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βFTZ-F1 and Broad-Complex positively regulate the transcription of the wing cuticle protein gene, *BMWCP5*, in wing discs of *Bombyx mori*

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ABSTRACT

The present study was undertaken to clarify the mechanism regulating cuticle protein gene expression. Expression of *BMWCP5* was strong at around pupation and weak at the mid-pupal stage in wing tissues of *Bombyx mori*. We analyzed the upstream region of the *BMWCP5* gene using a transient reporter assay with a gene gun system to identify the regulatory elements responsible for its unique expression pattern. We identified two βFTZ-F1 binding sites to be important *cis*-acting elements for the transcription activation of the luciferase reporter gene by an ecdysone pulse. Site-directed mutagenesis of these sites, followed by introduction into wing discs, significantly decreased the reporter activity. We also found that the regions carrying the binding sites for the ecdysone-responsive factor BR-C Z4 (BR-Z4) were responsible for the hormonal enhancement of the reporter gene activity in wing discs. Mutation of the BR-Z4 binding sites decreased the reporter activity. The nuclear proteins that bound to these βFTZ-F1 and BR-Z4 sites were identified by an electrophoretic mobility shift assay (EMSA). The results demonstrate for the first time that the BR-Z4 isoform can bind to the upstream region of the cuticle protein gene, *BMWCP5*, and activate its expression. The results also suggest that the *BMWCP5* transcription is primarily regulated by the ecdysone pulse through βFTZ-F1, and the stage-specific enhancement is brought about through BR-Z4.

1. Introduction

Insect cuticle proteins are major components of an exoskeleton. When insects molt, the exoskeleton is renewed via biosynthesis of new cuticle proteins and degradation of old ones. Insect cuticle is constructed from many cuticle proteins with different temporal and spatial patterns (Willis, 1996). Stage specific expression of the cuticle protein genes is induced by the fluctuation of hormones. Ecdysone up-regulates (Braquart et al., 1996; Noji et al., 2003) and down-regulates (Hiruma et al., 1991) or ecdysone pulse induces (Apple and Fristrom, 1991; Suzuki et al., 2002; Noji et al., 2003; Zhong et al., 2006) different ecdysone responsive cuticle protein genes, and juvenile hormone (JH) affects the type of ecdysis and cuticle proteins (Riddiford, 1982; Bouhin et al., 1992; Krämer and Wolbert, 1998; Zhou and Riddiford, 2002).

An ecdysone-responsive transcription factor, βFTZ-F1 has been suggested to be a regulator responsible for the stage-specific expression of cuticle protein genes during the prepupal stage (Kawasaki et al., 2002). The timing of βFTZ-F1 expression has been reported to be affected by DHR3 and Blimp-1 (Lam et al., 1997; White et al., 1997; Agawa et al., 2007). After being expressed, βFTZ-F1 has also been found to positively regulate the pupal cuticle protein gene, *Edg84A*, during the mid- to late- prepupal period (Murata et al., 1996). Studies with mutants have shown that βFTZ-F1 was required for normal larval cuticle production (Yamada et al., 2000). *BmβFTZ-F1*, an ortholog of *βFTZ-F1* of the silkworm *Bombyx mori*, was expressed during larval, pupal molts and adult development, in coincidence with an ecdysone pulse (Sun et al., 1994; Nita et al., 2009). Although an earlier study proposed that BmβFTZ-F1 was a possible factor directing the stage-specific expression of the peptide gene *BmACP-6.7* (Shiomi et al., 2000), the detailed role of BmβFTZ-F1 in cuticle formation has not been elucidated.

