Sequence-specific interaction between ABD-B homeodomain and castor gene in Drosophila

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We have examined the effect of bithorax complex genes on the expression of castor gene. During the embryonic stages 12-15, both Ultrabithorax and abdominal-A regulated the castor gene expression negatively, whereas Abdominal-B showed a positive correlation with the castor gene expression according to real-time PCR. To investigate whether ABD-B protein directly interacts with the castor gene, electrophoretic mobility shift assays were performed using the recombinant ABD-B homeodomain and oligonucleotides, which are located within the region 10 kb upstream of the castor gene. The results show that ABD-B protein directly binds to the castor gene specifically. ABD-B binds more strongly to oligonucleotides containing two 5'-TTAT-3' canonical core motifs than the probe containing the 5'-TTAC-3' motif. In addition, the sequences flanking the core motif are also involved in the protein-DNA interaction. The results demonstrate the importance of HD for direct binding to target sequences to regulate the expression level of the target genes. [BMB Reports 2014; 47(2): 92-97]

INTRODUCTION

Homeotic/Hox genes were first identified in Drosophila, and encode transcription factors that play a pivotal role in giving a unique identity to each segment, thereby setting up morphogenesis along the anterior-posterior axis. The homeotic genes are evolutionarily conserved in organisms ranging from ctenophores to mammals (1). Loss-of-function mutants transform one segment into a copy of another segment (2, 3). In Drosophila, the homeotic cluster consists of eight genes which are divided into two groups: Antennapedia complex (ANTP-C) and Bithorax complex (BX-C), on the third chromosome. In the BX-C, Ultrabithorax (Ubx), abdominal-A (abd-A), and Abdominal-B (Abd-B) are responsible for determining the identity of thoracic and abdominal segments (4, 5).

The homeotic genes contain a highly conserved 180-bp homeobox encoding homeodomain (HD), a DNA-binding domain consisting of 60 amino acid residues. Although the primary amino acid sequences are different from Hox proteins, the threedimensional structures are very similar, representing a well-conserved mode of HD-DNA interaction during evolution (1). The HD consists of a flexible N-terminal arm and three α-helices (6). The N-terminal arm is positioned in the minor groove, and is required for the specificity of the protein action. The second and third helices of the HD fold into a conformation similar to the helix-turn-helix DNA-binding motif, and bind in the major groove of the DNA, making them essential for DNA binding (6).

HD proteins recognize a canonical core sequence 5'-TTAT-3', which plays a major role in determining high binding affinity with an approximate $K_d$ value of $10^{-8}$ to $10^{-11}$ M, and regulate the expression of a target gene acting either as an activator or a repressor (7-9). In contrast, ABD-B HD binds preferentially to a sequence with an unusual 5'-TTAT-3' core motif instead of 5'-TAAT-3' for other HD proteins (10). It has been reported that the 10 bp sequence (5'-TTATGGCC-3') is the optimal DNA binding site for the ABD-B HD, and the N-terminal arm of ABD-B HD is responsible for the sequence-specific binding (11).

ABD-B protein is expressed in the ectoderm and mesoderm of parasegments 13-15, and strongly expressed in the parasegment 14 central nervous system (CNS) at germ band retraction (12, 13). Therefore, ABD-B functions primarily to assign identities to parasegments 10 to 14, and it is also involved in the formation of CNS (14).

Castor protein contains a zinc finger domain, and is expressed in a restricted set of neuroblasts and glia in the cephalic regions and the ventral nerve cord (15). A loss-of-function mutation of castor results in alterations to gene expression in CNS, followed by defects in axonogenesis (16). The castor is also involved in the development of mushroom body in post-embryonic stages (15), and in neuronal differentiation (17). The expression level of the castor transcripts is the highest in the CNS of embryos at stage 14, and is detectable only in a few cells of the thoracic and terminal abdomen in stage 15 embryos (16, 18). Recently, the roles of BX-C genes in the regulation of castor expression have been studied using anti-
body staining and in situ hybridization. The Ubx and abd-A genes negatively regulated castor expression, whereas the relationship between ABD-B and castor was positive (18).

In this study, to determine the effect of Ubx, abd-A, and Abd-B on castor expression, the expression levels of castor during embryogenesis were examined using real-time PCR. The results showed that the expression of castor was positively regulated by Abd-B, in contrast to Ubx and abd-A, which regulate castor expression negatively. The relationship between BX-C genes and castor are not changed during the embryonic stages 12 through 15. We also demonstrated that the recombinant ABD-B HD specifically binds to the upstream promoter sequence of castor, implying that ABD-B directly regulates the expression of the castor gene.

