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Drosophila castor is regulated negatively by the *Ubx* and *abdA* genes, but positively by the *AbdB* gene

JU-HYUN AHN[1], SIUK YOO[2], HYUN-JOO PARK[2], KEUK-IL JUNG[1], SANG-HEE KIM[3] and SANG-HAK JEON*[,1]

[1]Department of Biology Education, Seoul National University, Seoul, [2]Department of Biology, Yeungnam University, Gyeongsan and [3]Department of Chemistry, Konkuk University, Seoul, Korea

ABSTRACT The ventral nerve cord (VNC) of *Drosophila* exhibits significant segmental-specific characteristics during embryonic development. Homeotic genes are expressed over long periods of time and confer identity to the different segments. *castor (cas)* is one of the genes which are expressed in neuroblasts along the VNC. However, at late embryonic stages, *cas* transcripts are found only in head and thoracic segments and terminal abdominal segments, while Cas protein lasts longer in all segments. In this study, we investigated the regulation of temporal and spatial expression of *cas* by the bithorax complex genes. In the loss-of-function mutants of *Ultrabithorax (Ubx)* and *abdominal-A (abdA)*, *cas* transcripts were ectopically expressed in abdominal segments at late embryonic stage. However, unlike in *Ubx* and *abdA* mutants, in *Abdominal-B (AbdB)* loss-of-function mutant embryos, *cas* disappeared in the terminal region. Ectopic *Ubx* and *abdA* suppressed *cas* expression, but ectopic *AbdB* activated *cas* expression in most abdominal segments. Moreover, *cas* was co-expressed in the cells in which *AbdB* was normally expressed, and overexpressed in the ectopically expressed *AbdB* embryos. These results suggest that the expression of *cas* is segment-specifically regulated negatively by *Ubx* and *abdA* genes, but positively by the *AbdB* gene.

KEY WORDS: *Ubx, abdA, AbdB, castor, neuroblast*

Introduction

The development of the central nervous system (CNS) in the *Drosophila* begins with the delamination of neural progenitor cells, called neuroblasts (NBs), from the neuroectoderm. About 25% of the neuroectodermal cells delaminate into the embryo as neuroblasts (Hartenstein and Campos-Ortega, 1984; Doe, 1992). In each hemisegment, about 30 NBs segregate from the neurogenic region of the ectoderm, forming stereotyped spatiotemporal segmental patterns along the anterior to posterior axis.

The number and pattern of neuroblasts that initially delaminate from the neuroectoderm in the early *Drosophila* embryo are identical in thoracic and abdominal segments (Skeath, 1999). However, the homologous VNC lineages begin to specifically differ between thoracic and abdominal neuromeres (Udolph *et al.*, 1993; Bossing *et al.*, 1996; Schmidt *et al.*, 1997; Prokop *et al.*, 1998; Technau *et al.*, 2006). For example, neuroblast NB1-1 produces 2 motorneurons and about 10 interneurons in the thorax, while it gives rise to 1 motorneuron, about 6 interneurons, and three glial cells in the abdomen (Prokop and Technau, 1994). NB6-4 produces four to six interneurons and four glial cells (medialmost cell body glia, MM-CBG) in the thorax, but exclusively two glia in the abdomen (Schmidt *et al.*, 1997; Kang *et al.*, 2006). For NB7-3, differences between the thoracic and abdominal counterparts emerge later in development (Rogulja-Ortmann *et al.*, 2008).

The embryonic segment-specific characteristics are basically determined by the homeotic genes (Duncan, 1987; Kaufman *et al.*, 1990; McGinnis and Krumlauf, 1992). The *Drosophila* homeotic genes consist of two groups of genes, Antennapedia complex

Abbreviations used in this paper: abdA, abdominal A; AbdB, Abdominal B; ANT-C, antennpedia complex; BX-C, bithorax complex; cas, castor; CNS, central nervous system; eg, eagle; Hb, hunchback; Kr, Krüpple; NB, neuroblast; Pc, Polycomb; PcG, Polycomb group; PS, parasegment; Ubx, Ultrabithorax; Vnc, ventral nerve cord.

***Address correspondence to:** Sang-Hak Jeon. Department of Biology Education, Seoul National University, San 56-1, Shillim-dong, Gwanak-gu, Seoul 151-742, Korea. Fax: +82-2-886-2117. e-mail: jeonsh@snu.ac.kr

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(ANT-C) and Bithorax complex (BX-C). BX-C contains three transcriptional units, *Ultrabithorax* (*Ubx*), *abdominal-A* (*abdA*), and *Abdominal-B (AbdB)*, specifying segmental identity along the anterior-posterior body axis in *Drosophila*. Molecular genetic studies have revealed that the homeotic genes are strongly expressed in the nervous system, in the ectoderm, and in the visceral mesoderm. *Ubx* is expressed in parasegments (PS) 5-12 with the highest concentration in PS6 (Beachy *et al.*, 1985), *abdA* in PS7-13 (Karch *et al.*, 1990), and *AbdB* in PS10-14 (Celniker *et al.*, 1989). This expression pattern is maintained by the negative regulation of Polycomb group (PcG) genes (Dellino *et al.,* 2002). Mutations of PcG genes lead to homeotic transformations of segments into a more posterior identity (Jürgens, 1985; McKeon and Brock, 1991; Simon *et al.*, 1992).

