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Original Article

The *Drosophila pleiohomeotic* mutation enhances the *Polycomblike* and *Polycomb* mutant phenotypes during embryogenesis and in the adult

SEUNG-HAE KWON[1], SANG HEE KIM[2], HAE-MOON CHUNG[3], JACK R. GIRTON[4] and SANG-HAK JEON*[,5]

[1]Department of Biological Sciences and [2]Department of Chemistry, Konkuk University, Seoul, Korea, [3]School of Biological Sciences, Seoul National University, Seoul, Korea, [4]Department of Zoology and Genetics, Iowa State University, Ames, USA and [5]Department of Biology and Education, Seoul National University, Seoul, Korea

ABSTRACT In *Drosophila*, the spatially restricted expression of the homeotic genes is controlled by Polycomb group (PcG) repression. PcG proteins appear to form different complexes to repress this gene expression. Although the *pleiohomeotic* gene *(pho)* shares mutational phenotypes with other PcG mutations, which demonstrates that PHO binds directly with a Polycomb (Pc)-containing complex, the genetic interactions of *pho* with other PcG genes have not been examined in detail. Here we investigated whether *pho* interacts with *Polycomblike* (*Pcl*) and *Polycomb* (*Pc*) during embryonic and adult development using developmental and genetic approaches. *Pcl* and *Pc* strongly enhanced *pho* phenotypes in the legs and tergite of the adult fly. Embryonic cuticle transformation was also greatly enhanced in *Pcl*; *pho* or *Pc; pho* double mutant embryos. The double mutant phenotypes were more severely affected by the *pho* maternal effect mutation than in zygotic mutant background, suggesting dosage-dependent processes. Taken together, these results provide genetic evidence of an interaction between PHO with other Polycomb group proteins at the embryonic and adult stages, and of the functioning of PHO as a component of the PcG complex.

KEY WORDS: *pleiohomeotic, Polycomb, Polycomblike, Drosophila, homeotic genes*

Introduction

The homeotic genes are transcription factors that are involved in the specification of body segments, through their expression along the anterior-posterior (AP) axis of the embryo (MaGinnis and Krumlauf, 1992). These genes are regarded as mediators of positional information signals. The expression patterns of the homeotic genes in *Drosophila* is initiated by segmentation genes during early embryonic development, and their later expressions are regulated by two groups of genes, the trithorax group (trxG) and the Polycomb group (PcG) (Simon, 1995; Pirrotta, 1998). TrxG/PcG factors are part of a conserved cellular memory system that maintains the active or inactive state of many developmental regulators. To date, 14 PcG genes, which share many features, have been identified through genetic or biochemical screens based on the derepression of the homeotic selector genes, and it has been suggested that up to 40 more PcG members may exist (Jurgens, 1985).

The PcG proteins are involved in the maintenance of the correct expression patterns of homeotic genes. In PcG mutants, homeotic genes are misexpressed in body segments where they are normally repressed, and anterior larval cuticle may be transformed to posterior cuticle (Lewis, 1978; Jurgens, 1985). Although the homeotic genes are ectopically expressed in all PcG mutants, these domains of derepression are not similar (Mckeon and Brock, 1991; Simon *et al.*, 1992; Kennison, 1995). Most of the PcG genes function maternally as well as zygotically (Breen and Duncan, 1986). The maternal contribution is responsible for the weak or normal phenotypes of some PcG mutant embryos (Glicksman and Brower, 1990). Of the PcG loci, embryos lacking *Pc* or *extra sex combs* (*esc*) activity, display severe homeotic phenotypes (Lawrence *et al.*, 1983; Jurgens, 1985), while embryos lacking *Pcl* activity show a relatively weak posteriorly directed segmental transformation, though thoracic segments are unaffected (Breen and Duncan, 1986). Embryos lacking both

Abbreviations used in this paper: Abd-B, Abdominal-B; Antp, Antennapedia; esc, extra sex combs; Pc, Plycomb; PcG, Polycomb group; Pcl, Polycomblike; pho, pleiohomeotic; PRE, Polycomb response element; Scr, Sexcomb reduced; TrxG, trithorax group.

*Address correspondence to: Dr. Sang-Hak Jeon. Department of Biology Education, Seoul National University, San 56-1 Shinrim-Dong, Kwanak-ku, Seoul 151-742, Korea. Fax: 82-2-886-2117. email: jeonsh@snu.ac.kr

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maternal and zygotic *pho* activity also show weak cuticular transformations (Girton and Jeon, 1994).

