAmut}$ reporter gene was obtained by mutating two to three nucleotides per GAGAG motive as indicated on top of the sequence in Fig. 7. Sixteen independent transformant lines were obtained and analysed.

RESULTS AND DISCUSSION

Pho and Phol bind to the same DNA sequence

phol is located on chromosome 3 in polytene subdivision 67B and is designated by the *Drosophila* genome project as CG3445. This sequence is predicted to encode a protein of 669 amino acids with four zinc fingers that share 80% sequence identity with the four zinc fingers of Pho (Fig. 1A). Although this is less conservation than between Pho and human YY1, which have 96% sequence identity over the zinc-finger region (Brown et al., 1998), all amino acids involved in making important DNA contacts (Houbaviy et al., 1996) are conserved in Phol. In addition, a short region of the spacer, conserved between Pho and YY1 (Brown et al., 1998), is also conserved in Phol, although to a lesser extent (Fig. 1B). No other regions of conservation between Pho and Phol or between Phol and YY1 were detected.

As the amino acids contacting the DNA are identical in Pho and Phol, we expected Phol to have the same DNA-binding specificity as Pho. Gel shift assays with the Phol zinc-finger domain showed that this protein specifically bound an oligonucleotide containing a Pho-binding site. Binding was not competed by an oligonucleotide containing a mutated Pho-binding site (Fig. 1C,D). A gel shift using full-length Pho is shown for comparison. Pho and Phol can bind to the same DNA sequence with the same apparent binding specificity.

phol mutants are homozygous viable but female sterile

A *Drosophila* strain (EP0559) containing a P element insertion in the untranslated leader region of the *phol* transcription unit was obtained from the *Drosophila* genome project (Fig. 2A). Flies that are homozygous or hemizygous for this P-element

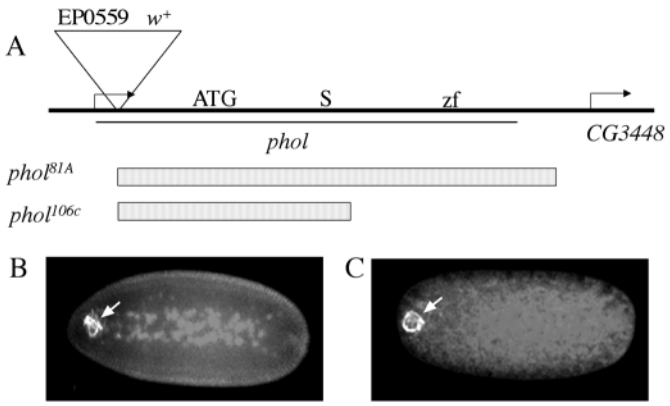

Fig. 2. Characterization of *phol* mutants. (A) The thick line indicates genomic DNA. The thin line indicates the extent of the *phol* transcription unit. The arrows indicate the start and direction of transcription of *phol* and the flanking transcription unit, *CG3348*. The transcription start sites are the first nucleotides of the ESTs from the *Drosophila* genome project. The shaded boxes indicate the extent of DNA deleted in the *phol* mutants. The approximate location of the start of the coding region (ATG), the conserved spacer region (S), and the zinc finger (zf) region are shown. (B,C) Embryos stained with DAPI and for the presence of the sperm tail (Karr, 1991). The embryo in B is from a wild-type mother and the embryo in C is from a *phol* mutant mother. The sperm tail is evident in both embryos (white arrows), but DAPI staining of nuclear DNA is evident only in the wild-type embryo (bright dots in centre of embryo).

insertion are viable and fertile. Two *phol* deletion alleles were generated by imprecise excision of the P-element (see Materials and Methods). In the $phol^{81A}$ null mutation, part of the P-element was deleted along with the entire *phol*-coding region. In $phol^{106C}$, the EP0559 element was completely deleted along with 1389 bp of the *phol* transcription unit, but leaving the *phol* promoter and the zinc-finger region intact. Therefore, it is possible that $phol^{106C}$ encodes a truncated Phol protein. Importantly, the flanking transcription unit, CG3448, is left intact in both alleles. In $phol^{81A}$, the deletion ends 846 bp upstream of the CG3448 mRNA. $phol^{106C}$ and $phol^{81A}$ are both homozygous and hemizygous viable; males are fertile but females are sterile. The female sterility of both mutant alleles is rescued by a *phol* transgene (data not shown). Homozygotes for either *phol* allele look phenotypically normal and the mutants show no obvious homeotic phenotypes. Eggs laid by mothers that are homozygous for either *phol* allele look normal, are fertilized, but do not develop (Fig. 2B,C). Embryos derived from germline clones from heterozygous $phol^{106C}$ and $phol^{81A}$ mothers have the same phenotype showing that *phol* is required in the germ cells (data not shown). The requirement for *phol* in the germline did not allow us to generate embryos that lack Phol protein and we therefore could not examine the role of *phol* in regulation of homeotic genes in embryos.