Several studies showed that the inter-segmental diversity within the CNS was also established by the homeotic genes. For example, the segmental specificity of NB1-1 was controlled by *Ubx* and *abdA* (Prokop and Technau, 1994). The abdominal fate of NB6-4 was specified by *abdA* and *AbdB*. Loss-of *abdA* or *AbdB* function transformed abdominal NB6-4 to thoracic NB6-4, while ectopic *abdA* or *Ubx* resulted in the reverse transformation (Berger *et al.*, 2005). *eagle* (*eg*) was one of the NB6-4 marker genes that showed segment-specific expression (Higashijima *et al.*, 1996).

castor (*cas*), which is also called *ming*, is also one of genes that show segment-specific expression. It encodes a putative zinc finger protein and is expressed in the subset of CNS neuroblasts but not in neurons, suggesting that it is necessary for the development of a subset of CNS neuronal precursors (Cui and Doe, 1992; Mellerick *et al.*, 1992). *cas* transcripts first appear in a subset of the midline CNS cells at stage 9. Soon after, it is expressed in one NB of every hemisegment and, by late stage 11, it is in 17 neuroblasts of each hemisegment of the CNS. *cas* transcripts begin to decrease from stage 14 and are detectable in only a few cells of the thoracic and terminal abdominal CNS of stage 15 embryos, which clearly shows the differential expression at the transcriptional level in thoracic and abdominal segments. The role of *cas* has not been thoroughly characterized yet. It seems to be involved in axonogenesis. For example, *cas* mutation caused a diminished CNS axonal network and embryonic lethality (Cui and Doe, 1992; Mellerick *et al.*, 1992). In a recent study*, cas* was found to specify late-born neuronal identity (Grosskortenhaus *et al.*, 2006). Embryonic neuroblasts generate an ordered sequence of neuronal progeny through sequential expression of Hunchback (Hb), Krüppel (Kr), Pdm, and Cas transcription factors. Hb and Kr specify early-born temporal identity, while Pdm and Cas regulate late-born motor neuron identity within the NB7-1 lineage. However, Tran and Doe (2008) suggested that *cas* does not specify the fourth temporal identity, but instead closes the third temporal identity window.

One issue that can be addressed regarding *cas* is how its segment-specific expression pattern is regulated along the anterior to posterior axis. In order to understand such molecular aspects, we investigated the role of the homeotic genes that are involved in the segmental identity along the anterior to posterior axis. We found that *Ubx* and *abdA* suppressed the expression of *cas* in abdominal segments, while *AbdB* activated its expression.

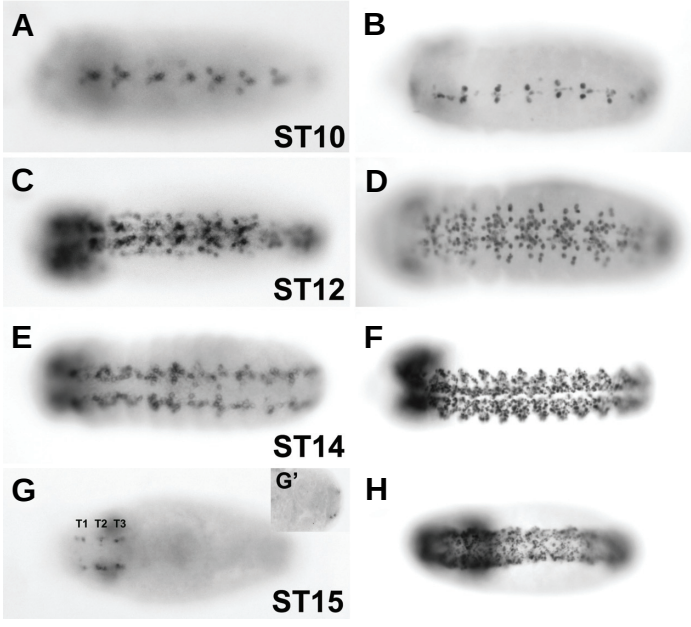

Fig. 1. *cas* expression patterns at the level of transcription and translation. (A,C,E,G) cas *mRNA expression pattern.* cas *transcripts appear in the midline and in one hemisegment at stage 10* **(A)** *and in more than 17 neuroblasts per hemisegment* **(C)**. *But it decreases after stage 14* **(E)** *and is mostly observed in thoracic segments at stage 15* **(G,G')**; *magnified in the terminal segment.* **(B,D,F,H)** *Cas protein expression pattern. Cas protein was observed throughout the central nervous system of stage-15 embryo* **(H)**.

Results

cas shows segment-specific expression at late embryonic stage

cas encodes a predicted zinc finger protein and is transcribed in a subset of NBs and GMCs, but not in neurons (Cui and Doe, 1992; Mellerick *et al.*, 1992). Cas protein first appeared in a subset of the midline CNS cells at stage 9. Soon after, it was observed in one NB of every hemisegment (Fig. 1 A,B) and, by late stage 12, it was found in more than 17 neuroblasts in every hemisegment of the CNS (Fig. 1 C,D). However, from stage 14, *cas* transcripts and protein showed different modes of expression. *cas* transcripts begin to decrease after stage 14 (Fig. 1E) and are detectable only in a few cells of the thoracic and terminal abdominal CNS in stage 15 embryos (Fig. 1 G,G'). These results clearly show the differential expression in thoracic and abdominal segments. However, Cas protein is observed through embryonic stage 15 (Fig. 1F and H).

As the segment-specific characteristics along the anterior to posterior axis are basically caused by the homeotic genes, we examined *cas* expression in loss-of-function and gain-of function mutants of the BX-C genes in order to better understand the molecular aspects causing segmental differences in *cas* expression.

