Mol. Cells, Vol. 10, No. 2, pp. 156–161

Molecules and Cells

Effects of *Polycomb* Group Mutations on the Expression of *Ultrabithorax* in the *Drosophila* Visceral Mesoderm

Seung-Hoon Choi, Chun Taek Oh, Sang Hee Kim[1], Yun-Taik Kim[2], and Sang-Hak Jeon*

Department of Biological Sciences, Konkuk University, Seoul 143-701, Korea;
[1] Department of Chemistry, Konkuk University, Seoul 143-701, Korea;
[2] Department of Life Science, Sogang University, Seoul 121-742, Korea.

(Received on November 5, 1999)

The *Polycomb* group (PcG) genes encode repressors of many developmental regulatory genes including homeotic genes and are known to act by modifying chromatin structure through complex formation. We describe how *Ultrabithorax* (*Ubx)* expression is affected by the PcG mutants in the visceral mesoderm. Mutant embryos of the genes *extra sex combs* (*esc*), *Polycomb* (*Pc*), *additional sex combs* (*Asx*) and *pleiohomeotic* (*pho*) were examined. In each mutation, *Ubx* was ectopically expressed outside of their normal domains along the anterior-posterior axis in the visceral mesoderm, which is consistent with the effect of PcG proteins repressing the homeotic genes in other tissues. All of these four PcG mutations exhibit complete or partial lack of midgut constriction. However, two thirds of *esc* mutant embryos did not show *Ubx* expression in parasegment 7 (PS7). Even in the embryos showing ectopic *Ubx* expression, the level of *Ubx* expression in the PcG mutations was weaker than that in normal embryos. We suggest that in PcG mutations the ectopic *Ubx* expression is caused by lack of PcG repressor proteins, while the weaker or lack of *Ubx* expression is due to the repression of *Ubx* by Abd-B protein which is ectopically expressed in PcG mutations as well.

Keywords: *Drosophila*; *Polycomb* Group Genes; *Ultrabithorax*; Visceral Mesoderm.

Introduction

During the development of *Drosophila*, the homeotic selector (HOM) genes of the bithorax complex (BX-C) and Antennapedia complex (ANT-C) perform numerous functions that are required to determine the proper identities of the different segments and tissues (Kaufman *et al*., 1980; Lewis, 1978; reviewed by McGinnis and Krumlauf, 1992). The HOM genes are expressed in the epidermis, neuroectoderm, and somatic as well as visceral mesoderm (Akam and Martinez-Arias, 1985; Beachy *et al*., 1985; White and Wilcox, 1985). The genes of BX-C, *Ultrabithorax* (*Ubx*), *abdominal-A* (*abd-A*) and *Abdominal-B* (*Abd-B*), specify the identities of the third thoracic (T3) and first through ninth abdominal segments (A1–A9) corresponding to parasegment 5 (PS5) through PS13 (reviewed by Duncan, 1987). Each of them exhibits a complicated expression pattern. For example, *Ubx* is first expressed at the germ band extended stage in the epidermis from PS5 to 13 with the strongest expression in PS6. The expression intensity is reduced posteriorly from PS7 to PS12. *Ubx* is also expressed in the visceral mesoderm of PS7, indicating that the patterns of homeotic genes in the visceral mesoderm are different from those of epidermis.

The stable expression of homeotic genes is essential for normal development. The PcG genes are known to be involved in the long-term maintenance of the repressed state of homeotic genes. The PcG genes include over a dozen described members. Genetic analyses suggested that there might be up to 30 to 40 members in the PcG genes (Jurgens, 1985; Landecker *et al*., 1994). Synergistic interaction between PcG mutations (Jurgens, 1985), co-precipitation (Franke *et al*., 1992), and co-localization of PcG proteins on the same chromosomal sites (Rastelli *et al*., 1993) indicate that their gene products are thought to function as a multimeric complex. It was recently suggested that the complex may be formed by the primary

* To whom correspondence should be addressed.
Tel: 82-2-450-3411; Fax: 82-2-456-1522
E-mail: jeonsh@kkucc.konkuk.ac.kr

Abbreviations: *Asx*, additional sex combs; *esc*, extra sex combs; *Pc*, Polycomb; PcG, Polycomb group; *pho*, pleiohomeotic; *Ubx*, Ultrabithorax.

role of the *pleiohomeotic* (*pho*) protein that is known as a unique DNA binding protein among PcG proteins (Brown *et al.*, 1998).