RESULTS AND DISCUSSION

Regulation of castor gene expression by Bithorax complex

The segment-specific expression patterns of castor suggest that its expression is regulated by BX-C. Therefore, in order to investigate the effect of BX-C on the expression of the castor gene, we generated BX-C mutant lines and measured the transcript level of castor. For the homozygous null mutant embryos for bithorax genes, the TM1 balancer chromosomes in BX-C mutant lines were replaced by TTG balancer chromosomes, and the null embryos lacking a fluorescence signal were collected. For overexpressing BX-C genes, sca-GAL4 was crossed with each UAS-Ubx, UAS-abdA, or UAS-AbdB line.

Total RNAs were extracted from the embryos either lacking or overexpressing BX-C genes at stage 15, and real-time PCR was carried out using the primers for BX-C genes and castor (Supplementary Table 1). The expression levels of BX-C genes

![Fig. 1. Expression levels of castor (cas) transcripts determined by real-time PCR. Total RNAs were extracted from stage 15 embryos in which each BX-C gene is mutated or overexpressed. The expression levels of each gene were compared to that in wild-type embryos. (A) Relative expression levels of castor in Ubx and abd-A mutant and Abd-B OE embryos. The expression of castor was down-regulated in Abd-B mutant embryos. (B) Relative expression levels of castor upon overexpression (OE) of BX-C genes. Expression of castor in Ubx and abd-A OE embryos was decreased. (C) Expression of castor in BX-C mutant embryos at stages 12 through 15. Expression of castor transcript did not depend on embryonic stage. The means and standard deviations from three independent experiments are shown.](http://bmbreports.org)
were decreased or increased by more than 3-fold in BX-C mutants or overexpressing embryos, respectively, compared to those in wild-type embryos (data not shown). The castor expression was increased in Ubx and abd-A mutant embryos, whereas it was decreased in Abd-B mutant embryos (Fig. 1A). Conversely, the ectopic expression of Ubx and abd-A suppressed the expression of the castor gene, but Abd-B enhanced the castor transcript level by approximately 2.5-fold (Fig. 1B). These results clearly demonstrate the positive regulation of ABD-B and the negative regulation of UBX and ABD-A on castor gene expression.

Since the expression patterns of castor are dramatically changed during stages 14 and 15 (18), we further examined whether the effect of BX-C on castor gene expression is dependent on the developmental stage. The BX-C mutant embryos were collected at each developmental stage from 12 to 15, and the castor transcript level was measured. The result displayed that the castor expression was up-regulated in both Ubx−/− and abd-A−/− embryos, and down-regulated in Abd-B−/− embryos during stages 12 through 15, implying that the action of BX-C on castor expression is not stage-specific (Fig. 1C).

Taken together, in agreement with a previous study (18), these results suggest that both UBX and ABD-A act as repressors, whereas ABD-B acts as an activator to control castor gene expression.

**Preparation of ABD-B homeodomain and analysis of castor promoter region**

To examine whether BX-C directly interacts with the castor gene, we performed electrophoretic mobility shift assays (EMSA) using the ABD-B HD and oligonucleotides, which are located approximately within the 10 kb upstream of the castor gene. The Abd-B fragment containing HD was cloned into the pET30b expression vector (Fig. 2A), and the recombinant protein was purified. Coomassie staining of the purified protein indicated purity greater than 90% (Fig. 2B), and immunoblot analysis using anti-His·tag antibody confirmed the approximately 11 kDa protein of recombinant ABD-B HD, as indicated by an arrow in Fig. 2C.

Recently, it was reported that ABD-B HD binds to the responsive elements containing a canonical 5’-TTAT-3’ core motif and a non-canonical 5’-TTAC-3’ motif derived from the promoter region of the yellow gene (19). In addition, it has been identified that the upstream region of the castor gene contains seven conserved DNA sequence clusters to regulate the expression of castor gene in the embryonic brain and ventral nerve cord (20). Thus, we searched for the ABD-B binding motifs in the castor promoter region up to −10 kb, and found seven 5’-TTATGG-3’ binding motifs and five 5’-TTACGA-3’ binding motifs for ABD-B HD (Supplementary Table 2). Among them, we selected the most putative ABD-B binding sites for EMSA analysis: the cas1 containing 5’-TTAC-3’ motif, the cas2 carrying 5’-TTAT-3’ motif, and the cas3 containing two canonical core motifs which are overlapped by one thymidine (Supplementary Table 1). The 8-base pair core sequences of cas1 and cas3 have been reported as ABD-B binding sites in the promoter region of the yellow gene (19), and the core sequence of cas2 was analyzed as an ABD-B HD binding site in cut, spalt, and unpaired genes (11).