Ubx and abdA, and AbdB loss-of-function mutations show the reverse results on ectopic cas transcripts in abdominal segments of stage 15 embryos

The homeotic proteins function in the morphological diversification of segments along the antero-posterior body axis (McGinnis

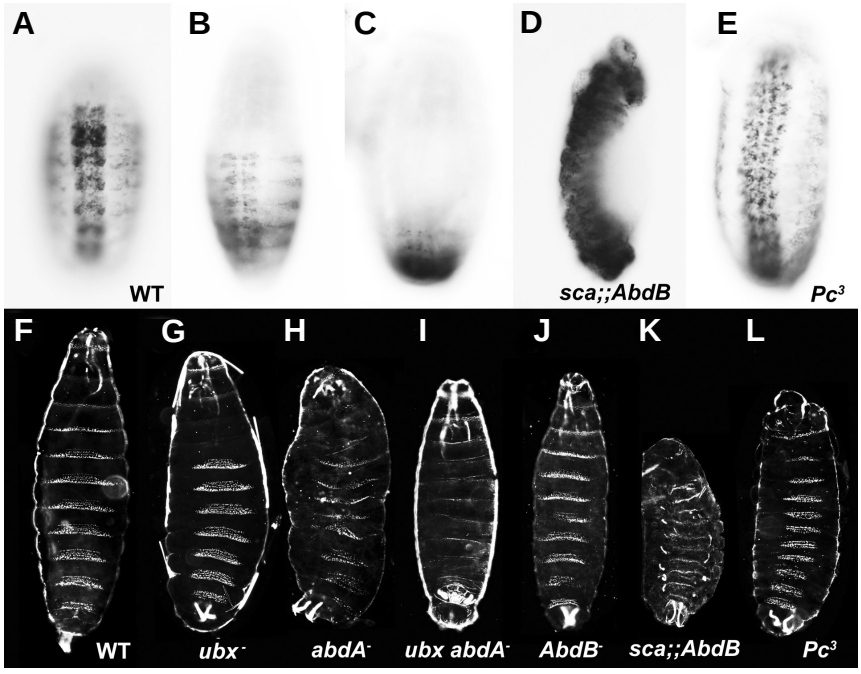

Fig. 2. Expression patterns of the bithorax complex genes and cuticle phenotypes of the bithorax complex gene mutation. (A-E) *Expression patterns of* Ubx, abdA *and* AbdB *in* Drosophila *embryos.* **(A)** *Wild-type Ubx expression. It is expressed from PS5 to PS12 with the highest concentration in PS6.* **(B)** *Wild-type* abdA *expression. It is expressed from PS7 to PS13.* **(C)** *Wild-type* AbdB *expression. It is expressed from PS10 to PS13.* **(D)** AbdB *expression in* sca;;AbdB *embryo.* AbdB *is ectopically expressed throughout the CNS.* **(E)** AbdB *expression in* Pc^3 *mutant embryo.* AbdB *is ectopically expressed throughout the CNS.* **(F-L)** *Embryonic denticle belt patterns.* **(F)** *Wild type. It has three finely thoracic and eight strong hooked abdominal denticle belts.* **(G)** *Ubx mutant. The third thoracic and first abdominal denticle belts are transformed to the second thoracic denticle belt.* **(H)** abdA *mutant. The second to fifth abdominal denticle belts are transformed to the first abdominal denticle belt.* **(I)** Ubx *and* abdA *double mutant. The third thoracic to the fifth abdominal denticle belts are transformed to the second thoracic denticle belt.* **(J)** AbdB *mutant. The fifth to eighth abdominal segments are transformed to the fourth abdominal segment.* **(K)** sca;;AbdB *embryo. Filzköpper, a terminal structure, appears in all abdominal segments.* **(L)** Pc^3 *mutant. The denticle belts of all segments were transformed to the eighth denticle belt.*

and Krumlauf, 1992). The homeotic genes begin to be expressed from blastoderm stages and their proteins are first seen at germ band elongation stages with a precise anterior boundary, their expression patterns are maintained throughout the embryonic stages (Supplementary Fig. 1). At later embryonic stage, the BX-C genes are strongly expressed in the central nervous system (Supplementary Fig. 1 B,C,E,F,H,I and Fig. 2 A-C). *Ubx* is expressed in parasegments (PS) 5-13 (Fig. 2A), *abdA* in PS7-13 (Fig. 2B), and *AbdB* in PS10-14 (Fig. 2C). For this study, *AbdB* was ectopically expressed in embryos produced from *sca-GAL4;;UAS-AbdB* (Fig. 2D), in which *AbdB* was spatio-temporally expressed in the *sca* expressing region (Mlodzik *et al.*, 1990). Ectopic *AbdB* expression was also observed in *Pc^3* mutant embryos (Fig. 2E). Prokop and Technau (1994) reported that the segmental specificity of NB1-1 is determined in the neuroectoderm under the control of *Ubx* and *abdA* genes.