Although the mutations of the PcG genes do not affect the initial expression of homeotic genes, they soon cause ectopic expression (Mckeon and Brock, 1991; Simon *et al.*, 1992; Struhl and Akam, 1985). For example, in most of the PcG mutants, the initial distribution of *Ubx* protein is indistinguishable from that of the wild type, but abnormal expression is soon shown in head through PS4 of the epidermis and CNS (McKeon and Brock, 1991). *abd-A* and *Abd-B* genes are also ectopically expressed throughout the anterior-posterior (A-P) axis (Simon *et al.*, 1992).

The visceral mesoderm adhering to the midgut consists of an internal germ layer that constitutes most of the A-P axis (PS 2–13). Unlike the epidermal cells, these cells exclusively express one of homeotic genes (Tremml and Bienz, 1988). For example, the ectodermal cells belonging to PS7 typically express high levels of *abd-A* (Karch *et al.*, 1990) as well as weak level of *Antp* (Carroll *et al.*, 1986) and *Ubx* (White and Wilcox, 1985), whereas cells in the visceral mesoderm mainly express high levels of the *Ubx* protein (Tremml and Bienz, 1988). These observations indicate that *Ubx* is expressed by different components and mechanisms in two germ layers.

In this study we demonstrated how four PcG mutations, *extra sex combs* (*esc*), *Polycomb* (*Pc*), *additional sex combs* (*Asx*) and *pleiohomeotic* (*pho*), affected *Ubx* expression in the visceral mesoderm using *UbxlacZ* reporter construct strain. Our results show that *Ubx* is ectopically expressed outside of their normal domains along the A-P axis in the visceral mesoderm, which is consistent with the effect of PcG proteins that repress homeotic genes in other tissues. However, a considerable number of PcG mutant embryos lack *Ubx* expression in PS7. The variable expression pattern of *Ubx* appears to be due to the incomplete penetrance of *Abdominal-B* in the visceral mesoderm of PcG mutant embryos.

Materials and Methods

***Drosophila* stocks and culture** Marker mutations and balancer chromosomes used are as described in Lindsley and Zimm (1992). Flies were reared in 20 mm-diameter vials containing a standard cornmeal/yeast medium seeded with live yeast (Kim *et al.*, 1999). Fly stocks were maintained at 19°C, and all crosses and egg collection were performed at 25°C unless experiments required a different temperature. pho^{cv}/pho^{cv} flies from $pho^{cv}/+$ stock were collected at 19°C and transferred to 25°C for egg collection.

PcG mutations used are *additional sex combs*, *Asx* (Sinclair *et al.*, 1992); *extra sex combs*, esc^2 and esc^5 (Struhl, 1981) [recovery of *esc* maternal effect embryos was as described in Glicksman and Brower (1990)]; *pleiohomeotic*, pho^{cv} (Girton and Jeon, 1994); *Polycomb*, Pc^3 (Denell and Frederick, 1983). Pc^3 *UbxlacZ* recombinant chromosome was produced and balanced by *TM3 ftzlacZ* to distinguish the Pc^3 homozygotes embryos from others. *Asx*; *UbxlacZ* was also balanced by *CyO wglacZ*. A strain carrying the *Ubx* visceral mesoderm reporter gene is described in Hursh *et al.* (1993).

Antibody and X-gal staining For antibody staining, embryos were dechorionated in 50% commercial bleach for 3 min, washed in NTX (0.4% NaCl, 0.03% Triton X-100), fixed on the interface of a heptane/4% paraformaldehyde solution for 30 min, then devitellinized by removing the lower aqueous fixative and adding an equal volume of methanol. After the embryos were washed with PT (1× PBS, 0.1% Triton X-100) several times, they were blocked with 5% goat serum for 30 min. Embryos were incubated overnight with primary anti-β-galactosidase (Cappell), anti-*Ubx*, anti-*abd-A* or anti-*Abd-B* antibodies. This was followed by a second incubation for 2 h with biotinylated anti-mouse antibody, and for 30 min with avidin and biotinylated horseradish peroxidase (HRP) (Vectorlabs, Vectastain ABC kit). The staining reaction was performed by adding DAB and H_2O_2 as HRP substrates. Stained embryos were then serially dehydrated in EtOH and mounted in methylsalicylate.

