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Analysis of ecdysone-pulse responsive region of *BMWCP2* in wing disc of *Bombyx mori*

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ABSTRACT

The present study was undertaken to clarify the regulation of cuticle-protein gene expression. *Bombyx* BAC library was screened to obtain the sequences of regulatory regions of cuticle protein genes isolated from wing discs of *Bombyx mori*. Two kb upstream of *BMWCP2* was cloned into a reporter plasmid, and a reporter assay was operated. Plasmids were introduced into wing discs and wing tissues using a gene gun. DNA introduction into wing discs was confirmed with plasmid pA3GFP. The upstream region of *BMWCP2* showed stage-specific activity: strongest at P0. EMSA analysis indicated the binding of BmβFTZ-F1. Ecdysone pulse-responsive sequences were examined *in vitro*. A luciferase assay was performed using reporter plasmids that contained different length upstream-regions of *BMWCP2*. With this method, we identified the ecdysone-responsive region. With deletion of the *BMWCP2* upstream region, mutagenesis of the BmβFTZ-F1 binding site and EMSA analysis, it was confirmed that the *BMWCP2* expression was regulated by BmβFTZ-F1 through the ecdysone pulse. This is first to apply the introduction of reporter plasmids into small organs to examine the developmental and hormonal regulation of the cuticle protein gene expression. We demonstrated that the binding of BmβFTZ-F1 facilitated the promoter activity of the *BMWCP2* cuticle protein gene *in vitro*.

1. Introduction

Insect cuticle is built up in layers, which are constructed with different proteins. Hundreds of cuticle proteins exist in insects, and combinations form the spatio- and temporal-specific cuticle (Willis, 1996). The cuticle protein genes have different stage-specific expression patterns, which indicate that they have different hormone-responsive characteristics. *BMWCP1-9* and *BmGRP1-3* in *Bombyx mori* have been reported to be ecdysone-pulse responsive (Noji et al., 2003; Zhong et al., 2006). By contrast, *BMWCP10* in *B. mori* (Noji et al., 2003) and *ACP-20* in *Tenebrio molitor* (Braquart et al., 1996) were up-regulated by 20-hydroxyecdysone, and *LCP-14* in *Manduca sexta* was down-regulated by it (Hiruma et al., 1991). Recently, the genome wide analysis enabled large-scale expression analysis of cuticle protein genes in *Anopheles gambiae* (Togawa et al., 2008), which demonstrated the several stage-specific expression patterns of cuticle protein genes. The regulatory mechanism controlling the expression of cuticle protein genes has not been clarified, even though some reports have identified cis-regulatory elements relating to the spatio- and temporal-specific expression of the cuticle protein genes in *Drosophila melanogaster* (Murata et al., 1996; Kawasaki et al., 2002; Bruey-Sedano et al., 2005; Kayashima et al., 2005) and *T. molitor* (Lemoine et al., 2004).

Ecdysone plays an essential role during the metamorphosis of insects. The fluctuation of hemolymph ecdysteroid titer at the initiation of metamorphosis is well known in insects (Riddiford and Truman, 1993; Thummel, 1995). Molecular aspects showed that ecdysone functioned as a signal to activate or repress target genes, which regulate morphogenesis in *D. melanogaster* (White et al., 1997; Natzle, 1993). Most ecdysone-responsive genes encode transcriptional regulators that recognize specific DNA sequences. Among these transcriptional regulators, βFTZ-F1 has been suggested to be a regulator responsible for the stage-specific expression of cuticle protein genes during the prepupal stage. After being expressed, DmβFTZ-F1 has also been found to positively regulate the pupal cuticle protein gene *EDG 84A* and *EDG74E* during the mid- to late-prepupal period in *D. melanogaster* (Murata et al., 1996). Studies using mutants have shown that DmβFTZ-F1 is required for normal larval cuticle production (Yamada et al., 2000). *BmβFTZ-F1*, an ortholog of *DmβFTZ-F1* in silkworm *B. mori*, is expressed during larval, pupal molts and adult development in coincidence with ecdysone pulse; *BmβFTZ-F1* expression requires ecdysone signaling, but it is only activated as hemolymph ecdysteroid levels begin to decline (Sun et al.,