Before we used the flies with mutant homeotic genes, the mutant lines were checked by observing the mutant phenotypes in loss-of-function (Carratala *et al.*, 1989) and gain-of-function mutations of the bithorax complex genes and one of the Polycomb group (PcG) genes, *Polycomb* (*Pc*). Wild-type embryos show three thoracic fine denticle belts and eight abdominal thick denticle belts (Fig. 2F). *Ubx* mutant embryos showed transformation of T3 and A1 segments to T2 segments (Fig. 2G), and *abdA* mutant embryos showed transformation of A2~A5 segments to A1 segments (Fig. 2H). *Ubx* and *abdA* double mutation caused the transformation of most of the abdominal segments to the second thoracic segments (Fig. 2I). *AbdB* mutant embryos showed transformation of A5 through A8 segments to A4 segments (Fig. 2J). Ectopic *AbdB* expression by *sca-GAL4;;UAS-AbdB* caused the filzköpper structure in all abdominal segments (Fig. 2K), which is the most distal structure, but the transformation of denticle belts was not clear. In *Pc^3* mutant embryos, all denticle belts were transformed to the eighth abdominal denticle belt.

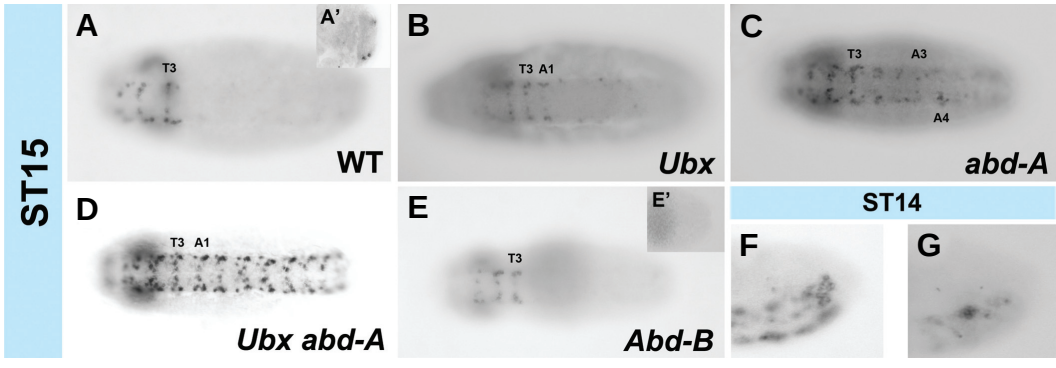

Fig. 3. *cas* mRNA expression pattern in the loss-of-function bithorax complex mutant embryos at stage 15. (A) *Wild-type embryo.* cas *transcripts were observed in thoracic and posterior abdominal segments (magnified in A').* **(B)** Ubx *mutant embryo. Ectopic* cas *appeared in the first abdominal segments and in some spots in other abdominal segments.* **(C)** abdA *mutant embryo. The first to fourth segments showed* cas *expression spots.* **(D)** Ubx abdA *double mutant embryo.* cas *was strongly expressed in abdominal segments as in wild-type embryo.* **(E)** AbdB *mutant embryo.* cas *expression in posterior abdominal segments disappeared (magnified in E').* **(F,G)** *Stage 14 embryos. Compared to wild type embryos* **(F)**, *the number of cells expressing cas was reduced in the* AbdB *embryo* **(G)**.

In stage 15 wild-type embryos, *cas* transcripts were observed mostly in thoracic segments (Fig. 3A) and mostly four spots of the posterior abdominal segments (Fig. 3A'). In *Ubx* mutant embryos, *cas* was ectopically expressed in

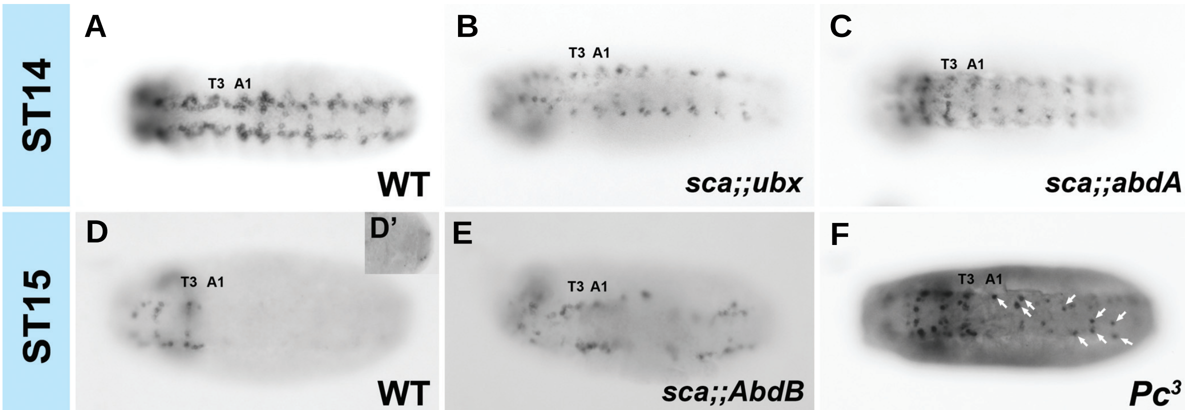

Fig. 4. *cas* mRNA expression pattern in the gain-of-function bithorax complex mutant embryos. (A) *Wild-type embryo at stage 14.* cas *transcripts were shown in abdominal segments at stage 14.* **(B)** *Ectopic* Ubx *embryo at stage 14 and* **(C)** *Ectopic* abdA *embryo at stage 14. Ectopic* Ubx *and* abdA *partially repressed* cas *expression in abdominal segments.* **(D)** *Wild-type embryo at stage 15.* cas *transcripts were observed only in thoracic segments and in the terminal segments (magnified in D') at stage 15.* **(E)** *Ectopic* AbdB *embryo at stage 15.* cas *was ectopically expressed throughout the abdominal segments.* **(F)** Pc^3 *embryo at stage 15.* cas *was also ectopically expressed with a few spots per segment throughout the abdomen.*