Pho and Phol act redundantly to silence homeotic gene expression in imaginal disks

pho homozygotes die as pharate adults with weak homeotic transformations (Gehring, 1970), while *phol* homozygotes survive and are phenotypically normal adults. By contrast, *phol, pho* double mutants die as third instar larvae and fail to pupate. Examination of *phol, pho* larvae showed that the brain is smaller than normal, the disks are misshapen and smaller than wild-type disks, and the salivary gland polytene chromosomes were enlarged (data not shown). The larger salivary gland polytene chromosomes may be due to additional rounds of endoreplication in the double mutants. To test whether *phol* functions in PcG repression, we examined *Ubx* and *Abd-B* expression in wing imaginal disks from single and double mutants of *phol* and *pho* (Fig. 3A). As expected, no Ubx or Abd-B expression was observed in wild-type or *phol* mutant wing disks. *pho* mutants showed misexpression of Ubx in a few cells in the wing pouch, but did not misexpress Abd-B (Fig. 3A) (Fritsch et al., 1999). By contrast, *phol, pho* double mutants strongly misexpress Ubx and Abd-B in the wing disk (Fig. 3A). This suggests that Phol and Pho redundantly repress homeotic genes in imaginal disks and can partially substitute for each other. We note that *Ubx* misexpression is confined to the wing pouch in *phol, pho* double mutants; the lack of *Ubx* misexpression in more peripheral areas of the disk possibly reflects downregulation by Abd-B, which is strongly misexpressed in these regions of the disk (Fig. 3A).

We next tested whether removal of *phol* during larval development would also cause derepression of *Ubx* and *Abd-B* by generating clones of *phol* mutant cells in imaginal wing disks of *pho* mutant larvae. In these experiments, *phol* mutant cells were identified by the absence of a GFP marker. Strong misexpression of Ubx and Abd-B was observed in double mutant cells in the wing pouch similar to the misexpression observed in wing disks from the *phol, pho* double mutant larvae (Fig. 3B and data not shown). These observations suggest that either Phol or Pho is required throughout development to keep homeotic genes repressed.

A re-examination of the role of *pho* in embryos

It has recently been suggested that *pho* may not play a role in PRE function in embryos (Poux et al., 2001a). This was surprising to us given previous reports showing misexpression of *engrailed*, *abd-A* and *Abd-B* in *pho* mutant embryos (Moazed and O'Farrell, 1992; Simon et al., 1992). In addition, the severe defects observed in embryos lacking maternal *pho* function suggested a strong requirement for Pho during oogenesis and/or embryogenesis (Breen and Duncan, 1986; Girton and Jeon, 1994). We therefore re-examined the role of *pho* in embryos by testing the requirement for *pho* and Pho binding sites in the regulation of PRE-containing reporter genes.

We did not see any additional mis-expression of *Ubx* or *AdbB* in *phol, pho* double mutant embryos over what was seen in *pho* single mutants. Thus, we have conducted our embryonic experiment in *pho* single mutants. We looked at *lacZ* expression from a *Pbx-Bxd-Ubx-lacZ* (BP01) reporter gene (Müller and Bienz, 1991). This reporter is derepressed in *Pc* mutant embryos (Müller and Bienz, 1991). Similarly, we found that it is derepressed in a *pho* mutant (Fig. 3C). This shows that Pho protein is required for the silencing of this reporter gene in the embryo. Next, we looked at whether mutation of the Pho-binding sites within a PRE disrupts silencing. We used a construct containing PRE_D, a 567 bp fragment from the *Ubx* gene. We have previously shown that mutation of Pho-binding sites in PRE_D inactivated its silencing capability in imaginal disks (Fritsch et al., 1999). Poux et al. (Poux et al., 2001a) reported that mutation of Pho-binding sites did not cause a loss