PcG genes have been found in many vertebrates and invertebrates since the PcG genes were originally identified in *Drosophila*. One important observation based on their widespread existence is that PcG proteins form large chromatin-associated multimeric protein complexes that silence the expression of target genes. Several pieces of evidence show that the PcG proteins form a complex. First, when more than one PcG gene is mutated, the homeotic transformations are strongly enhanced (Jurgens, 1985), which suggests that the PcG complexes function in a dosage-dependent manner. Second, the different PcG proteins bind in the same bands on polytene chromosomes in salivary gland cells (Zink and Paro, 1989; Decamillis *et al.,* 1991). Third, PcG proteins are co-immunoprecipitated and co-fractionated in lysates from *Drosophila* embryos (Franke *et al.,* 1992). There are mammalian PcG homologs, found in similar complexes to those of flies (Brunk *et al.,* 1991; Alkema *et al*., 1997). Human Bmil and HPh1 proteins co-immunoprecipitated and were found to be co-localized in large nuclear domains of mammalian cell lines. There are two distinct types of PcG complexes and probably more PcG complexes. Two types of PcG complexes identified in flies and in vertebrates are the PRC1 (containing PC) and the ESC-EZ complexes (Shao *et al*., 1999; Ng *et al*., 2000). The existence of multimeric PcG protein complexes may explain the different biological functions of the PcG proteins (Satijn and Otte, 1999).

Of the 14 molecularly characterized PcG proteins, only PHO has been found to have a DNA binding domain (Brown *et al*., 1998). Recently it was been shown that PHO directly binds to a Polycomb (Pc)-containing complex as well as to the Brahma (BRM) chromatin-remodeling complex (Mohd-Sarip *et al*., 2002). And, the vertebrate PHO homolog, YY1, rescued *pho* mutant phenotypes and strongly interacted with other PcG genes in *Drosophila* (Atchison *et al*., 2003). Taken together, these results suggest that PHO could be the initial recruiter of the PcG complex and so the means for recruiting PcG complexes.

However, a number of labs have published evidence showing that PHO is not sufficient to recruit other PcG proteins (Girton and Jeon, 1994; Brown *et al*., 1998; Fritsch *et al*., 1999; Shimell *et al*., 2000; Poux *et al*., 2001a). Furthermore, its interaction with other PcG genes has hardly been investigated. In this study, we show that *pho* interacts with other PcG genes during embryonic and adult development. The double mutant phenotypes were more severe in *pho* maternal effect mutant than in zygotic mutant background, indicating that *pho* works in a dosage-dependent manner.

Results

Interaction of pho *with* Pcl and Pc *during embryonic development*

Homeotic transformation of the embryonic denticle belts, is a commonly observed phenotype in PcG mutants. Three thoracic and eight abdominal denticle belts are distinguished by their unique features (Fig. 1A), and these denticle patterns were used to elucidate the interaction between *pho* and *Pcl* or *Pc* genes during embryogenesis.

pho homozygous mutant embryos from pho^{cv} heterozygous flies are normal. *Pcl* / *Pcl* mutant embryos are embryonically lethal, showing partial posteriorly-directed transformations of the abdominal segments (Fig. 1B). The head and thorax are unaffected. The phenotypes of embryos obtained from crossing *Pcl* /+; pho^{cv} / + heterozygous males and females were examined. It was assumed that the strongest phenotypes in the collection of embryos were the double mutants. *Pcl* and *pho* double mutations greatly enhanced the transformation of the abdominal denticle belts to the eighth belt (Fig. 1C). Thoracic segments showed relatively weak transformations as compared with the abdominal segments and the head appeared only marginally affected.

Homozygotes for the *Polycomb* mutation die as late embryos showing homeotic transformation of head, thoracic and abdominal segments. As shown in Fig. 1D, Pc^{1} mutant embryos showed partial transformation of thorax to the eighth abdominal segment, while the Pc^{3} mutant embryos showed complete transformation of all segments to the eighth (Figure not shown). Therefore, Pc^{1} was selected to examine the interaction with *pho*. Embryos that are heterozygous for Pc^{1} and homozygous for pho^{cv} produced by heterozygous mothers died as pupa. It is assumed that the strongest phenotypes came from the homozygous double mu-