the first abdominal segment (Fig. 3B). In *abdA* mutant embryos, *cas* was ectopically expressed from the first to the fourth abdominal segments (Fig. 3C). In the *Ubx* and *abdA* double mutant embryos, *cas* was expressed in virtually all abdominal segments and in more cells per segment compared to the single mutant embryos of *Ubx* or *abdA* gene (Fig. 3D). These results suggest that UBX and ABDA normally suppress the expression of *cas* at late embryonic stages. However, *cas* expression was not observed in the terminal segments of *AbdB* mutant embryos at stage 15 (Fig. 3 E,E'). Although *cas* mRNA expression begins to disappear from stage 14, it can still be observed in a number of cells of the most posterior abdominal segments at stage 14 embryos. However, in *AbdB* mutant embryos (Fig. 3G), *cas* mRNA expression was observed in fewer cells than in wild type embryo (Fig. 3F), also suggesting that ABDB positively regulates *cas* expression.

Ectopic UBX and ABDA repress cas, while ectopic ABDB activates it

To confirm that UBX and ABDA repress *cas* expression in abdominal segments and ABDB activates it, the homeotic genes were expressed in the GAL4 and UAS system (Brand and Perrimon, 1993). The GAL4 transcription factor activates the UAS regulatory site, which subsequently leads to the expression of a gene adjacent to UAS. In this study *Ubx, abdA* and *AbdB* genes were ectopically derived by *sca-GAL4,* in which the homeotic genes were expressed in the *sca* domain (Fig. 2D for *AbdB*; data not shown for *Ubx* and *abdA*). When *AbdB* was ectopically expressed in the *sca* domain, the embryo showed the filzköpper, a terminal structure, in all abdominal segments, indicating that all abdominal segments were transformed to the posterior abdominal segment (Fig. 2K) (Lamka *et al.*, 1992).

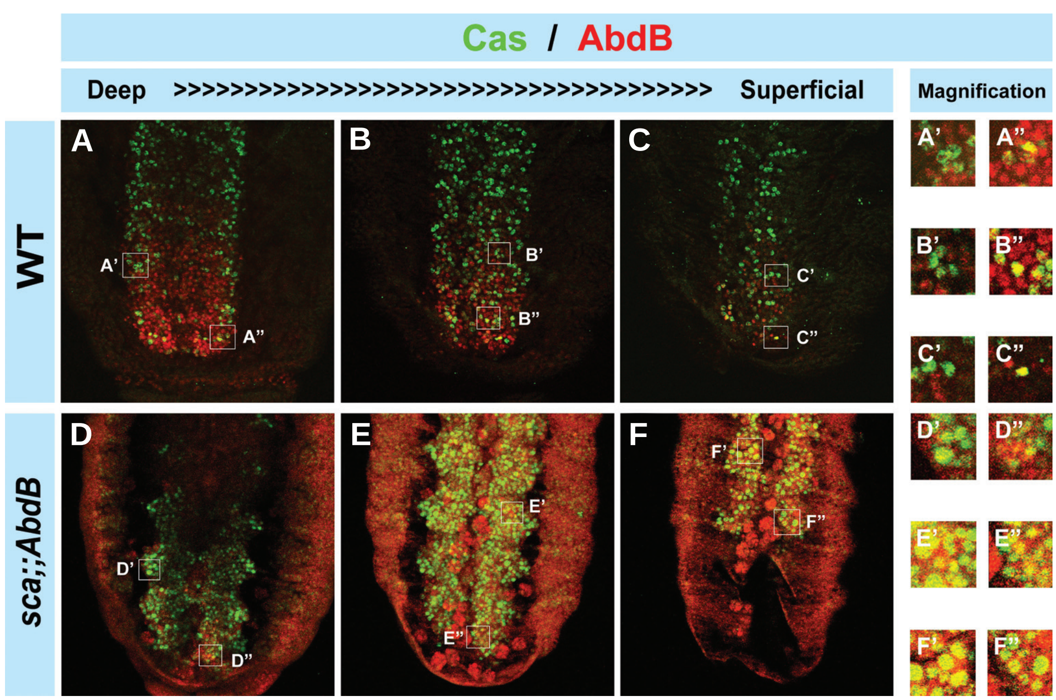

Fig. 5. Co-localization of Cas and ABDB. (A,B,C) *Wild-type embryos at stage 14. Cas (green) and ABDB (red) are co-localized in the ABDB expression domain from the deep layer to the superficial layer* **(A',B',C')**. *Cas and ABDB were coexpressed only in a few cells of the terminal segment* **(A",B",C")**. **(D,E,F)** sca;;AbdB *embryos.* cas *was expressed in the ectopic ABDB expression region* **(D",E")**, *as well as the endogenous ABDB region* **(D',E',F')**. *The superficial region of the most posterior abdominal segment does not seem to show the co-localization due to the loss of cuticle in the posterior abdominal region* **(F)**.