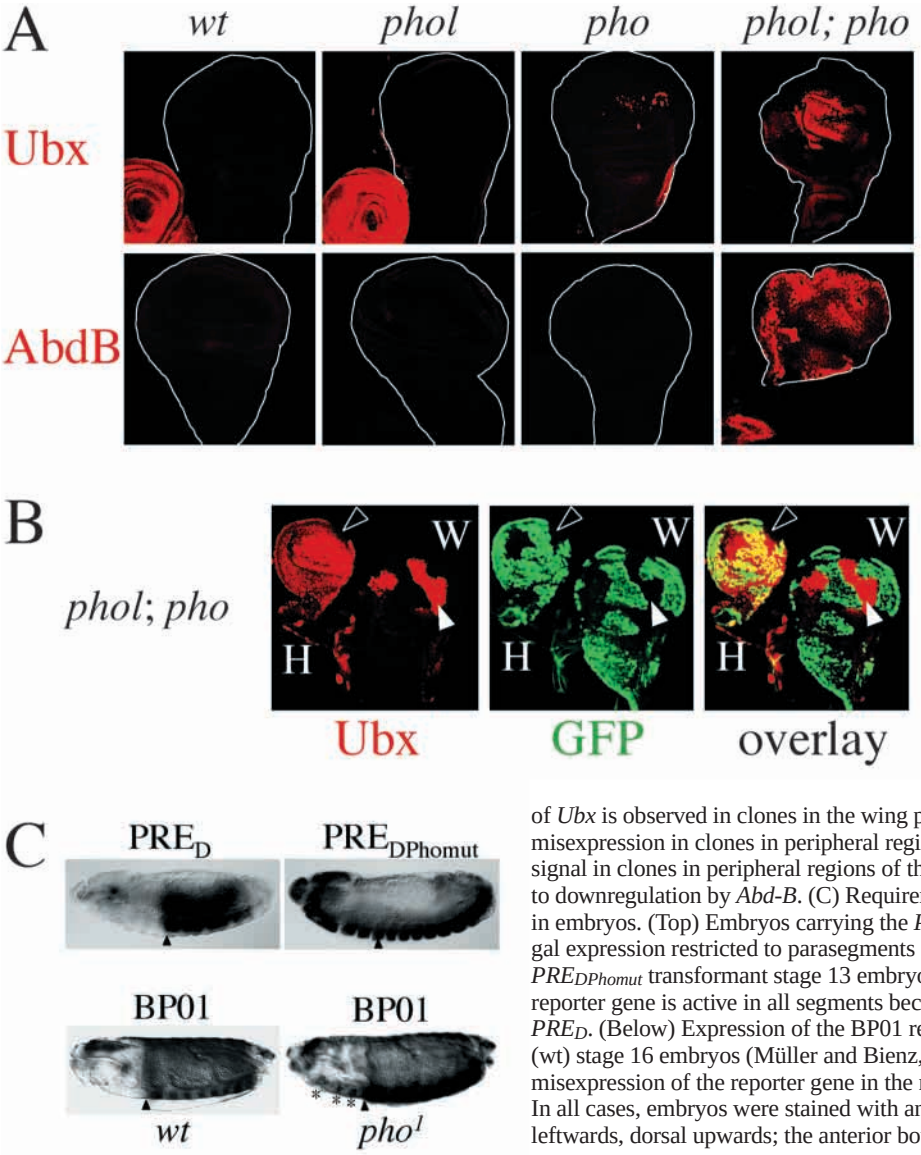

Fig. 3. Essential role for *pho and phol* in the repression of homeotic genes in embryos and imaginal disks. (A) Wing imaginal disks from larvae stained with Ubx or Abd-B antibody (red in both cases). No *Ubx* or *Abd-B* expression is detected in wing imaginal disks of wild-type larvae or in *phol* homozygotes. In *pho* homozygotes, *Ubx* is misexpressed in some cells in the wing pouch; but *Abd-B* is not misexpressed. Strong misexpression of *Ubx* and *Abd-B* is observed in *phol, pho* double homozygous disks; misexpression of *Ubx* is confined to the wing pouch, the lack of misexpression in more peripheral parts of the wing disk is possibly the result of downregulation by Abd-B protein, which is expressed at higher levels in these regions. (B) *phol* function is required throughout development. Wing (W) and haltere (H) imaginal disks with clones of *phol* mutant cells, marked by the lack of GFP signal (green), were induced in *pho* homozygous larvae and disks were analysed 96 hours after clone induction by staining with antibody against Ubx (red). The Minute technique was used in this experiment to generate *M+ phol–/ M+ phol–* cells that carry two copies of a wild-type Minute allele; this gives them a growth advantage relative to their *M+ phol–/ M– phol+* neighbours. Strong misexpression of *Ubx* is observed in clones in the wing pouch (filled arrowhead). The lack of misexpression in clones in peripheral regions of the wing disk and the reduction of *Ubx* signal in clones in peripheral regions of the haltere disk (empty arrowhead) might be due to downregulation by *Abd-B*. (C) Requirement for *pho* and Pho-binding sites for silencing in embryos. (Top) Embryos carrying the PRE_D reporter gene (Fritsch et al., 1999) show β-gal expression restricted to parasegments 6-12 (ps 6-12) in late (stage 13) embryos. In $PRE_{DPhomut}$ transformant stage 13 embryos, repression anterior to ps 6 is lost and the reporter gene is active in all segments because of mutation of the six Pho-binding sites in PRE_D. (Below) Expression of the BP01 reporter gene is restricted to ps 6-12 in wild-type (wt) stage 16 embryos (Müller and Bienz, 1991). *pho* mutant stage 16 embryos show misexpression of the reporter gene in the nervous system (asterisks) and in the epidermis. In all cases, embryos were stained with anti-β-gal antibodies and are oriented anterior leftwards, dorsal upwards; the anterior boundary of ps 6 is indicated by an arrowhead.

of PRE_D silencing in embryos. However, we obtained different results using the same lines. We looked at expression from three wild-type PRE_D lines and five $PRE_{DPhomut}$. All wild-type PRE_D lines gave the expression pattern shown. Two out of five $PRE_{DPhomut}$ lines gave expression similar to that shown in Fig. 3C, including two out of three lines examined by Poux et al. (Poux et al., 2001a). A third $PRE_{DPhomut}$ line also showed unrestricted expression in embryos but the levels were lower compared with the other lines. A fourth line showed no silencing in the embryonic epidermis and in discs, but maintained restricted expression in the embryonic CNS. A fifth line showed restricted expression similar to the wild-type PRE_D control lines. These results show that Pho protein and Pho-binding sites do play a role in repression during embryogenesis.