**EMSA analysis and kinetic study**

Generally, BX-C genes are mutually exclusive, so posteriorly expressed BX-C genes down-regulate the more anteriorly expressed ones on the embryonic surface. However, in the development of male and female sex organs and appendages, BX-C genes cooperatively interact with one another (21, 22).
To examine the relationship between BX-C genes, the ABD-B binding site within the abd-A promoter region was analyzed by employing EMSA (Fig. 3A). The amount of protein-probe complexes is elevated as the concentration of ABD-B HD increases, demonstrating physical interaction between the recombinant ABD-B HD and abd-A probe.

Next, we explored whether ABD-B directly binds to the castor promoter region. The result showed that ABD-B HD interacted with all cas probes examined (Fig. 3B-D). In the competition assay adding a non-biotinylated cold probe with 200-fold excess molar concentration, no protein-probe complex was detected, indicating that the proteins specifically recognize the probe sequences. The specific interaction of ABD-B HD and cas probes was further confirmed using the cas(m) probes containing mutations within core binding motifs. The formation of the protein-probe complexes was significantly diminished in the cas(m) probes shown in the left panels in Fig. 3B-D, representing a sequence-specific interaction of ABD-B HD with the castor gene.

The binding affinities of the recombinant protein with its target sequences were measured by a luminescent image analyzer, and the equilibrium dissociation constant (Kd) was determined (Supplementary Fig. 1). The highest affinity of ABD-B HD was observed for the cas3 probe containing two 5'-TTAT-3' canonical core motifs (Table 1). This is consistent with a study in which a probe carrying more than one motif displayed higher affinity for ABD-B protein than that carrying only one motif (19). In addition, we revealed that the binding affinities for 5'-TTAT-3' and 5'-TTAC-3' core motifs did not differ much since cas1 and cas2 showed similar binding affinities. It should be noted that the binding affinity of cas1 for ABD-B HD is higher than that of the abd-A probe, although both probes contain the same 8-base pair core motif, implying that the flanking nucleotides are also involved in the interaction with ABD-B HD. It has been reported that the flanking bases contribute to the overall affinity and sequence specificity, possibly by interacting with the N-terminal flexible arm of the HD (6, 23). Therefore, we speculate that the flanking nucleotides of the abd-A probe are not favorable to the access of ABD-B HD. Taken together, we suggest that ABD-B can regulate the expression of castor transcripts by interacting with the castor promoter region.

In summary, we showed that BX-C genes are involved in regulating the expression of the castor gene by real-time PCR. Consistent with a previous study (18), ABD-B positively regulates castor gene expression, whereas UBX and ABD-A regulate castor gene expression negatively during embryonic stages 12 through 15. From the analysis of the castor promoter region, we selected the most putative ABD-B binding sites containing 5'-TTTTATGG-3' and 5'-TTTTACGA-3' core motifs for ABD-B HD. EMSA results showed that ABD-B HD specifically recognizes these sequences and directly binds to them. This is the first demonstration of the binding of ABD-B to the castor gene. The precise role of ABD-B in the regulation of castor expression needs to be addressed in future experiments.

### MATERIALS AND METHODS

**Drosophila strains and cross**

UbxB<sup>2.32</sup>, abd-A<sup>Am</sup>, and Abd-B<sup>M2</sup> carrying a loss-of-function mutation of BX-C genes, scabrous-GAL4 (sca-GAL4) expressing GAL4 transcription factor in some proneural clusters and sensory organ precursor cells, and Dr/TTG (stock number 6663) were obtained from the Bloomington Drosophila Stock Center (Indiana). UAS-UBX, UAS-abdA, and UAS-AbdB were kindly provided by S. H. Jeon. To generate the homozygous null mutant embryos for bithorax genes, the TM1 balancer chromosomes in the Ubx<sup>9.22</sup>, abd-A<sup>Am</sup>, and AbdB<sup>M2</sup> lines were replaced by TTAG balancer chromosomes carrying P-element containing twi-GAL4 and UAS-2xEGFP. The flies carrying one copy of bithorax mutant gene and TTAG were crossed with each other, and the null embryos lacking a fluorescence signal were collected under a dissecting fluorescent microscope. To overexpress bithorax complex genes, sca-GAL4 was crossed with each UAS-Ubx, UAS-abdA, or UAS-AbdB line. The Oregon-R strain was used as a wild-type control.