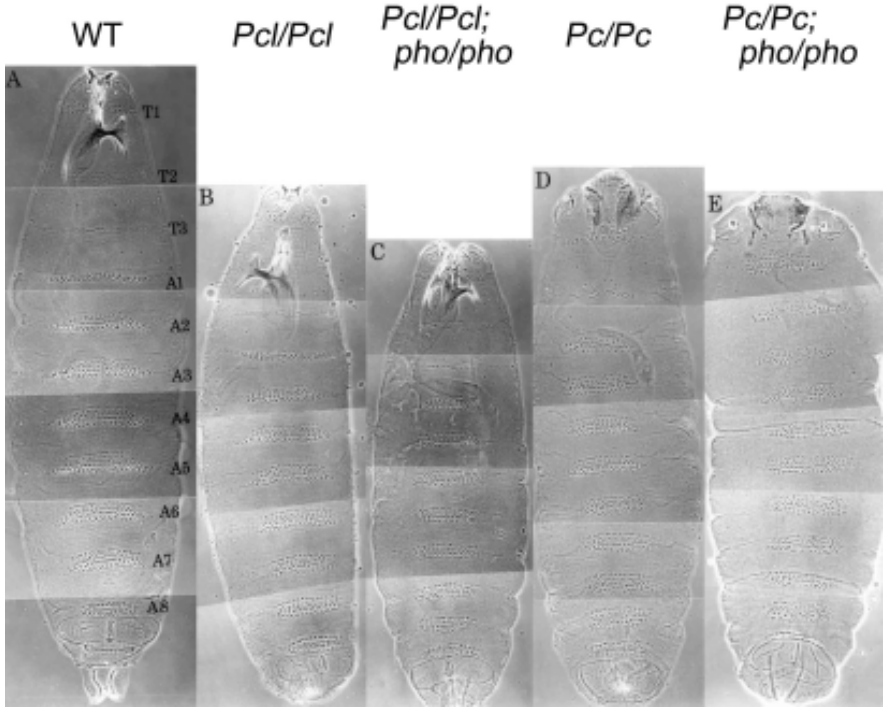

Fig. 1. Light micrographs showing embryonic cuticle aspects. **(A)** *Wild type. The wild type larvae have three thoracic (T1-T3) and eight abdominal denticle belts (A1-A8).* pho^{cv} / pho^{cv} *embryos from* pho^{cv} *heterozygotes are normal.* **(B)** Pcl/Pcl. *Abdominal denticle belts are weakly transformed to those of the more posterior segments.* **(C)** Pcl/Pcl; pho^{cv} / pho^{cv} *(Z). Double mutant embryos have largely hooked denticles in the thorax, indicating the transformation of the thorax to the abdominal segments. Abdominal segments are strongly transformed to the eighth segment.* **(D)** Pc/Pc *mutants showing that the head is disrupted. Thoracic denticle belts are partially transformed, while all ventral denticle belts are transformed to the eigth denticle belt.* **(E)** Pc/Pc; pho^{cv} / pho^{cv}. *The head is severely disrupted and the thoracic belts resemble the eighth abdominal belt.*

tants. Embryos doubly mutant for *Pc*^{1} and *pho*^{cv} produced from heterozygotes for both genes had all thoracic and abdominal denticles nearly completely transformed to the eighth abdominal segment (Fig. 1E). This result suggests that the zygotic *pho* functions during embryonic development.

pho homozygous mutant embryos from *pho*^{cv} heterozygote flies are normal (Fig. 2A). However, *pho/pho* embryos from *pho*^{cv} homozygous flies show maternal effects, which cause transformations of the thoracic segments to the first abdominal segment, and the sixth and seventh segments to the eighth abdominal segment (Fig. 2B). *pho* maternal effect embryos have a severely affected head. This result indicates that the normal phenotype of *pho* zygotic mutant embryo is due to maternally produced functional *pho* products. Although most of the *Pcl*/+; *pho*^{cv}/ *pho*^{cv} flies were pharate adult-lethal, a few eclosed to adults. *Pcl*/ *Pcl*; *pho*^{cv} / *pho*^{cv} produced from *Pcl*/+; *pho*^{cv}/ *pho*^{cv} females were embryonically lethal, showing severe head defects and the transformation of all thoracic segments to the eighth abdominal segment (Fig. 2E). The head was almost disrupted and segment defects increased in the double mutant (Fig. 2E, arrow).