Binding of PcG proteins to polytene chromosomes in *phol, pho* double mutants

The experiments described above suggest that the DNA-binding proteins Pho and Phol play important and redundant roles in PcG repression. One possible role of these two proteins may be to anchor other PcG proteins to PREs. To test this hypothesis, we analysed binding of five different PcG proteins to polytene chromosomes in *phol, pho* double mutants.

First, we examined the localization of Pho proteins on polytene chromosomes of wild-type larvae. We previously reported binding of Pho to about 35 chromosomal sites (Fritsch et al., 1999). Using a new Pho antiserum, combined with immunofluorescent techniques, we now detect Pho binding to about 100 sites on polytene chromosomes (Fig. 4). Psc colocalizes with Pho at about 65% of these sites (Fig. 4). Psc has also been reported to bind to 65% of the Pc sites (Rastelli et al., 1993).

Next, we looked at the distribution of the PcG proteins Pc, Psc, Polyhomeotic (Ph), Sex combs on midleg (Scm) and Enhancer of zeste (E(z)) on polytene chromosomes. Pc, Ph and Psc are all core components of the PcG protein complex called PRC1 (Shao et al., 1999; Saurin et al., 2001; Francis and Kingston, 2001). Scm has also been reported to co-purify with PRC1 (Shao et al., 1999). Scm and Ph may also be present in

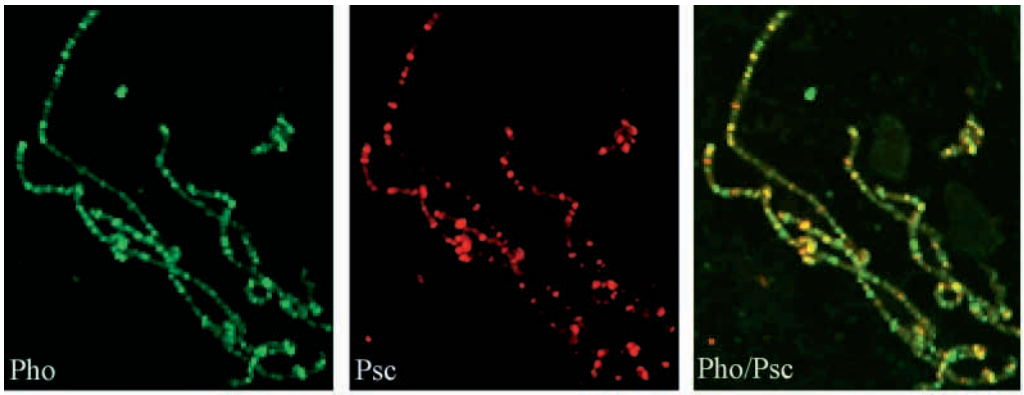

Fig. 4. Pho and Psc colocalize to many sites on polytene chromosomes. This figure shows a partial spread of polytene chromosomes from a wild-type larva labelled with Pho (Cy2 labelled, green) and Psc (Cy3 labelled, red) antibodies. The individual Pho and Psc patterns are shown, together with an overlay of the two patterns in the final panel. Of the Pho sites, roughly 65% of them are also sites for Psc. Almost all of the Psc sites overlap with the Pho sites.

protein complexes other than PRC1 (Roseman et al., 2001; Hodgson et al., 2001). E(z) is a component of the Esc-E(z) complex, which is distinct from PRC1 (Ng et al., 2000; Tie et al., 2001). We focused our analysis on PcG protein binding sites on the X chromosome and on the right arm of chromosome 3, which includes the bithorax and Antennapedia gene complexes (BXC and ANTC).

Fig. 5A shows binding of Pho, Pc, Psc, Ph and Scm to chromosomal sites in the distal region of the X chromosome in wild type and *phol, pho* double mutants. Three sites that are bound by all five PcG proteins in wild-type chromosomes are indicated. As expected, in *phol, pho* double mutants, no Pho protein is detected (Fig. 5A). Binding of Pc, Psc and Scm is lost at polytene subdivision 2D (Fig. 5A, arrow) in *phol, pho* double mutants; binding of these proteins to all other sites on the X chromosome is unaffected (Fig. 5A and data not shown). Binding of Ph is completely unaffected in *phol, pho* double mutants. In particular, the Ph signal at 2D is present, suggesting that Ph can bind at this site even if other PcG proteins are removed. We also find that Pc binding to 2D is not lost in either *pho* or *phol* single mutants (Fig. 5C), suggesting that the presence of either of these two proteins is sufficient for Pc to bind to this site.