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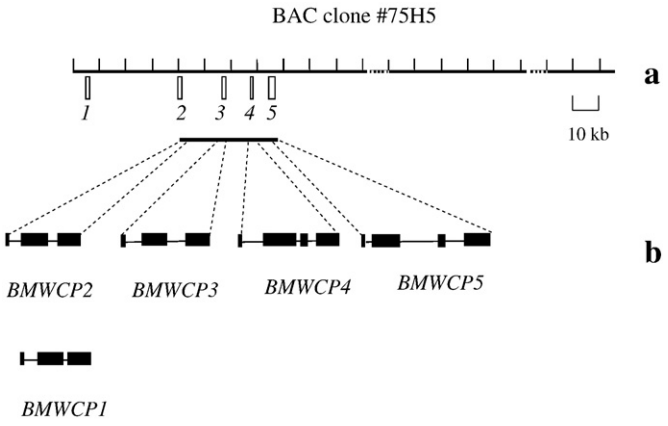

Fig. 1. Genomic structure of *BMWCP1-5* cuticle protein genes of *B. mori*. (a), Schematic map of *BMWCP1-5* located in the BAC clone, #75H5. All the genes were located on the minus strand of the BAC clone. The bold lines indicate the sequenced areas. The open boxes indicate the areas of *BMWCPs* genes located at the genome. (b), Enlarged region of *BMWCP2*-5 and *BMWCP1* and their exon/intron structures. Left hand is 5′ direction. The closed boxes indicate exons of *BMWCPs*.

1994). However, the detailed role of BmβFTZ-F1 in cuticle formation remained to be elucidated.

We had identified several cuticle protein genes in the wing disc of *B. mori* and separated them into groups with different developmental profiles. Among them, *BMWCP2*, *3*, *7*, *8* and *9* are expressed only during pupation. In contrast, *BMWCP1*, *4*, *5*, and *6* are expressed twice, during pupation and in the mid-pupal stage (Takeda et al., 2001). *BMWCP1-9* are suggested to be targets of BmβFTZ-F1 and to have BmβFTZ-F1 binding sites, since the expression of them was related with that of BmβFTZ-F1; their expression was observed after the peak of hemolymph ecdysteroid (Sun et al., 1994; Takeda et al., 2001), and *BMWCP2*, one of them, was induced by the 20-hydroxyecdysone (20E) withdrawal *in vitro* (Noji et al., 2003). *BMWCP2* required ecdysone signaling, but it was only activated as 20E levels began to decline (Noji et al., 2003), as well as *BmβFTZ-F1* in wing discs or wing tissues (Shiomi et al., 2000; Zhong et al., 2006). BMWCP2 is likely to be indirectly induced by 20E (through intermediate factors), in contrast to a related gene *BMWCP10* (Noji et al., 2003). To address these mechanisms, we tried to obtain the genomic sequences of *BMWCPs* and determine the BmβFTZ-F1-regulatory region in the present study. Wing disc seems to be an appropriate tool for the study of ecdysone function and the regulatory mechanism of spatio- and temporal-specific gene expression, since the wing disc is suggested to produce various types of cuticle protein and is easy to be cultured. Moreover, cuticle protein mRNAs are abundant, short in size, and transcribed in a short period of time (Takeda et al., 2001).

Using *Drosophila* cells (Rudolph et al., 1991) or organs (Chen et al., 2002), the promoter activity has been estimated with reporter assays. Lemoine et al. (2004) applied a transfection method to the introduction of the promoter region of *ACP20* in the pupal wing tissue of *T. molitor*. In contrast, wing discs are surrounded with the wing sac and the basement membrane, so another method is required for the introduction of DNA to wing discs. Since a gene gun system with silk glands of *B. mori* has been examined and it was successful in silk glands (Takahashi et al., 2003; Shimizu et al., 2007), we tried to apply this method to wing discs and wing tissues. The present study was operated to make the system to clarify the regulation and the interaction of transcription factor with regulatory region of cuticle protein genes of *B. mori*, and confirm the BmβFTZ-F1 interaction with *BMWCP2* regulatory region by using this system. In this study, we performed a transient promoter assay of *BMWCP2* in wing discs by using a gene gun, thereby we analyzed the transcriptional regulation that controlled the stage-specific expression. We established the optimal conditions of the gene gun system in wing discs and showed that the stage-specific and ecdysone-pulse inducible expression of *BMWCP2* was regulated by BmβFTZ-F1.