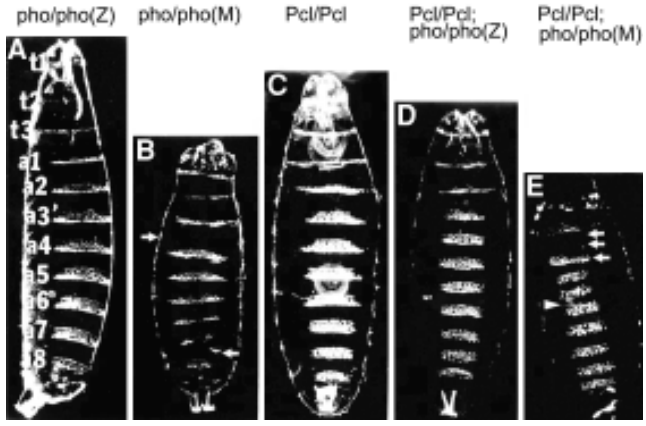

Fig. 2. Darkfield micrographs showing embryonic cuticle aspects. **(A)** pho *zygotic mutant embryos have a normal embryonic morphology.* **(B)** phocv *maternal effect embryo showing partial homeotic transformation. Head involution is abnormal. Thoracic segments are transformed to the first abdominal segment. A1 to A5 segments are normal, while A6 and A7 segments are transformed to the A8 segment. Segment defects can be observed (arrow).* **(C)** Pcl *zygotic mutant embryos display the transformation of posterior abdominal segments A6 and A7 to the A8 segment.* **(D)** *Zygotic mutant embryo for both* Pcl *and* pho *shows strong transformation of all abdominal segments to A8, but weak transformation of thoracic segments. Head involution is partially abnormal.* **(E)** Pcl *mutation in the background of* pho *maternal effect mutation causes the transformation of all ventral denticle belts to the A8 segment. Segment defects were often observed (arrow). Extradenticles outside the normal denticle belt boundary can be observed (arrow head). 'Z' indicates the zygotic background of the* pho *mutation, and 'M' the maternal background* pho *mutation.*

Effects of Pcl *and* pho *mutations on the expression of homeotic genes*

Ultrabithorax (*Ubx*) was first expressed at the germ band extended stage in the parasegment (PS) 5 to PS12 (Fig. 3A). PS6 showed the highest level of staining, which subsequently decreased posteriorly from PS7 to PS12 (White and Wilcox, 1984; Duncan, 1987; Bienz and Tremml, 1998). *pho* zygotic mutations did not affect the expression of *Ubx*, but *pho* maternal effect mutations caused the ectopic expression of *Ubx* in the CNS and in the epidermis (Fig. 3B). *pho* mutant embryos showed ectopic *Ubx* expression in PS2-4, which was relatively weak compared to other PcG mutants (Mckeon and Brock, 1991). *Pcl* mutant embryos showed ectopic *Ubx* expression in the brain and PS1-4, and PS6 was not distinguishable from the other parasegments (Fig. 3C). This result shows that although *Ubx* is ectopically expressed in the brain and thorax, it does not affect the development of the head and thorax (Fig. 1B).

Pcl; pho double mutant embryos showed ectopic expression patterns, similar to those caused by a single mutation of *Pcl* (Fig. 3D). Accordingly, *pho* mutation appears to contribute little to the ectopic expression of *Ubx* in the *Pcl; pho* double mutation.

This pattern was also observed when *Abdominal-B* (*Abd-B*) expression was examined in *Pcl, pho* and the *Pcl; pho* double mutation. The expression of the *Abd-B* gene has been investigated by Celinker *et al.* (1989), and is expressed from PS10 through PS15 in wild-type embryos (Fig. 4A). In *Pcl* mutant embryos, *Abd-B* was ectopically expressed from the brain to PS9 in the CNS (Fig. 4B). Although sparse mis-expression of