For X-gal staining, embryos were dechorionated and fixed as in antibody staining. After fixation, all the solution was removed, and the embryos were dried for about 30 s. The embryos were then washed with PT twice and incubated in X-gal staining buffer for 10 min. X-gal solution was then added and the mixture incubated for more than 3 h. Stained embryos were serially dehydrated in EtOH and mounted in methylsalicylate and then viewed with an Olympus BX50 microscope.

Embryonic cuticle preparation To observe cuticular patterns in late embryos, eggs were collected at 6 to 12-h intervals and incubated for 24 h at 25°C. Embryos with a pharyngeal skeleton were collected and transferred to double-sided cellophane tape for manual dechorionation. Embryos were then mounted in 1:1 mixture of Hoyer's mountant and lactic acid, and devitellinized with a fine tungsten needle. Embryonic internal structures were cleared at 60°C on a slide warmer for several days (Girton and Jeon, 1994). The embryos were examined with dark field microscopy.

Results and Discussion

Generation of PcG mutant and *UbxlacZ* recombinants Pc^3, *Asx*, esc^2, esc^5 and pho^{cv} mutants were combined with a strain containing *Ubx* reporter gene. Each combined mutant was double-checked by phenotypes and by crossing with the original stocks. The recombinant of Pc^3 and *Ubx* reporter gene was produced by chromsomal recombination since both are on the third chromosome.

The denticle patterns were examined to collect the right recombinant of PcG mutant and *Ubx* reporter strain (Denell and Frederick, 1983; Lohs-Schardin *et al.*, 1979) (Fig. 1A). The *Asx* mutant embryos showed the transformation of the thorax to the first abdominal segment and the abdominal segments to more posterior ones (Fig. 1B). *pho* maternal effect mutant embryos showed a partial transformation of the thoracic segments to the first

abdominal segment and the posterior abdominal segments to the eighth segment (Fig. 1C). Pc^3 zygotic mutation caused a very strong homeotic transformation of all abdominal denticle belts to more posterior abdominal segment (Fig. 1D). esc^2/esc^5 maternal effect mutation caused all denticle belts to have a rectangular shape, indicating the transformaton of all segments to the eighth abdominal one (Fig. 1E). The cuticular phenotypes of four PcG mutants suggested that *Ubx* expression in the visceral mesoderm might be affected by the PcG mutant embryos. Anti-Ubx antibody-stained embryos showed the signal in epidermis, central nervous system (CNS) and visceral mesoderm (Fig. 2A). Unlike *Ubx* expression seen in Fig. 2A, it was barely dectectable in the visceral mesoderm of many embryos stained with anti-Ubx antibodies. A *Ubx* reporter strain, in which *Ubx* is expressed in the visceral mesoderm of PS4 and 7 but not in epidermis and CNS, was selected to observe the effects of PcG on *Ubx* expression in the visceral mesdoerm.

***Ubx* expression in the visceral mesoderm of PcG mutant embryos** *Ubx* was first expressed at the germ band extended stage in the epidermis from PS5 to PS13 with the strongest expression at PS6 (Fig. 2A, PS6; arrowhead). The intensity of *Ubx* expression was reduced posteriorly from PS7 to PS12. *Ubx* was also expressed in the PS7 of the visceral mesoderm (Fig. 2A; arrow), whose