Another early responsive gene, *Broad-Complex* (*BR-C*), has been reported, and some target genes of BR-C (Hodgetts et al., 1995; Cakouros et al., 2002; Cao et al., 2007) and stage and tissue specific isoforms of BR-C (Emery et al., 1994; Mugat et al., 2000) have been identified in *Drosophila melanogaster*. BR-C also has been reported to be critical for specifying pupal program and for suppressing both larval and adult program in *Manduca sexta* (Riddiford et al., 2003) and *Tribolium castaneum* (Konopova and Jindra, 2008; Parthasarathy et al., 2008). Z1 is the predominant BR-C isoform (BR-Z1) during

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the time of pupal cuticle formation in *D. melanogaster* (Bayer et al., 1996) and can direct the epidermis to a program of pupal cuticle production in *M. sexta* (Zhou and Riddiford, 2001). Misexpression of the *BR-C* isoforms demonstrated that BR-Z1 could suppress the larval cuticle gene *Lcp65A-b* and activate the pupal cuticle gene *Edg78E* in *D. melanogaster*, whereas other BR-C isoforms either promoted pupal cuticle formation or inhibited adult cuticle protein gene expression to varying degrees (Zhou and Riddiford, 2002). In contrast, BR-C Z4 (BR-Z4) was strongly expressed and was a predominant isoform in the *Manduca* imaginal discs and epidermis during pupal cuticle formation (Zhou et al., 1998; Zhou and Riddiford, 2001). The *Manduca* BR-Z4 transgene resulted in weak re-induction of *EDG78E* and partial suppression of the adult cuticle protein gene *Acp65A* (Bayer et al., 2003). In the *Bombyx* wing disc, BR-Z4 seems to be the predominant isoform through the prepupal period of pupal cuticle formation (Ijiro et al., 2004; Reza et al., 2004). Knock-down of BR-Z4 by RNAi resulted in the failure of animals to complete the larval–pupal transition or brought about later morphogenetic defects in *B. mori* (Uhlirova et al., 2003). However, the regulatory mechanisms by which BR-C controls the expression of cuticle protein genes at a molecular level is not well understood.

Imaginal discs have been studied as targets of ecdysone for a long time. So far, we have isolated several cuticle protein genes with different developmental profiles and hormonal responses in wing disc of *B. mori*. Among them, *BMWCP2*, *3*, *7*, *8*, and *9* are observed only at the stage of pupal wing formation. By contrast, *BMWCP1*, *4*, *5*, and *6* are expressed twice, during pupation and the mid-pupal stage (Takeda et al., 2001). *BmGRP1*, *2*, and *3* are expressed at the fourth molting in the epidermis, in addition to pupation and the mid-pupal stage (Zhong et al., 2006). All of them were expressed after an ecdysone pulse *in vivo* and induced by the removal of 20-hydroxyecdysone (20E) after exposure to it *in vitro* (Noji et al., 2003; Zhong et al., 2006). In the present study we selected *BMWCP5* for the examination of the promoter assay, since we found putative binding sites of βFTZ-F1 and BR-Z4 in the upstream region of *BMWCP5*. We performed a detailed analysis of *BMWCP5* promoter region and showed a strong transcriptional activity by ecdysone pulse. Furthermore, EMSA experiments indicated that βFTZ-F1 and BR-Z4 could bind to the *BMWCP5* promoter and mutagenesis of either one of these sites resulted in a reduction of the promoter activity. These results suggest that βFTZ-F1 and BR-Z4 positively regulate the promoter of the *BMWCP5* in a stage-specific manner.

2. Materials and methods

2.1. Insects

A hybrid strain of *B. mori* (N124 X C124) was reared at about 25 °C under a 12 h light:dark cycle. Larvae began wandering after the 6th day of the 5th larval instar, and pupation occurred 3 days later. Here, we designate the day of the fourth ecdysis as V0, the day of wandering as W0, and the day of pupation as P0.

2.2. Northern blot analysis of BMWCP5 mRNA

Total RNA was extracted from wing discs of the fifth instar larvae and wing tissues of pupae using ISOGEN reagents (Nippongene). The RNA concentrations were determined by absorbance at 260 and 280 nm. Total RNA (15 μg) was separated by 1.2% agarose–formaldehyde gel electrophoresis and blotted to a nylon membrane (Hybond N+; Amersham Biosciences). Plasmids having insert cDNAs of *BMWCP5* were digested with EcoRI and XhoI. Inserts were randomly labeled using the DIG-DNA Labeling Kit (Roche Applied Science). Hybridization was performed for 16 h at 42 °C. The membranes were washed twice with 2× SSC containing 0.1% SDS for 5 min each at 25 °C and then three times with 0.1× SSC containing 0.1% SDS at 68 °C for 15 min each. Chemiluminescent detection was performed according to Roche's protocol.