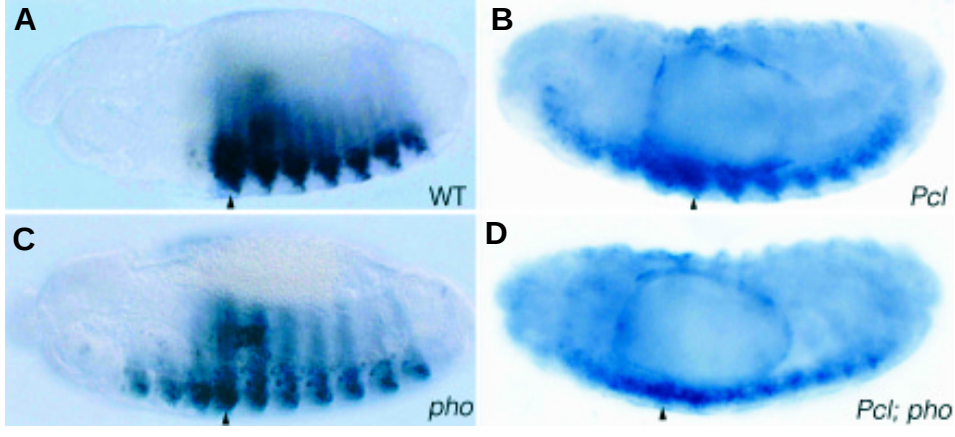

Fig. 3. Expression of the *Ubx* gene in wild type and PcG mutant embryos. **(A)** *Wild type.* Ubx *is expressed in the parasegments PS5 to PS12. PS6 shows the highest level of staining, and then the intensity decreases posteriorly from PS7 to PS12.* **(B)** phocv / phocv *maternal effect mutant embryo.* pho *zygotic mutant embryos show normal expression of* Ubx, *but* pho *maternal effect mutation causes a weak misexpression at PS3-PS4.* **(C)** Pcl/Pcl *mutant embryo. Embryos have ectopic* Ubx *expression in the brain and PS1-4, but PS6 is indistinguishable from the other parasegments.* **(D)** Pcl/Pcl; phocv / phocv *double mutant embryo. In this mutant,* pho/pho *is a zygotic mutation. As the* Pcl/Pcl *mutation alone causes the strong ectopic expression of* Ubx, pho *zygotic mutation may contribute little to the misexpression of* Ubx *in the double mutation.*

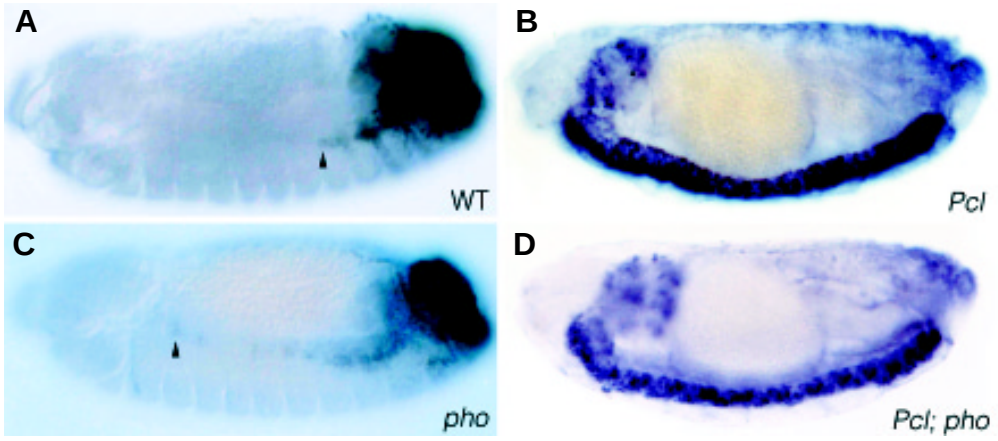

Fig. 4. Expression of the *Abd-B* gene in wild type and PcG mutant embryos. **(A)** *Wild type.* Abd-B *is expressed from PS10 through PS15 in wild-type embryos.* **(B)** Pcl/Pcl *mutant embryo.* Abd-B *is ectopically expressed from brain to PS9 in the CNS.* **(C)** pho^{cv} /pho^{cv} *mutant embryo.* Abd-B *is very weakly misexpressed in the CNS of PS7 to PS9, and in all the visceral mesoderm* **(D)** Pcl/Pcl*;* phocv /phocv *double mutant embryo.* Abd-B *has an expression pattern almost similar to that by a single mutation of* Pcl *in the CNS, but with a stronger expression in the visceral mesoderm.*

Abd-B was observed in *pho^{b}* null mutant embryos, *Abd-B* was normally expressed in most *pho^{cv}* zygotic mutant embryos. However, in some *pho* maternal effect mutant embryos, *Abd-B* was weakly misexpressed, with a few spots in the epidermis and in the CNS of PS7 to PS9, and throughout the visceral mesoderm (Fig. 4C). In *Pcl; pho* double mutant embryos, *Abd-B* was found to have an expression pattern similar to that caused by a single mutation of *Pc*/in the CNS, but with stronger expression in the visceral mesoderm.