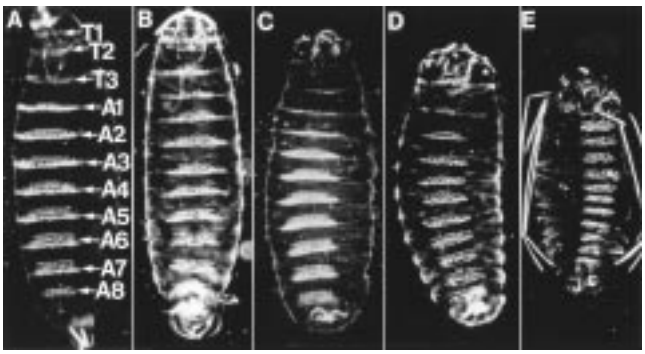

Fig. 1. Phenotypes of late embryos formed by the wild type and *Polycomb* group mutations. wild type (**A**); *Asx* (**B**); pho^{cv}/pho^{cv} (**C**); Pc^3 (**D**); esc^2/esc^5 (**E**). Anterior is up. **A.** Wild type embryo. Each of thoracic and abdominal segment exhibits the characteristic pattern of ventral denticle belts close to their anterior margin. The mesothorax and metathorax carry bands of fine hairs, while abdominal segments have largely hooked denticles. **B.** *Asx* mutant embryo. The thoracic segments are transformed to the first abdominal one and the abdominal segments to more posterior one. **C.** pho^{cv}/pho^{cv} maternal effect mutant embryo. The denticle belts of thoracic segments are transformed to the first abdominal segment and the posterior segments to the eighth one. **D.** Pc^3 mutant embryo. All of the thoracic and abdominal segments are strongly transformed to more posterior abdominal segments. **E.** esc^2/esc^5 mutant embryo. All denticle belts have a rectangular shape, indicating that they are transformed to the eighth abdominal one.

anterior boundary was shifted one PS toward the posterior. *Ubx* reporter gene exhibited a normal expression pattern in wild type background, where *UbxlacZ* expression was detected in the visceral mesoderm at PS4 (Figs. 2B–2D; arrowheads) and at PS7 (Fig. 2B, arrow). The second midgut constriction was formed at stage 15 and coincided with the boundary between *Ubx* and *abd-A* expression (Bienz and Tremml, 1988) (Figs. 2C and 2D).

While esc^2/esc^5 transheterozygotes are viable, embryos from esc^2/esc^5 females are lethal, showing maternal effects. *Ubx* was ectopically expressed at PS5 and 6 (Fig. 2E). The *Ubx* expression shown in Fig. 2E was relatively strong compared to that of Pc (Fig. 2F) and Asx (Fig. 2G), but such an expression was observed only in less than 1% *esc* mutant embryos. Other embryos showed very weak ectopic expression as shown in Fig. 2F. Pc^3 is an antimorphic allele and caused ectopic expression of *Ubx* at PS5 (Fig. 2F; first arrowhead) and 6 (Fig. 2F; second arrowhead). In order to distinguish the Pc^3 homozygous chromosomes from others, Pc^3 was balanced with *TM3 ftzlacZ*. While *Ubx* was ectopically expressed throughout the body in the epidermis and CNS (Struhl and White, 1985), it was relatively weakly misexpressed in the visceral mesoderm in *esc* and *Pc* mutant embryos (Fig. 2F). *Ubx* was also ectopically expressed at PS5 and 6 of *Asx* zygotic mutant embryos (Fig. 2G). Embryos from pho^{cv}/pho^{cv} females produced maternal effects and showed ectopic expression of *Ubx* in PS5 and 6 (Fig. 2H). As *Pc* and *esc* mutations caused the strong ectopic expression from head to PS4 in epidermis and CNS, it was somewhat suprising that the anterior boundary moved just two PS forward in the visceral mesoderm. Although *Pc* and *esc* mutations caused a strong homeotic transformation in the epidermis (Figs. 1D and 1E), they showed a very similar level of ectopic expression of *Ubx* with *Asx* and *pho*. So far, the ectopic expression of homeotic genes has not been examined yet in any tissue of *pho* mutants. Unlike other PcG zygotic mutants, *pho* maternal effect mutant embryos frequently showed a distinct abnormal development, which made it hard to count the exact number of segments. Some *pho* embryos frequently showed several large holes throughout the body, suggesting that many cells may die during embryogenesis (data not shown). In each PcG mutation, *Ubx* was ectopically expressed outside of their normal domains along the A-P axis in the visceral mesoderm, which is consistent with the PcG proteins, in which the homeotic genes are repressed (Glicsman and Brower, 1990; Lewis, 1978; Struhl and White, 1985).