In order to visualize the effects of ectopic *Ubx* and *abdA*, stage 14 embryos were examined because wild-type embryos still showed *cas* expression in the abdominal segments at this stage (Fig. 4A). *cas* expression mostly disappeared in the ectopic *Ubx* and *abdA* embryos, although a few spots still remained, suggesting that *cas* expression was partially suppressed in those embryos (Fig. 4 B,C). However, in order to visualize the positive activity of *AbdB*, stage 15 embryos were selected because *cas* was not expressed in the abdominal segments, except in the terminal region, in wild-type embryos at this stage (Fig. 4 D,D'). Ectopic ABDB caused ectopic expression of *cas* in the abdominal segments (Fig. 4E). *Polycomb* group (PcG) genes function as negative regulators of the homeotic genes once the initial expression pattern is established. So the homeotic genes are ectopically expressed in the PcG mutant embryos. *Polycomb*^{3} (*Pc*^{3}), one of PcG gene mutants, causes the strong homeotic transformation from thoracic segments to abdominal segments (Lewis, 1978) and induces the ectopic expression of all three genes belonging to the BX-C (McKeon and Brock, 1991; Simon *et al.*, 1992; supplementary Fig. 2). So the abdominal segments of *Pc*^{3} embryos have a complicated situation where cas may be repressed by the ectopic UBX and ABDA, and activated by ABDB. As the all abdominal denticles of *Pc*^{3} mutant showed the transformation to the eighth one that is the *AbdB*-expression domain, this suggests that *cas* mRNA in the abdominal segments of *Pc*^{3} mutant might be activated by the ectopic *AbdB* (arrows in Fig. 4F) and ABDB dominated the effect of ectopically expressed UBX or ABDA.

cas is co-localized with ectopically expressed AbdB

In order to determine whether *cas* and *AbdB* are co-localized in the wild-type and *AbdB* gain-of-function mutant embryos, embryos were double-stained with anti-Cas and anti-ABDB antibodies, and subsequently treated with fluorescent conjugated secondary antibodies. Stage 15 embryos were examined by confocal microscopy of the deep to superficial layers. In order to clearly see the co-expression of both genes, Cas protein was examined instead of mRNA transcripts and both proteins were found in the nuclei.

In wild-type embryo, ABDB was relatively expressed in deeper cells than in superficial cells (Fig. 5 A–C), while Cas was expressed in more superficial cells than in deep cells. Cas appears as green and ABDB as red. In wild-type, Cas and ABDB are expressed in different cells in PS10-12 (Fig. 5 A',B',C'), while they are coexpressed only in a few cells of PS13 (Fig. 5 A",B",C"). Some of *sca;;AbdB* gain-of-function mutant embryos had a curved shape or a slightly twisted (Fig. 4E) or incomplete ventral closure (Fig. 5F), so that the microscopic section might not be horizontal as in wild type. Cas was expressed in more cells in the deep layer of *sca;;AbdB* gain-of-function mutant embryos than in that of wild type (Fig. 5D, magnification in D' and D''). However, in the middle and superficial layer, Cas and ABDB were co-expressed along the entire abdominal segment in which ABDB was ectopically expressed (Fig. 5 E and F, magnification in E',E" and F',F"). These results suggest that ABDB activates the expression of Cas.

Real-time PCR shows that ectopic AbdB causes overexpression of the cas gene

The *in situ* hybridization and antibody staining data demonstrated that *Ubx* and *abdA* down-regulates the expression of the *cas* gene, however, *AbdB* up-regulates *cas* gene expression. Therefore, we determined the expression level of *cas* transcript to investigate the effect of bithorax complex genes on the regulation of *cas* expression. cDNAs synthesized from total RNAs extracted from stage 15 mutant and wild-type embryos were subjected to real-time PCR. The expression level of *cas* of each mutant was compared to that of wild-type. Since we used heterozygous mutant lines containing one copy of *Ubx, abdA,* or *AbdB* gene, the expression of each gene was reduced to approximately 50 % of the wild-type carrying two copies of bithorax complex genes (Fig. 6A). The result showed that *cas* expression was increased in *Ubx* and *abdA* mutant embryos, whereas it was decreased in *AbdB* mutant embryos, suggesting possible roles of bithorax complex genes in the regulation of *cas* expression. It should be noted that the *cas* expression in *Ubx abdA* double mutant was higher than that in single mutants.

The effects of ectopic expression of bithorax complex genes on the regulation of *cas* expression was also examined using GAL4/