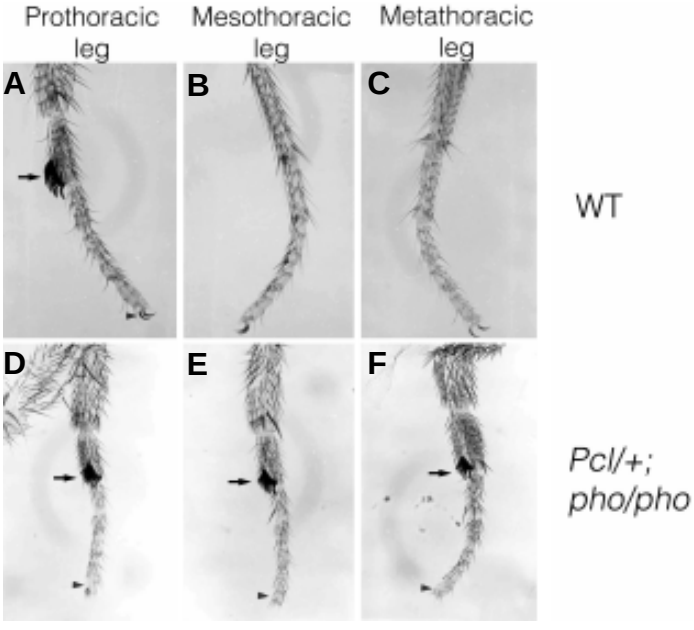

Fig. 5. Leg transformation of adult males. **(A,B,C)** *Wild type male legs. Wild type males have sexcombs on the prothoracic legs and each leg has 5 tarsal segments.* **(D,E,F)** Pcl/*+;* pho^{cv} /pho^{cv} *male legs. The mesothoracic and metathoracic legs have almost the same number of sexcomb bristles as the prothoracic legs. All three legs showed the transformation of claw to tarsal segment.*

Interaction of pho *with* Pcl or Pc *during adult development*

pho alleles produce a variety of adult homeotic transformations. In wild types, each leg has 5 tarsal segments and claws, and the first legs of males have sexcombs with an average of 10 bristle teeth (Fig. 5 A,B,C). *pho^{b}*/*pho^{b}* flies invariably die as pharate adults, and show a few sexcomb bristles on the second and third legs (Girton and Jeon, 1994). Claws were partially transformed on to the 6th tarsal segments. However, *pho^{cv}*/*pho^{cv}* males remain viable, and have normal legs and a few dark spots in the fourth tergum, which are much weaker than those shown in the *pho^{b}*/*pho^{b}* null mutant males. The claws of *pho^{cv}*/*pho^{cv}* are normal. *Pcl*/*Pcl* homozygotes are embryonically-lethal and *Pcl*/+ flies don't have extra sexcomb bristles on the second and third legs, but out-crossed males occasionally show a few sexcomb bristles on the second legs. *pho^{cv}* was crossed with *Pcl* to obtain viable *Pcl*/+; *pho^{cv}*/*pho^{cv}* flies. Although most *Pcl*/+; *pho^{cv}*/ *pho^{cv}* genotypes were lethal, a few eclosed. Pharate and eclosed male flies were examined. The second and third legs of *Pcl* /+; *pho^{cv}*/ *pho^{cv}* males showed sexcombs with almost same number of bristles as the first legs, and all claws were transformed to resemble those of the 6th tarsal segments, indicating a synergistic interaction between *Pcl* and *pho* (Fig. 5 D,E,F). Embryos heterozygous for *Pc^{1}* and homozygous for *pho^{cv}* produced by heterozygous mothers died as lethal pupa, and showed enhanced homeotic transformations of adult structures, which were more extreme transformations than observed for either alleles of the single mutant individuals. The second and third legs had extra sex combs and an average of 5.3 and 3.7 teeth, respectively.

In wild-type flies, the dorsal side of the male abdomen, tergite, has a dark color on the fifth and sixth segments (Fig. 6A). This feature was used to investigate altered tergite identity. The seventh and eighth segments become the genital system. In *Pcl*/+ (Duncan, 1982) or *pho*/*pho* adult males (Girton and Jeon, 1994), the cuticle of the fourth tergite showed small dark patches, indicating that the fourth tergum was transformed to the fifth/sixth tergite (Fig. 6B). In *Pcl* /+; *pho^{cv}*/ *pho^{cv}* double mutant males, the A1 segment has large bristles, which are characteristic of A2, and the A2 to A4 tergite had the dark patch of A5/A6, indicating that the first to fourth segments had been transformed to the fifth/sixth segments. The flies also showed the partial or complete absence of the A4, A5 and A6 tergites, indicating transformation of the A7/A8 segments (Fig. 6C). Both legs and tergite phenotypes in the *Pcl; pho* double mutants clearly showed that *pho* synergistically interacts with *Pcl*. Males heterozygous for both *Pc^{1}* and *pho^{cv}* also showed a transformation of the abdominal segment A3 to A5/A6, the claws of which were transformed to tarsal segments.