2. Materials and methods

2.1. Experimental animals

The larvae, hybrids of the N124 and C124 *B. mori* strains, were reared at 25 °C. Wandering occurred on day 6 of the 5th larval instar, pupation occurred 3 days thereafter, and adults eclosed 10 days after pupation. The periods (in days) corresponding to the developmental stages of larval ecdysis to fifth instar, wandering, pupation, and eclosion were designated as V0, W0, P0, and A0, respectively.

2.2. Screening of the BAC library of B. mori and sequence analysis

The probes for the screening of BAC library were labeled using the ECL Direct Nucleic Acid Labeling and Detection System (Amersham, UK) just before hybridization. A silkworm high-density filter of the BAC library (Koike et al., 2003) was screened. Sequencing of BAC clone #75H5 was performed by BAC shotgun sequencing as described previously (Koike et al., 2003).

2.3. Plasmid constructs

The construction of plasmids and used primers in the present study are shown in Fig. 2 and Table 1. The 1708 bp fragment containing the upstream region of *BMWCP2* was amplified by PCR using 1708 forward primer, *BMWCP2* reverse primer and BAC clone #75H5 (Acc. No. AB262389) of the *Bombyx* P50-strain as a template. After digestion with KpnI and Hind III, this fragment was cloned into the KpnI-Hind III sites of PGV-B (Toyo Inc.), and the construct was named *WCP2-1708*. Fragments of 942 bp and 442 bp were made by PCR using a 942 or 442 forward primer and a *BMWCP2* reverse primer, and *WCP2-1708* was used as a template. Fragment of 170 bp was amplified by PCR using a 170 forward primer and a PGV-B reverse primer derived from the vector's sequence and *WCP2-1708* was used as a template. After digestion with KpnI and Hind III, fragments of 942 bp, 442 bp, and 170 bp were cloned into the KpnI-Hind III site of PGV-B, and these

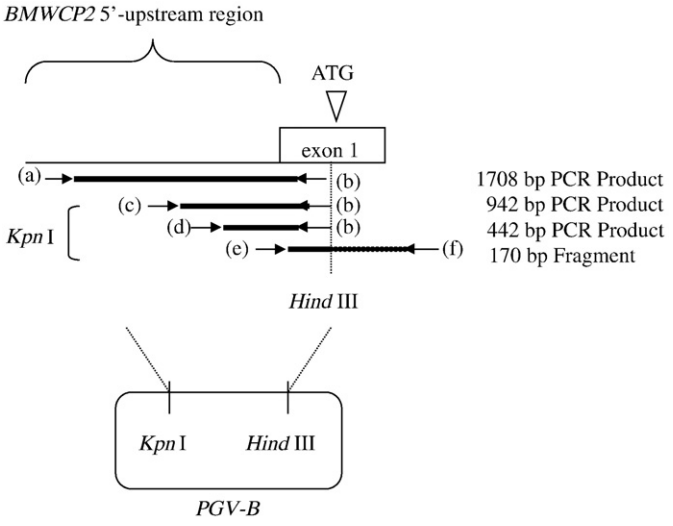

Fig. 2. Schematic representation of reporter constructs. Constructs of *WCP2-1708*, *WCP2-942*, *WCP2-442* and *WCP2-170* are shown. Upstream sequences of *BMWCP2*, amplified by PCR, were inserted into PGV-B between the KpnI and HindIII sites. The arrows indicate the used primers, and letters (a–f) indicate primers in Table 1. The numbers are from PCR product size. ATG indicates the translation start site. The reverse primer (b) was designed between the putative transcription start site and the translation start site. The reverse primer of WCP2-170 (f) was designed from the sequence of the vector.