We were particularly interested in knowing whether E(z) protein distribution would change in the double mutants because the vertebrate homologues of Pho and Esc interact in in vitro binding experiments (Satijin et al., 2001) and Pho co-immunoprecipates with Esc in early embryos (Poux et al., 2001b). One attractive hypothesis is that Pho might be required for the binding of E(z)/Esc protein complexes to chromatin. However, we did not detect changes in any E(z) chromosomal

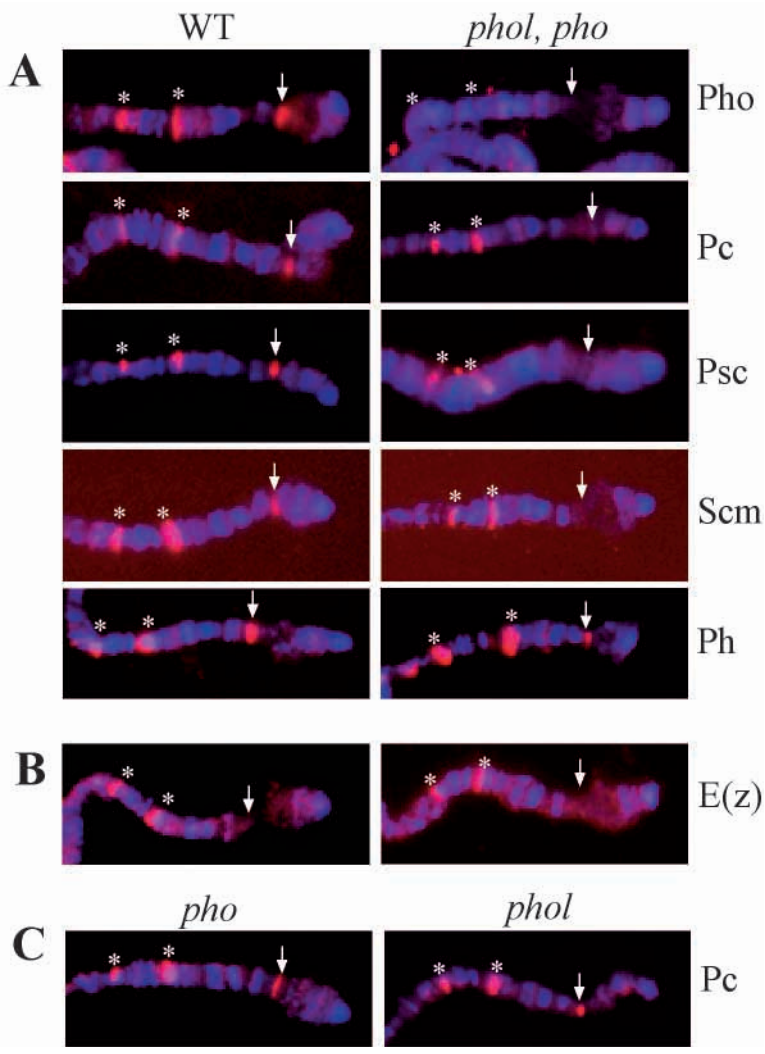

Fig. 5. Analysis of PcG protein binding sites on the X chromosome. PcG-binding sites on polytene chromosomes were detected by immunofluorescent staining using primary antibodies directed against specific members of the PcG of genes. Secondary antibodies were Cy3-conjugated at 1:100 dilution (red). The DNA was labelled with DAPI (blue). An asterisks represents binding positions on the end of the X chromosome that do not change in the single or double mutants (with the exception of Pho, since all Pho bands are lost in the *phol, pho* double mutant). The arrow indicates the position of the 2D subdivision. (A) The binding pattern on chromosomes from wild-type (WT), or *phol, pho* double mutant larvae are shown for Pho, Pc, Psc, Scm and Ph. The Pc, Psc and Scm bands at the 2D position are lost in the *phol, pho* double mutants. (B) In E(z), a signal at 2D was seen only in about 20% of the wild-type chromosomes, and is not seen here. (C) Binding of Pc to the end of the X chromosome in *pho* and *phol* single mutants. The Pc 2D signal is not lost in *pho* or *phol* single mutants.

sites on either the X chromosome (Fig. 5B) or on 3R in *phol, pho* double mutants (data not shown). It was reported that E(z) bound to chromosomal subdivision 2D (Carrington and Jones, 1996); however, we were able to detect E(z) at this site on only about 20% of the wild-type chromosomes. Although we never saw E(z) at 2D on *phol, phol* double mutant chromosomes, we cannot definitely conclude there is a difference between this and wild type.

The patterns of binding of Psc, Ph, Scm and E(z) proteins on chromosome arm 3R were indistinguishable in wild type and *phol, pho* double mutants (data not shown). In particular, these PcG proteins were still bound to regions that include the BXC and ANTC loci in *phol, pho* double mutants. The binding of Pc to the BXC and ANTC, and most other loci was also unaltered in the double mutant, but we found that binding to two specific chromosomal sites was lost (Fig. 6). Interestingly, Psc, Scm and E(z) were not detected at these sites on wild-type chromosomes (data not shown) (Rastelli et al., 1993; Carrington and Jones, 1996), suggesting that Pc binds independently of these proteins at these sites. Ph was present at one of these two sites, but its binding was not altered in *phol, pho* double mutants (data not shown).