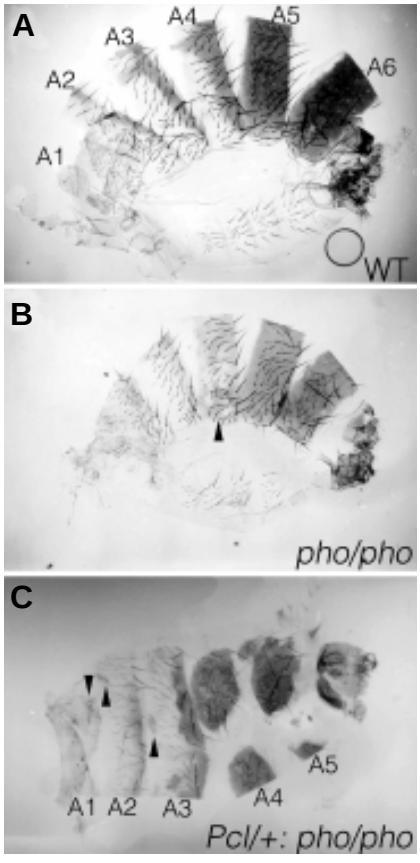

Fig. 6. Tergite transformation of adult males. **(A)** *Wild type. The dorsal side of the tergite has a dark color on the fifth and sixth segments.* **(B)** pho^{cv}/pho^{cv}. *The fourth tergite shows small dark patches.* **(C)** Pcl/+; pho^{cv}/pho^{cv}. *The A1 segment has large bristles, which are characteristic of A2, and the A2 to A4 tergites have the dark patch of A5/A6. The flies also show the partial or complete omission of the A4, At and A6 tergites.*

Discussion

In *Drosophila*, PcG proteins function as high molecular weight complexes that bind to the chromatin of specific *cis*-regulatory sequences called Polycomb response elements (PREs) (Pirrotta, 1997; Satijn and Otte, 1999). There are at least two distinct multimeric complexes that each contain different PcG proteins. One complex, PRC1, consists of the Pc, Psc, Ph, and Scm proteins (Shao *et al*., 1999), and is associated with the chromatin of PREs (Strutt and Paro, 1997). A second complex contains the Esc and the E(z) proteins (Ng *et al.,* 2000). So far, it has not been determined what initiates the complexes and what hierarchy is involved in forming such complexes. A single PcG protein, PHO, has been shown to bind to DNA specifically (Brown *et al*., 1998), and therefore, is considered as an initiator of the nucleation of PcG complexes on DNA. PHO binds to many PRE regulatory sites and mutations in PHO binding sites disrupt PcG silencing (Girton and Jeon, 1994; Fritsch *et al*., 1999). PHO can physically bind to PC protein to generate a PcG ternary structure on DNA (Mohd-Sarip *et al*., 2002).

Although mutations in Pcl and Sce enhanced mutations in pho in adults (Camphell *et al.,* 1995), there is no genetic evidence of the interaction between *pho* and other PcG genes during embryogenesis. Here we present genetic evidence based on the interaction between *pho* and *Pcl* or *Pc* during the embryonic and adult development.

pho null mutant homozygotes produced from *pho^{b}* heterozygotes show normal embryonic phenotypes, but homeotic transformation during the adult stage (Girton and Jeon, 1994). LexA-Pho chimeric protein was also found to be incapable of repressing the transcription of a lexA-Ubx-LacZ reporter (Poux *et al*., 2001b). In addition, unlike other PcG mutations, the *pho* mutant embryos from the *pho^{b}* heterozygote showed normal expressions of *Sexcomb reduced* (*Scr*) and *Antennapedia* (*Antp*), and subtle misexpressions of *Ubx*, *abd-A* and *Abd-B* (McKeon and Brock, 1991; Simon *et al.,* 1992). Taken together, these findings suggest that PHO may not act to repress these mutations during at early embryonic development.

However, there is considerable evidence that PHO works during embryogenesis and may have an essential role in nucleating the PcG complexes. The normal phenotypes of *pho* mutant embryos appear to be due to maternally produced PHO. Germ-line mosaic analysis (Breen and Duncan, 1986) and characterization of the *pho^{cv}* mutant allele showing maternal effects (Girton and Jeon, 1994) demonstrated that without maternal PHO products the embryonic head is very abnormal, the thoracic denticle belts transform to the first abdominal segment, and the posterior abdominal segments transform to the eighth segment. Our genetic data support the reported roles of PHO during embryonic and adult development. *Pcl*/+; *pho*/+ was embryonically normal, but homeotic transformation was found in the adult stage. Sexcomb and tergite phenotypes were dramatically increased in *Pcl*/+; *pho*/*pho* flies. These dosage-dependent interactions were also observed during embryogenesis. *Pcl*/*Pcl* mutant embryos in the background of *pho* maternal effect mutation showed much more severe transformation of embryonic denticle belts than in a background of *pho* zygotic mutation, which is demonstrated for first time by this study. These results clearly demonstrate that PHO functions during embryogenesis and works as a member of the PcG complex. Fritsch *et al*. (1999) demonstrated a direct physical link between PHO and PRE and that PHO may act to recruit anchor PcG proteins to DNA. Recent biochemical work suggesting that PHO appears to physically interact with PC showed that PHO is a member of PcG complexes (Mohd-Sarip *et al*., 2002). More indirect evidence that *pho* interacts with other PcG genes, came from the characterization of a vertebrate PHO homolog, YY1, in *Drosophila* (Atchison *et al*., 2003). The homology between YY1 and PHO resides in two YY1 domains: sequences 298-414 constituting of four zinc fingers (95% identical), and a short segment between residues 205-226 (82% identity). YY1 rescued *pho* mutant phenotypes in *Drosophila*, indicating structural and functional conservation between two genes. YY1 transcriptional repression was ablated in mutations of Pc, Pcl, Scm, Sce, Asx, Su(Z) Psc, and esc genes, which are all members of PcG, suggesting that YY1 functions as a PcG protein. This indirectly suggests that PHO interacts with other PcG proteins and is a member of the PcG system.

