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Ecdysone Receptor Binds the Promoter of the *CPR28* Gene and Regulates its Expression

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Abstract

We tried to clarify the mechanism that regulates the *CPR28* expression in the feeding stage. *CPR28* expression was high when ecdysteroid in the hemolymph was low and decreased when it started to be secreted in the hemolymph. Therefore, we first examined the ecdysone responsiveness of *CPR28*. Expression of *CPR28* was inhibited by the addition of 20E in the culture medium and decreased during the culture even in the medium without 20E. We performed reporter assay to clarify the effect of ecdysone on the promoter activity of *CPR28*. *CPR28* promoter showed difference in its promoter activity with and without 20E. The addition of 20E decreased the promoter activity. Then, we mutated the putative EcRE, resulted in the decrease of promoter activity in the 20E free medium. Thus, through the genomic information and transient reporter assay, we found the regulating mechanism of *CPR28* expression in wing discs in the final larval instar of *B. mori.*

Keywords: Cuticular protein; Ecdysone; Wing disc; *Bombyx mori;* EcRE

Introduction

Insect development is punctuated by the steroid hormone ecdysone that is secreted before ecdysis and brings about ecdysis [1,2]. Ecdysone, an insect steroid hormone, is well known as an inducer of ecdysis and metamorphosis of insect, and its functional mechanism has long been studied [2,3]. Ecdysone functions for the ecdysis by the activation of the ecydsone-signaling cascade [3] and induces ecdysis with high concentration [1,2]. Ecdysone binds with ECR/USP heterodimer, and this complex induces the expression of primary responsive genes, resulting in transcriptional activation of the secondary responsive genes [4]. The fluctuation of the hemolymph ecdysteroid titer at the initiation of metamorphosis is well known in insects [5,6]. Ecdysone signaling functions through ecdysone-responsive transcription factors, and several transcription factors affect each other and activate the target genes [3]. Thus, according to the ecdysone fluctuation in the hemolymph, different transcription factors are produced and bring about insect metamorphosis.

Among these transcriptional regulators, βFTZ-F1 has been suggested to be a regulator responsible for the stage-specific expression of cuticle proteins during the prepupal stage [7,8]. Recently, research data showed that ecdysone-responsive transcription factors determine the expression of cuticular protein genes [7-15]. βFTZ-F1 increased the promoter activity of the cuticular protein gene *BMWCP9*, which was expressed around pupation, when the ecdysteroid titer decreased after its peak [8]. Moreover, it bound upstream of the promoter region of *CPR55* [7].

Broad-Complex (BR-C) isoforms were expressed stage and tissue specifically [16,17]. BR-C also has been reported to be critical for specifying pupal program and for suppressing both larval and adult program in *M. sexta* [2] and *T. castaneum* [4]. Ecdysone receptor directly binds the promoter of the Drosophila caspase dronc, regulating its expression in specific tissues [18]. These studies have been easily operated by using the genomic database. Thus, transcription factors bind to their binding sites upstream of the target cuticular protein genes and bring about the stage-specific expression of cuticular protein genes in wing discs and epidermis. Thus, cuticular protein genes are targets of ecdysone-responsive transcription factors as described above.

Expression of stage specificity of cuticlular protein genes has been reported in *A. gambiae* [19] and *B. mori* [20]. RR2 was expressed around the pupation RR1 showed three different types of expression pattern in wing discs of *B. mori.* One group was expressed at the same time as RR2,

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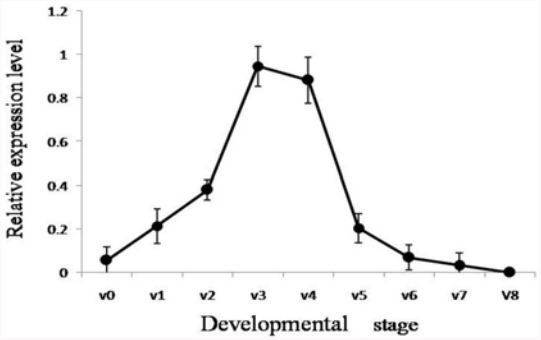

Figure 1: The expression profiles of *CPR28*. Total RNA was isolated at each time point and analyzed by qRT-PCR. Each datum was calculated from three independent experiments. RNA was extracted from wing discs and reverse-transcribed to cDNA for use in qRT-PCR. The data were normalized by determination of the amount of Bmrpl. Values were first normalized to rpl and then plotted relative to the highest value for each panel. Developmental profiles of *CPR28* RNA in wing discs during the fifth larval instar. Wing discs were dissected at the indicated time.