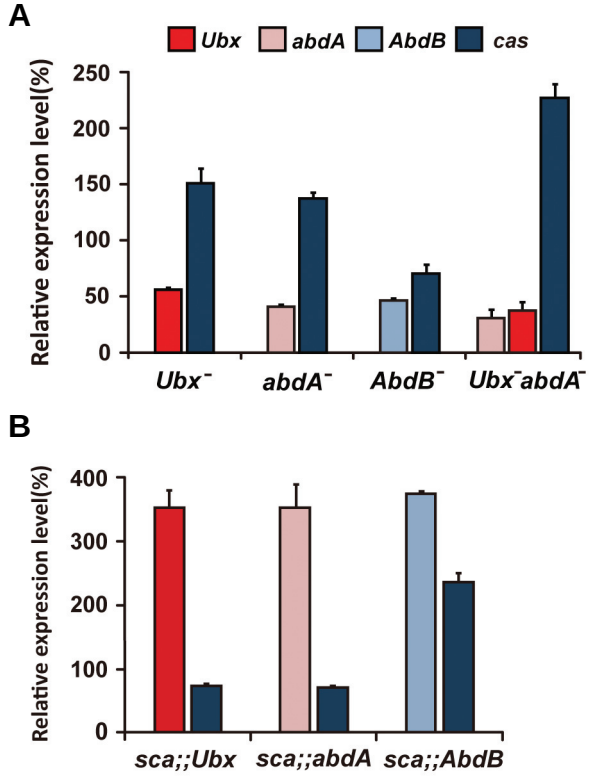

Fig. 6. *cas* mRNA expression level in stage 15 embryos determined by real-time PCR. (A) *Expression level of* cas *in the loss-of-function bithorax complex mutant embryos. Expression level of each gene in mutant embryos was compared to that in wild-type embryos.* cas *expression level was increased in* Ubx *and* abdA *mutants, whereas it was decreased in* AbdB *mutant. Note that cas expression in* Ubx abdA *double mutant significantly enhanced compared to either Ubx or* abdA *single mutant.* **(B)** *Expression level of* cas *in the gain-of-function bithorax complex mutant embryos. After crossing* sca-GAL4 *with each UAS bithorax complex lines, the embryos were collected, and incubated until stage 15. In contrast to down-regulation of* cas *in ectopic expression of* Ubx *and* abdA*, the expression of* cas *was dramatically increased in ectopic expression of* AbdB*. Each lane represents the mean plus standard deviation of three independent real-time PCR experiments.*

UAS system. Each gene driven by *sca-GAL4* was shown to have approximately 3~4 fold higher expression level compared to wild-type (Fig. 6B). The result clearly shows that the ectopic expression of *Ubx* and *abdA* suppress the *cas* expression, however, *AbdB* enhances the *cas* expression more than 2-fold, implying that UBX and ABDA may act as a repressor, whereas ABDB may function as an activator in *cas* gene expression.

Discussion

cas is transiently expressed in a subset of neuroblasts in their cell lineage. Its transcripts are present with homologous patterns in thorax and abdomen at early embryonic stages. At late embryonic stages, *cas* transcripts are found in only a few cells per hemisegment in thoracic and posterior abdominal segments, but not in other abdominal segments. This indicates that *cas* is expressed in segment-specific mode during late embryonic stages (Cui and Doe, 1992; Mellerick *et al.*, 1992). We investigated how this regional diversity was produced.

The segment-specific expression of *cas* was regulated by the homeotic genes. *Ubx* or *abdA* mutation caused the homeotic transformation of abdominal cuticle belts to the more anterior ones (Ducan, 1987). These transformation patterns were also observed in *cas* expression. Mutations in *Ubx* or *abdA* genes caused ectopic *cas* expression in abdominal segments, which was normally observed in the thoracic segments at stage 15 of wild-type embryo, suggesting transformation of the thoracic pattern to an abdominal pattern at that stage. This transformation was synergistically enhanced in the *Ubx* and *abdA* double mutants. *cas* was ectopically expressed in A1 segment in *Ubx* mutant embryos. This result is coincident with the function of *Ubx* to specify the posterior thorax and a portion of A1 segment (Beachy, 1990). *cas* was also ectopically expressed in A1 to A4 segments in *abdA* mutant embryos, which coincide with the function of *abdA* (Karch *et al.*, 1990). In *Ubx abdA* double mutant embryos, *cas* was expressed in virtually all abdominal segments and in more cells than in each single mutant embryo. The roles of *Ubx* and *abdA* on *cas* expression in the abdominal segments were confirmed in the ectopically expressed *Ubx* and *abdA* mutant embryos. For this experiment, proper embryonic stages were very important because *cas* expression changed dramatically in the abdominal segments between stages 14 and 15. We investigated whether ectopic *Ubx* or *abdA* repressed *cas* expression in the abdominal segments at this stage. The GAL4-mediated induction of *Ubx* or *abdA* suppressed *cas* expression in the abdominal segments.

However, in contrast to the *Ubx* and *abdA* mutations, *AbdB* mutation caused reverse effects on *cas* expression in the abdominal segments, which have never been reported. Loss-of *AbdB* function caused lack of *cas* expression, while ectopic ABDB activated *cas* expression in the abdominal segments. This phenotype was also observed in *Polycomb* mutant embryos. Although *Polycomb* mutation induced ectopic expression of *Ubx*, *abdA* and *AbdB* at the same time (McKeon and Brock, 1991; Simon *et al.*, 1992), *cas* was ectopically expressed in the abdominal segments of stage15 embryos, suggesting that ABDB dominated the effects of ectopic UBX or ABDA (Lamka *et al.*, 1992). The co-localization of Cas and ABDB is found in a few cells in the posterior abdominal segments, supporting the positive regulation of *cas* by ABDB. This idea was further intensified by the appearance of the ectopic *cas* mRNA in the numerous abdominal cells with the ectopic *AbdB* expression. Real-time PCR experiment showed the overexpession of *cas* mRNA in the ectopically expressed *AbdB* embryos, also supporting the positive regulation of *cas* by ABDB. Furthermore, seven AbdB DNA binding sites were found within 5kb upstream from the *cas* transcription start site enhancing the possibility that ABDB directly regulates the *cas* expression. ABDB binds preferentially to a sequence with an unusual 5'-TTAT-3' core (Ekker *et al.*, 1994).

One of questions was why all the cells with the *AbdB* expression does not show *cas* mRNA expression. In wild-type embryos, all *AbdB*-expressing cells does not show *cas* mRNA. Only a few cells among *AbdB*-expressing cells could maintain the expression of *cas* and the other cells lost it. This might be that the homoetic proteins carry out their function by interacting with other cofactors to regulate distinct sets of downstream genes (Moens and Selleri, 2006; Laurent *et al.*, 2008; Foronda *et al.*, 2009).