However, we frequently observed ectopic expression as well as lack of *Ubx* expression in the *esc* mutant embryos. *Ubx* expression was examined by X-gal and antibody staining. More than 50% of embryos did not show any Ubx signal at PS7 (Figs. 3B and 3C). Midgut constriction was partial (Figs. 3D and 3E) or not formed (Fig. 3F). The weak expression as shown in Fig. 3F could not be dectected with anti-Ubx antibody staining in the previous

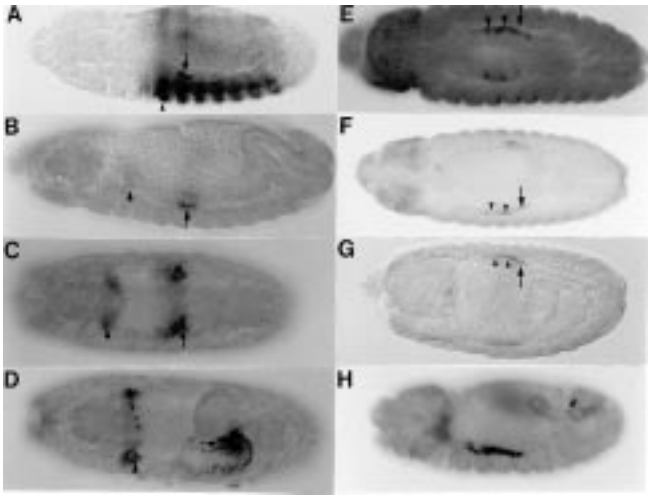

Fig. 2. Immunohistochemical detection of Ubx protein expression **(A)** and β-galactosidase expression directed by a *Ubx*-reporter construct **(B-H)**. Anterior is to the left. **A, B** and **H**, lateral views and **C–F**, horizontal views. **A–D**, wild type embryos. **A** and **B**, stage 14; **C**, stage 15; **D**, stage 16; **E–F**, stage 14-15. Ubx protein is observed in the epidermis, central nervous system and visceral mesoderm **(A)**. The reporter gene is expressed only in the ventral mesoderm both in PS4 (arrowhead) and in PS7 (arrows) **(B–D)**. **E**, esc^2/esc^5; **F**, Pc^3; **G**, *Asx*; **H**, *pho* mutant embryos. The first and second arrowheads indicate PS5 and 6, respectively, while the arrows represent PS7. *Ubx* reproter gene was ectopically expressed in PS5 and 6. Its level of expression was weak in most mutant embryos although some embryos showed relatively strong expression as shown in Figs. 2E and 2H.

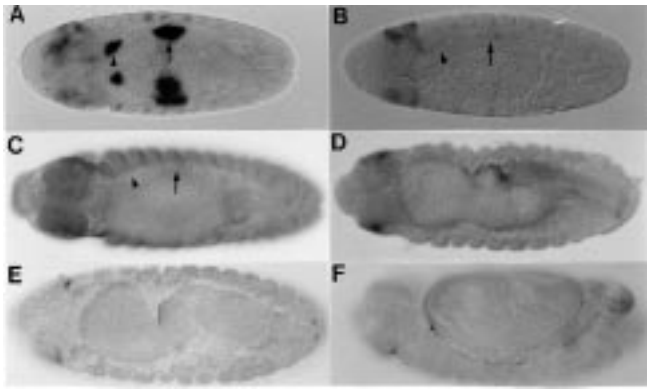

Fig. 3. The variable expression of *Ubx* in the visceral mesoderm of *esc* mutant embryos. *Ubx* expression was detected with X-gal **(A** and **B)** and anti-β-gal antibody staining **(C–F)**. Anterior is to the left. **A**, wild type embryo. The arrowhead indicates PS4 and arrow PS7. **B–F**, *esc* maternal effect mutant embryos. **B** and **C**, stage 13 embryo. *Ubx* is not expressed in PS4 and PS7. **D** and **E**, stage 16 embryo. The second midgut constriction is partially formed. **F**, stage 16 embryo. It lacks the midgut constriction, but *Ubx* is expressed with a very weak signal in the visceral mesoderm covering the midgut.

study. When several lines of esc^2; *UbxlacZ* and esc^5; *UbxlacZ* were examined to check if the variable pattern of *Ubx* expression could be seen in *esc* mutant embryos, we obtained almost the same result from all the cases examined.