**Real-time PCR**

Embryos at each stage (12 through 15) 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 an RNeasy Mini Kit (Qiagen). The purity and the amount of RNA were determined by nano micro-volume spectrophotometer (Maestro). cDNA was gen-

### Table 1. Summary of relative affinity of the recombinant ABD-B homeodomain for various target sequences

| Probe   | Binding motif       | Nucleotide position<sup>a</sup> | K<sub>d</sub> (nM)<sup>b</sup> | Fold compared to abd-A<sup>c</sup> |
|---------|---------------------|---------------------------------|------------------------------|-----------------------------------|
| abd-A   | 5'-TTTACGA-3'       | −4,489                          | 5.48 × 10<sup>−9</sup>       | 1X                                |
| cas1    | 5'-TTTACGA-3'       | −7,410                          | 2.98 × 10<sup>−9</sup>       | ↑ 1.84X                           |
| cas2    | 5'-TTTTATGG-3'      | −7,461                          | 3.23 × 10<sup>−9</sup>       | ↑ 1.70X                           |
| cas3    | 5'-TTTATAG-3'       | −10,169                         | 2.30 × 10<sup>−9</sup>       | ↑ 2.38X                           |

<sup>a</sup>Position of the 5' end of core motif is represented by the nucleotide number from the transcription start site according to flybase annotation. The binding motif of cas1 and cas3 is shown in reverse orientation. <sup>b</sup>The affinity was shown by K<sub>d</sub> value, equilibrium dissociation constant. <sup>c</sup>The affinities for each cas probe are compared to that for abd-A probe.
PCR was performed using a StepOnePlus™ Real-time PCR System with Power SYBR Green PCR Master Mix (Applied Biosystems). The real-time PCR was performed using a StepOnePlus™ Real-time PCR System according to the manufacturer's protocol with a minor modification. The thermal cycling conditions were 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 60 sec. 

Immunoblot analysis

The samples from each column fraction were mixed with equal volumes of SDS sample buffer and boiled for 10 min. Ten micrograms of proteins were electrophoresed on an 8-14% gradient denaturing polyacrylamide gel, and transferred to PVDF membrane (Immobilon) by electroblotting. The membrane was soaked in PBS buffer containing 0.2% casein and 0.1% Tween-20 for 1 h, and incubated with anti-His-tag antibody (1: 250 dilution) (Vector Laboratories) for 30 min. After incubation with the secondary antibody conjugated with biotin (1: 500 dilution) for 30 min, the membrane was treated with streptavidin conjugated with alkaline phosphatase (1: 1,000 dilution) (Vector Laboratories) for 30 min. After washing the membrane with PBS containing 0.05% Tween-20, the membrane-bound ABD-B HD was visualized by adding NBT (Nitroblue tetrazolium chloride)/BCIP (5-bromo-4-chloro-3-indolyl phosphate) in neutralization buffer (0.1 M Tris-HCl, 0.1 M NaCl, pH 9.5).

EMSA

The biotinylated oligonucleotides containing ABD-B HD binding sequence were prepared (IDT) by analyzing the region 10 kb upstream of the castor gene. The forward and reverse oligonucleotides for each probe are shown in supplementary Table 1. For the preparation of the EMSA probe, biotinylated forward oligonucleotide was mixed with reverse oligonucleotide at equal molar concentrations. The mixture of oligonucleotides was annealed by heating at 95°C for 10 min, and slowly cooled down to room temperature to generate a double-stranded probe. In each reaction, 10 fmol probes were mixed with the purified ABD-B HD up to 10 nM in binding buffer containing 2.5% glycerol, 5 mM MgCl₂, 50 ng/μl poly (dI-dC), and 0.05% NP-40. After incubation of the reaction mixture at 25°C for 20 min, the samples were run on a non-denaturing 7% polyacrylamide gel in 0.5X TBE (45 mM Tris-HCl, 45 mM boric acid, 1 mM EDTA, pH 8.3), and transferred to a nylon membrane (Whatman).

EMSA reactions were carried out with a LightShift Chemiluminescent® EMSA Kit according to the instructions (Pierce). In short, after cross-linking the probe to the membrane at 120 ml/cm² using a crosslinker (Spectronics Corp.), the membrane was incubated in streptavidin conjugated with horseradish peroxidase for 15 min. After washing the membrane, the protein-probe complex and free probe were detected by a Chemiluminescent Nucleic Acid Detection Module (Pierce), and visualized by X-ray film. The competition assay was performed by adding competitor DNA with 2 pmol of unlabeled oligonucleotide (200-fold excess) to the reaction mixture.

To determine the binding affinity of ABD-B HD with the probes, the band intensities were measured using an LAS-3000 luminescent image analyzer (Fuji Film), and the equilibrium dissociation constants (Kd) were determined using GraphPad Prism 4 software.

ACKNOWLEDGEMENTS

We would like to thank S. H. Jo for analysis of the data, and S. J. Kim for preparation of the manuscript. Special thanks to S. H. Jeon for providing Drosophila stocks. This research was supported by the Yeungnam University Research Grant (209A356024).
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