Taken together, the immunolocalization data suggest that binding of PcG proteins to most sites is unaltered in the absence of Pho and Phol protein, but that these two proteins are redundantly required for PcG protein binding at a few specific sites. Intriguingly, it appears that all PcG proteins tested here are still associated with the BXC and ANTC loci. Nevertheless, we found that the BXC genes *Ubx* and *Abd-B* were derepressed in *phol, pho* double mutant wing disks. We propose several different explanations for this paradox. First, derepression of homeotic genes and binding of PcG proteins were not assayed in the same tissues. We were not able to detect derepression of *Ubx* in salivary gland cells of *phol, pho* double mutants (data not shown). Second, Pho and Phol may only be required for anchoring PcG proteins at some PREs in the BXC. Different DNA-binding proteins may provide this function at other PREs. This is supported by our finding that binding of PcG proteins is lost at some sites in *phol, pho* double mutants (see Figs 5, 6). Moreover, several different PREs have been identified in the *Ubx* gene (Chan et al., 1994; Christen and Bienz, 1994; Müller, 1995; Chang et al., 1995; Orlando et al., 1998; Hodgson et al., 2001). The resolution of antibody signals on polytene chromosomes is not refined enough to resolve distinct PREs in a single gene and, hence, loss of only a fraction of PcG protein complexes may not be detectable. Finally, Phol and Pho may not be necessary for the anchoring of PcG protein complexes to the DNA, but may confer the actual transcriptional repression mediated by PREs in imaginal disks, while the PcG protein complexes might function in the propagation and memory of the repression. Thus, PcG protein complexes might serve to recruit Phol and Pho or their co-repressors to the DNA.

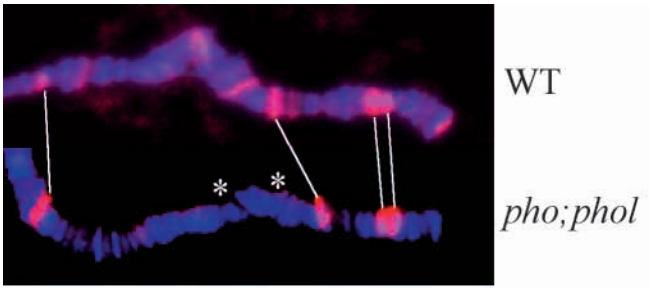

Fig. 6. Analysis of Pc binding sites on 3R. This figure shows the telomere end of 3R. Bands that are present in both wild-type and mutant chromosomes are linked with a white line. Bands that are absent in *phol, pho* double mutants are marked with an asterisk.

Trl is not redundant with *pho* for repression of homeotic genes in imaginal disks

The GAGA factor protein is encoded by the *Trithorax-like* (*Trl*) gene. A hypomorphic *Trl* allele was originally isolated due to mutant phenotypes that suggested a requirement for activation of homeotic gene expression (Farkas et al., 1994). The proposal that *Trl* functions in PcG repression was based on the observations that Trl protein bound to PRE sequences, that it co-immunoprecipitated with Pc, and that mutation of Trl-binding sites caused a loss of mini-*white* silencing and PRE function in reporter genes (Horard et al., 2000; Mishra et al., 2001; Busturia et al., 2001; Americo et al., 2002; Poux et al., 2002). These conflicting data prompted us to analyse the role of *Trl* in homeotic gene regulation by generating clones of *Trl* mutant cells in imaginal disks. In these experiments, we used Trl^{R85}, a null allele (Farkas et al., 1994), and the mutant cells were again marked by the absence of a GFP marker protein.

We first analysed *Trl* mutant clones in the wing disk for misexpression of *Ubx* and *Abd-B* and found no evidence for such misexpression (Fig. 7A and data not shown). As PREs often contain Pho- and Trl-binding sites in close proximity, and Busturia et al. (Busturia et al., 2001) reported a weak genetic interaction between *pho* and *Trl* heterozygous mutants, we tested whether removal of *Trl* in *pho* mutant wing disks would exacerbate the misexpression of *Ubx* observed in *pho* mutants. This was not the case. *pho* mutant wing disks with clones of *Trl* homozygous cells showed no additional misexpression of *Ubx* compared with *pho* single mutants (compare Fig. 7A with Fig. 3A). Thus, we find no evidence for a genetic interaction between *Trl* and *pho*.

We also analysed the effects of removing *Trl* on the silencing capabilities of two different PRE-containing *Ubx-LacZ* reporter transgenes; $PRE_{1.6}$ contains a PRE from the *Ubx* gene (Fritsch et al., 1999) and *MCP725* contains a PRE from the *Abd-B* gene (Busturia et al., 1997). In wild-type flies, expression of both transgenes was confined to the posterior compartments of the haltere and third leg disks, and both transgenes were misexpressed in a variety of PcG mutants (Busturia et al., 1997; Fritsch et al., 1999) (M. Bakala, Diploma thesis, University of Tübingen, 2001). By contrast, we observed no misexpression of either transgene in *Trl* mutant clones in wing imaginal disks (Fig. 7B).