The variable expression of *Abd-B* in PcG mutant embryos could be a reason for ectopic expression or repression of *Ubx* in the visceral mesoderm. It has been suggested that the complex pattern of *Ubx* gene expression normally depends on the activities of other bithorax complex genes, *abd-A* and *Abd-B* (Sanchez-Herrero *et al*., 1985). Through directing the ectopic expression of the BX-C using *esc* mutation, Struhl and White (1985) also found that the *abd-A* and *Abd-B* genes reduced *Ubx* expression in the epidermis and CNS. To find a similar kind of down-regulation of *Ubx* expression by the Abd-B protein in the visceral mesoderm, we examined the *Abd-B* expression in four PcG mutant embryos. In wild type, the anterior boundary of the *Abd-B* expression in the visceral mesoderm is at PS11 (Celinker *et al*., 1989; DeLorenzi and Bienz, 1990) (Fig. 4A, arrowhead). However, *Abd-B* was ectopically expressed throughout the whole visceral mesoderm of *esc* (Fig. 4B), *Pc* (Fig. 4C), *Asx* (Fig. 4D), and with relatively weak signal in *pho* mutant embryos (Fig. 4E).

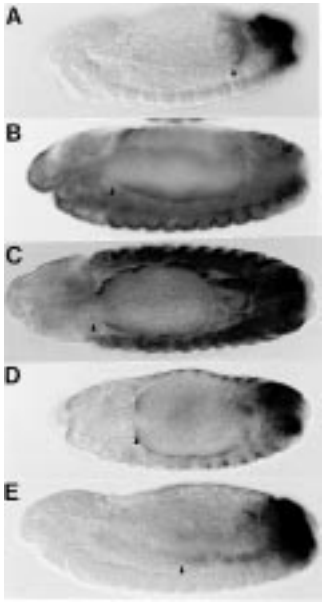

Fig. 4. *Abd-B* expression in the visceral mesoderm of the wild type and PcG mutant embryos. Anterior is to the left. Arrowheads indicate the anterior boundary of *Ubx* expression in the visceral mesoderm. **A**, wild type embryo. *Abd-B* is expressed from PS11 to the posterior end in the visceral mesoderm. **B**, *esc* maternal effect mutant embryo. **C**, *Pc* embryo; **D**, *Asx* embryo; **E**, *pho* maternal effect mutant embryo. *Abd-B* was expressed all the way in the visceral mesoderm covering the midgut of *esc*, *Pc* and *Asx*, but anteriorly in reduced level in *pho*.

We assumed that the ectopic expression of *Ubx* in the visceral mesoderm could be due to the escape from the regulation of the *Abd-B* gene. Thus, we quantified the expression of *Abd-B* in *esc* mutant embryos of stage 13 or 14. Although *Abd-B* was strongly misexpressed in most of *esc* mutant embryos, it was not expressed beyond the normal anterior boundary in about 20% of embryos. Neither was it misexpressed in more than 50% of *pho* mutant embryo. In such PcG mutant embryos, *Ubx* could be ectopically expressed due to the repression of *Abd-B*. Struhl and White (1985) suggested that from the combinational work on the effect of *abd-A* and/or *Abd-B* mutation on the *Ubx* expression, *Ubx* was largely repressed by *Abd-B*. However, in the case of the lack of *Abd-B*, *Ubx* was repressed by *abd-A* gene function. So the examination of *Ubx* in consideration of *abd-A* and *Abd-B* mutation by constructing the quadruple recombinant of *UbxlacZ*, *esc*, *abd-A* and *Abd-B* would give a complete picture of PcG mutation on *Ubx* expression in the visceral mesoderm.

Acknowledgments This study was supported by the Grants from the Konkuk University Foundation (1998). We would like to thank Dr. Ian Duncan for providing the Ubx antibody and Dr. Celinker for the Abd-B antibody. We would also like to thank Dr. William Gelbart for providing a strain containing the *Ubx* reporter gene. We also thank Sun Mee Yang and Kyoung-Ja Jeon for their assistance in the maintenance of fly stock.

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