2.3. Western blot analysis

Cuticle protein extraction was carried out according to previous description (Hopkins et al., 2000). Wing discs and wing tissues were excised and washed three times with ice-cold phosphate-buffered saline (PBS). Protein extracts were prepared by homogenizing tissues in 2× SDS sample buffer [100 mM Tris–HCl (pH 6.8), 5% SDS, 0.2% bromophenol blue, 20% glycerol and 200 mM DTT] and a mixture of several protease inhibitors [1 μg/ml pepstatin (Sigma), 5 μg/ml leupeptin (Sigma), 0.1 mM PMSF (Sigma)]. Then the extracts were centrifuged at 13,000 × *g* for 10 min at 4 °C. The supernatants were collected and the protein concentrations of the supernatants were determined using the BCA Protein Assay Reagent Kit (Pierce).

According to the specific finger domain of *Bombyx* BR-Z4, the synthesis of a peptide (CHHNIDFYKFKDQFNV) and the production of a rabbit antiserum were performed by ATP, L. C. (Japan). The antiserum was further affinity-purified using a HiTrap NHS-activated HP affinity column (Amersham Biosciences) according to the manufacturer's instructions. Extracted 15 μg proteins were loaded on 8% SDS-polyacrylamide gel. Proteins were transferred to a nitrocellulose membrane (Hybond-C, Amersham Biosciences). The membrane was blocked in 5% non-fat milk in TBS (20 mM Tris pH 7.6, 140 mM NaCl). After washing by TBST (TBS +0.2% Tween 20), the membrane was incubated with antiserum against BmBR-C Z4 for 16 h at 4 °C. The membrane was washed for 5 min with TBST, 3 times before incubation for 2 h with anti-rabbit IgG (H + L)-alkaline phosphatase (Vector Laboratories), diluted 1:4000, then was washed for 5 min with TBST 3 times again. The detection of BmBR-C Z4 antibodies was performed with nitroblue tetrazolium (NBT) (Sigma) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) (Sigma).

2.4. Plasmid construction

All nucleotide positions of the *BMWCP5* gene given below are numbered with respect to the putative transcription start site as +1. The promoter region spanning from −1977 to +36 was amplified with the forward primer (5′-GCGGGTACCCACATGGGCGGTCGCA TTCACTTG-3′) and the *BMWCP5* reverse primer (5′-CTAGCTAGC GTTAAGATGCCTTACTGAAGCGTGG-3′), using *B. mori* P50 strain BAC clone #75H5 (Acc. No. AB262389) as the template. The restriction enzyme sites are underlined. The amplified DNA fragment was digested with Kpn I and Nhe I and then ligated into the Kpn I/Nhe I sites of the pGL3-basic Firefly luciferase reporter vector (Promega) to generate WCP5-1977. Deletion constructs were obtained by PCR using the following forward primers:

WCP5-1458: 5′-CGGGGTACCAAGATTATACGGTGACCTTCAAGTG-3′,
WCP5-669: 5′-AAGGGTACCAGAACGCTACTTTCTTACCGGCTG-3′,
WCP5-417: 5′-CGGGGTACCAATCCAAGTGCAACATCTTC-3′,
WCP5-198: 5′-CGGGGTACCAATATGAATCGTGTGAAAAGAG-3′

combined with *BMWCP5* reverse primer. The PCR products were digested with Kpn I and Nhe I and then subcloned into a pGL3-basic vector to generate WCP5-1458, WCP5-669, WCP5-417, and WCP5-198. The Renilla luciferase vector, PhRG-hsp, was made by ligation of Kpn I and the *Hind III* fragment in hsp-LacZ (Tomita et al., 2001) into PhRG – B (Promega). The Renilla luciferase activity was used to normalize the data.