However, PHO does not seem to have a main role in nucleating PcG complexes, as its phenotypes are very weak compared to the other PcG mutations. Since maternal PHO is insufficient for the

complete silencing of the homeotic genes, it was suggested that PHO might play a role in the continuously anchoring PcG proteins to DNA, rather than have a role in the initiation of PcG nucleation (Fritsch *et al.*, 1999). PHO does not appear to physically interact with the ESC-EZ complex. So there remains a possibility that there is a second unidentified DNA binding protein in *Drosopohila*.

In summary, we present strong genetic evidence that PHO interacts with PCL and PC in a dose dependent manner during the embryonic and adult development. This finding supports the recently formed view that PHO physically binds PC.

Materials and Methods

Fly stocks and culture

pho^{cv} strain was recovered as a revertant of *pho*^{c} (Girton and Jeon, 1994), and both *pho*^{c} and *pho*^{cv} were produced by inserting mdg4/gypsy elements (Brown *et al.*, 1998). *Pcl*^{W6} and *Pc*^{1} are described in Sato *et al.* (1984) and Denell (1982), respectively. Other mutations and balancers are described in (Lindsley and Zimm, 1992) and the embryonic fate map in (Robert, 1986). Flies were reared in 20 mm-diameter vials containing a standard cornmeal/yeast medium seeded with live yeast. Stocks were maintained at 20°C, but experimental flies were reared at 25°C and eggs were also collected at 25°C.

Generation of Pcl; pho *and* Pc; pho *double mutants*

Pcl^{W6}/*SM6a* was crossed with *pho*^{cv}/*ci*^{D}. From the first progenies, *Pcl*^{W6}/+ ; +/*ci*^{D} flies were crossed with +/*SM6a* ; *pho*^{cv}/+. From the second progenies, flies with both *Cy* and *ci*^{D} phenotypes were selected and single-mated to get the *Pcl*^{W6}/*SM6a* ; *pho*^{cv}/*ci*^{D} double mutant. The final stock was confirmed by crossing with the original mutant lines.

Pc^{1}/*TM3, Sb Ser* was also crossed with *pho*^{cv}/*ci*^{D}. *Pc*^{1}/*TM3, Sb Ser;* and *pho*^{cv}/*ci*^{D} double mutant was obtained in the same way as the *Pcl; pho* double mutant.

Immunocytochemical staining

Embryos were collected and dechorionated, fixed and devitellined for staining. Immunocytochemical staining was carried out with Ubx or Abd-B primary antibodies and detected with a Vectastain ABC kit (Vectorlabs), described in (Jeon, 2002). If necessary, nickel chloride was added to the final coloring reactant to enhance the signal. Whole-mount embryos were viewed and photographed using an Olympus microscope BX51 with Normaski.

Embryonic cuticle preparation

Eggs were collected at 12 hr intervals and further incubated for 24 hrs at 25°C. Embryos with a pharyngeal skeleton were collected and transferred to double-sided cellophane tape for manual dechorionation with a fine tungsten needle. Dechorionated embryos were placed and devitellinized in a 1:1 mixture of Hoyer's mounting solution and lactic acid (Choi *et al.*, 2000). Embryos were viewed and photographed using an Olympus microscope equipped with phase optics.

Adult cuticle preparation

Eclosed or pharate male adult flies were collected under a low-power dissecting microscope and preserved in 70% ethanol. The flies were boiled in hot 1N KOH for 5-10 minutes to remove their inner body parts. The samples were then serially dehydrated, and the head, abdomen and six legs were separated for better observation in euparal on slide glass. The samples were then coverslipped, dried, and flattened on a heated slide-warming tray under weights.

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