We also tested whether mutation of Trl protein binding sites (i.e. GAGAG sequences) in a PRE from the *Ubx* gene would compromise its silencing capability. For this experiment we used a previously described reporter gene, PRE_D, that is stably silenced in the wing imaginal disk due to the presence of the 567 bp long PRE core fragment (Fig. 7C) (Fritsch et al., 1999). Previous studies showed that mutation of Pho protein-binding sites within PRE_D abolished repression of this reporter transgene in wing imaginal disks (Fig. 7C) (Fritsch et al.,

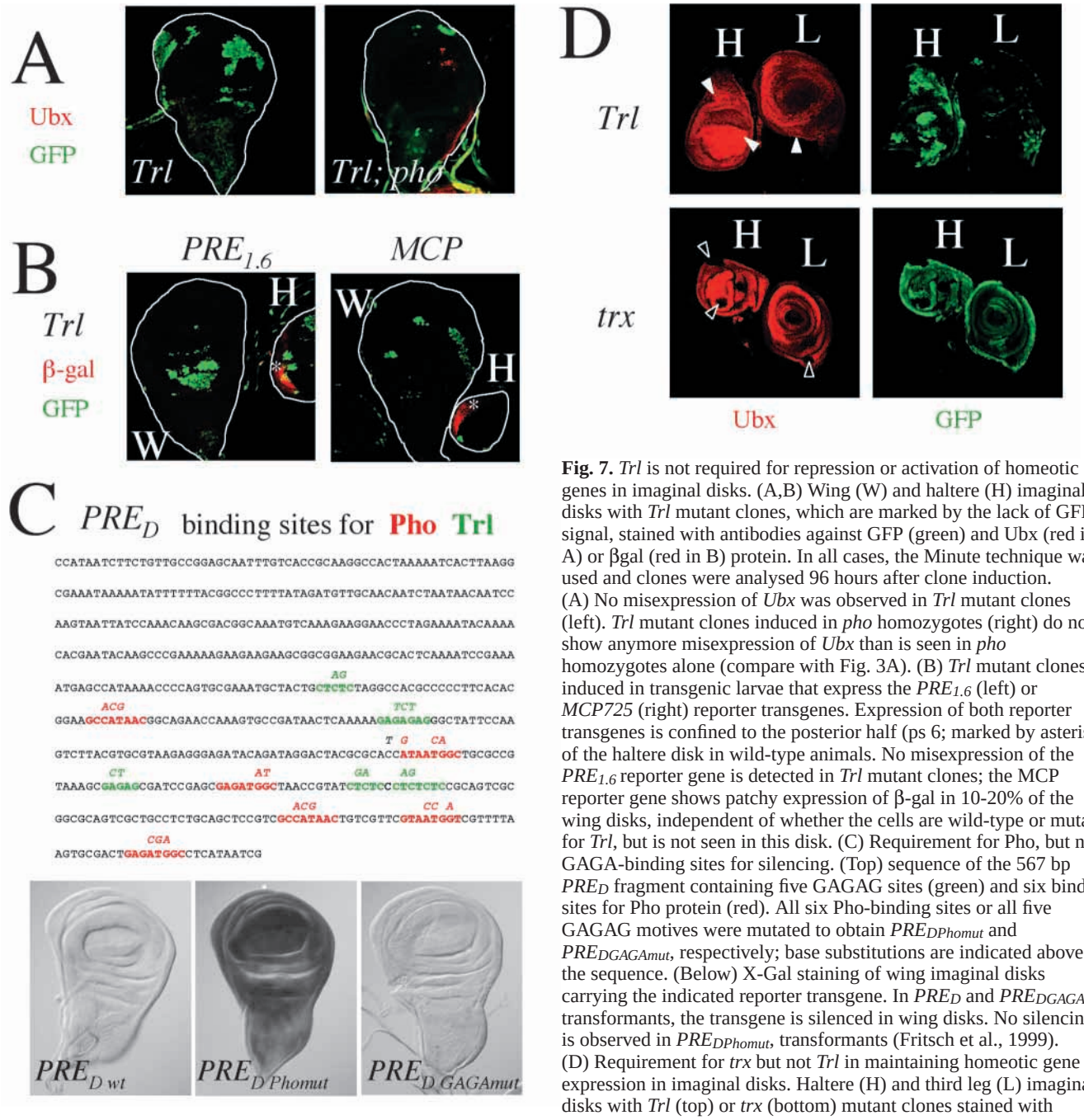

Fig. 7. *Trl* is not required for repression or activation of homeotic genes in imaginal disks. (A,B) Wing (W) and haltere (H) imaginal disks with *Trl* mutant clones, which are marked by the lack of GFP signal, stained with antibodies against GFP (green) and Ubx (red in A) or βgal (red in B) protein. In all cases, the Minute technique was used and clones were analysed 96 hours after clone induction. (A) No misexpression of *Ubx* was observed in *Trl* mutant clones (left). *Trl* mutant clones induced in *pho* homozygotes (right) do not show anymore misexpression of *Ubx* than is seen in *pho* homozygotes alone (compare with Fig. 3A). (B) *Trl* mutant clones induced in transgenic larvae that express the $PRE_{1.6}$ (left) or *MCP725* (right) reporter transgenes. Expression of both reporter transgenes is confined to the posterior half (ps 6; marked by asterisk) of the haltere disk in wild-type animals. No misexpression of the $PRE_{1.6}$ reporter gene is detected in *Trl* mutant clones; the MCP reporter gene shows patchy expression of β-gal in 10-20% of the wing disks, independent of whether the cells are wild-type or mutant for *Trl*, but is not seen in this disk. (C) Requirement for Pho, but not GAGA-binding sites for silencing. (Top) sequence of the 567 bp PRE_D fragment containing five GAGAG sites (green) and six binding sites for Pho protein (red). All six Pho-binding sites or all five GAGAG motives were mutated to obtain $PRE_{DPhomut}$ and $PRE_{DGAGAmut}$, respectively; base substitutions are indicated above the sequence. (Below) X-Gal staining of wing imaginal disks carrying the indicated reporter transgene. In PRE_D and $PRE_{DGAGAmut}$ transformants, the transgene is silenced in wing disks. No silencing is observed in $PRE_{DPhomut}$, transformants (Fritsch et al., 1999). (D) Requirement for *trx* but not *Trl* in maintaining homeotic gene expression in imaginal disks. Haltere (H) and third leg (L) imaginal disks with *Trl* (top) or *trx* (bottom) mutant clones stained with antibodies against GFP (green) and Ubx (red). In both cases mutant clones are marked by the lack of GFP signal and clones were analysed 96 hours after clone induction, the Minute technique was only used in the case of *Trl*. *Ubx* expression is unaffected in *Trl* mutant clones (white arrowheads), whereas *trx* mutant clones show a complete loss of Ubx signal (empty arrowheads).

1999). By contrast, mutation of all five GAGAG motifs in PRE_D caused no misexpression of this reporter transgene (Fig. 7C). Sixteen lines were obtained, five produced expression caused by positional effects and could not be analysed. The other eleven all showed silencing in the wing disk similar to that shown in Fig. 7C.