Accumulating evidence shows that the bithorax complex genes are involved at different steps in the segment-specific divergence of the CNS. *Ubx* or *abdA* activity is required for the abdominal pathway of the NB1-1 lineage. Both ectopic induction of *Ubx*- or *abdA* expression until several hours after gastrulation and homeotic de-repression in *Polycomb* mutants, override thoracic determination of NB1-1 (Prokop and Technau, 1994). The abdominal NB6-4 lineage is also specified by the *abdA* and *AbdB*. *abdA* is expressed in the NB6-4 lineage of abdominal segments A1-A6, whereas *AbdB* is expressed in the NB6-4 lineage of segments A7-A8 (Berger *et al.*, 2005). They specify the abdominal NB6-4 lineage by down-regulating levels of G1 Cyclin (*CycE*).

In summary, UBX and ABDA suppress *cas* expression in abdominal segments, so that mutation in both genes causes ectopic expression of *cas* in abdominal segments at late embryonic stage. However, ABDB activates *cas* expression, which is supported by co-localization of Cas and ABDB in cells ectopically expressing *AbdB*, and real-time PCR in ectopically expressed AbdB embryos.

Materials and Methods

Fly stocks

Oregon R was used as the wild-type strain. We used the following mutant strains: $Ubx^{9.22}$ (Capdevila and García-Bellido, 1981), $abdA^{MX1}$, and $AbdB^{M2}$ (Sánchez-Herrero *et al.*, 1985), *Df(3R)Ubx109/Dp3;3(P5)* (*Ubx abdA* double mutant) (Bloomington *Drosophila* stock center), Pc^{3} (Lewis, 1978). Ectopic expression was performed using the UAS/GAL4-system as described in Brand and Perrimon (1993). *Scabrous-GAL4* (*sca-GAL4*) was used to express *UAS-Ubx, UAS-abdA,* and *UAS-AbdB* (Castelli-Gair *et al.*, 1994) for ectopic expression of *Ubx, abdA* or *AbdB*, respectively. Such embryos were named *sca;;Ubx, sca;;abdA* or *sca;;AbdB*, respectively. *sca* is expressed in virtually all nervous cells (Mlodzik *et al.*, 1990).

Antibody staining

Antibody staining was performed under standard conditions as described elsewhere (Patel, 1994; Jung *et al.*, 2008). Staged embryos were collected, dechorionated, fixed, devitellinized, and then stained with antibodies. After embryos were preincubated with 5% goat serum, they were treated with the primary antibody. Primary antibodies used were: rabbit anti-Cas (1: 1,000, kindly provided by C. Doe, University of Oregon, USA). The color reaction was developed with the Vectastain ABC kit (Vector Labs). Embryos were viewed and photographed with an Olympus

microscope BX51. Embryonic stages are as described by Campos-Ortega and Hartenstein (1985).

To visualize the coexpression of Cas and ABDB, embryos were double-stained with anti-Cas and anti-ABDB antibodies (Leuyer *et al.*, 2008). The primary signal was amplified and detected with Tyramide 488 and 594 conjugated anti-IgG. Whole-mount embryos were photographed using a Carl Zeiss-LSM510 confocal microscope.

Whole-mount in situ *hybridization*

Whole-mount *in situ* hybridization was performed using digoxigenin-labeled antisense RNA probes (Tautz and Pfeifle, 1989; Kim *et al.*, 2008). Proper stages of embryos from Oregon-R and each mutant were collected, fixed in formaldehyde, and prepared for hybridization. The embryos were then treated with 5-mg/ml proteinase K; this activity was later repressed with 2-mg/ml glycine. Embryos were incubated for hybridization at 55ºC. The following day, the embryos were treated with AP-conjugated anti-Dig antibody and a color reaction was initiated with NBT and BCIP, after which the stained embryos were examined using the Olympus BX51.

Embryonic cuticle preparation

Wild-type embryos were collected for an hour and then incubated. Once they were enclosed to larvae, they were transferred to a 1:1 mixture of Hoyer's mounting solution and lactic acid (Kwon *et al.*, 2003) for a day at room temperature and then another day on the slide warmer. For mutants, eggs were collected for a day and further incubated for 24 hrs at 25ºC. Brown-colored lethal embryos with a pharyngeal skeleton were collected and transferred to double-sided cellophane tape for manual dechorionation with a fine tungsten needle. The internal structures of dechorionated embryos were removed in the same way as from wild-type embryos. Embryos were viewed and photographed using an Olympus BX51 equipped with dark-field.

Real-time PCR

Stage 15 embryos were collected on an agar-grape juice plate with yeast paste. After dechorionation using 50 % bleach solution, embryos were homogenized and total RNA was extracted using RNeasy Mini Kit (Qiagen). The purity and the amount of RNA were determined by spectrophotometer. cDNA was generated from the total RNA by reverse transcription using oligo $(dT)_{18}$ as a primer and reverse transcriptase (Promega). The cDNA was subjected to quantitative real-time PCR using ABI 75100 sequencer detector (Applied Biosystems) to detect the expression level of the genes, *Ubx, abdA, AbdB* and *cas*. Forward and reverse primers are shown in Supplementary Table 1. The quantitative real-time PCR was performed by StepOnePlus™ Real-time PCR System (Applied Biosystems) using power SYBR green PCR master Mix (Applied Biosystems). The thermal cycling conditions were 95ºC for 10 min, followed by 40 cycles of 95ºC for 15 sec and 60ºC for 60 sec. *Actin5C* was used as an endogenous (internal) control. The expression level of *Ubx*, *abdA*, *AbdB*, and *cas* from wild-type embryos was considered as 100 %, and the expression level of the genes from the mutant embryos was compared to the expression level of the genes those from wild-type embryos.

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