Finally, we tested the requirement for *Trl* in maintaining expression of *Ubx* and *Abd-B* in their normal expression domains. Intriguingly, we observed no obvious reduction of *Ubx* or *Abd-B* expression in *Trl* mutant clones in the haltere and third leg disk (Ubx) or in the genital disk (Abd-B) (Fig. 7D and data not shown). By contrast, clones of *trithorax* (*trx*) mutant cells showed a dramatic reduction in Ubx protein levels (Fig. 7D).

These results fail to support a role for *Trl* in PcG repression in imaginal disks. However, we cannot exclude the possibility that *Trl* is playing a role in the establishment of PcG repression in the embryo. The requirement for *Trl* function in the germline and the early embryo (Liaw et al., 1995; Bhat et al., 1996) does not allow an analysis of embryos lacking Trl protein.

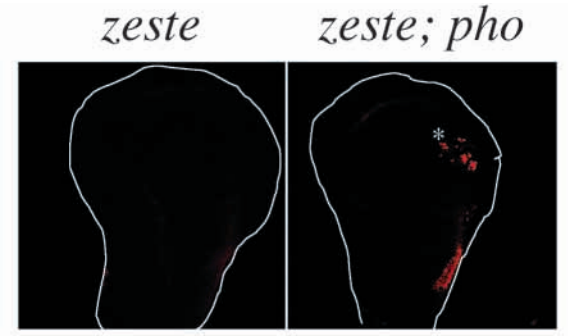

Fig. 8. *zeste* is not required for repression of homeotic genes in imaginal disks. Wing imaginal disks from *z* homozygotes (left) or *z, pho* double homozygotes (right) stained with antibody against Ubx protein. No misexpression is detected in *z* mutant disks and *z, pho* double mutants do not show more misexpression (asterisk) than do *pho* homozygotes alone (compare with Fig. 3A).

zeste is not redundant with Pho for repression of homeotic genes in imaginal disks

Another protein that has been proposed to function in PcG repression is Zeste (Hur et al., 2002). *zeste* (*z*) null mutants are viable and fertile and show no obvious homeotic phenotypes (Goldberg et al., 1989). However, Zeste co-purifies with the PcG protein complex PRC1 (Saurin et al., 2001) and Zeste protein binding sites have been implicated in PcG function of an embryonic reporter gene (Hur et al., 2002). To test whether *z* might interact genetically with *pho* in repression of homeotic genes, we examined the expression of Ubx in z^{v77h} and in z^{v77h}, pho^{1} double mutant wing disks. Both z^{v77h} and pho^{1} are presumed null mutants (Goldberg et al., 1989; Brown et al., 1998). *z* mutant wing disks showed no misexpression of Ubx (Fig. 8) and *z, pho* double mutants showed no more misexpression of Ubx than that seen in *pho* mutant wing disks (compare Fig. 3A with Fig. 8).

Concluding remarks

Our results show a strong requirement for the DNA-binding proteins Pho and Pho-like in homeotic gene silencing in imaginal disks. In fact, the strong misexpression of homeotic genes observed in *phol, pho* double mutant imaginal cells is comparable with that seen in imaginal disk clones mutant for *Pc*, *Scm*, *Sce* or *Pcl* (Beuchle et al., 2001). The loss of PcG protein binding at only a small number of sites in *phol, pho* polytene chromosomes is consistent with the idea that Phol and Pho are required to recruit PcG protein complexes at only a subset of PREs. Alternatively, Phol and Pho may be required for transcriptional repression mediated by PREs, but not for anchoring of PcG protein complexes.

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