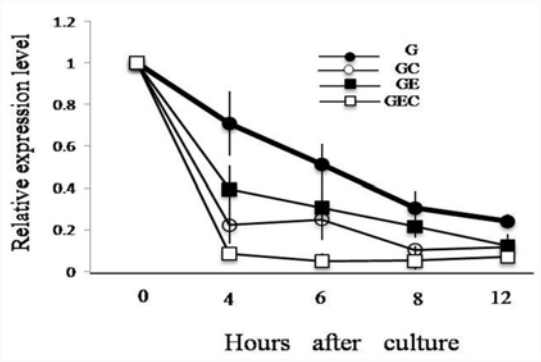

Figure 2: The expression profiles of *CPR28* . Total RNA was isolated at each time point and analyzed by qRT-PCR. Each datum was calculated from three independent experiments. RNA was extracted from wing discs and reverse-transcribed to cDNA for use in qRT-PCR. The data were normalized by determination of the amount of Bmrpl. Values were first normalized to rpl and then plotted relative to the highest value for each panel. *CPR28* expression in wing discs after cultured in the indicated condition. 20E (E) and cycloheximide (C) were applied to the culture medium at a concentration of 2 and 50 μg/ml, respectively. Level of mRNA after cultured for indicated time in Grace medium (G), Grace medium containing cycloheximide (GC), Grace medium containing 20E (GE) and Grace medium containing 20E and cycloheximide (GCE).

the second one was expressed when the hemolymph ecdysteroid titer was high, while the third one was expressed in the early final larval stage [20]. About first two, the mechanism of the regulation of their expression was demonstrated. The first one was induced by ecdysone pulse through transcription factors; βFTZ-F1 and BR-C [8,15]. The second one is up-regulated by ecdysone through EcR/USP.

The third group that was strongly expressed in wing discs in the mid fifth larval stage [20], which left to be resolved. They belong to RR1, and RR1s are found in several kind of EST library of *B. mori,* but their function in the insect development is unclear. In the present study, we tried to clarify the mechanism of *CPR28* expression in the fifth larval stage of *B. mori*. In this stage, larvae and wing discs grow by the nutrient from the hemolymph, and ecdysone starts to be secreted in the hemolymph after cessation of feeding. Expression of *CPR28* decreased at the same time when ecdysone starts to be secreted. Therefore, we tried to examine the relatedness of *CPR28* and ecdysone.

Materials and Methods

Experimental animals

Bombyx mori larvae were reared at 25°C under a photoperiod of 12h of light and 12h of dark. Under these conditions, most larvae began wandering in the late photophase of day 6 and then pupated after 3 days. The first day of the fifth larval instar was designated as V0, with subsequent feeding phases from V1 to V6. The three days before pupation were designated as V7-V9.

BLAST search of genomic sequences of cuticular protein *gene CPR28*

The cDNA sequences of *CPR28* were used for BLAST search analysis. BLAST search was operated using genomic database of *B. mori* (http://kaikoblast.dna.affrc.go.jp/). The binding sites of EcRE were identified through a website (http://www.genomatix.de/en/index.html).

In vitro culture of wing discs

Wing disc of larvae at the V4 stage was prepared for the *in vitro* culture. For wing disc preparation, the fat body, muscle, and trachea were carefully removed under a microscope. Wing discs were cultured according to Ali et al., [14].

Recombinant plasmid construction

The promoter region of the *CPR28* gene (-2138+22 and -297+22 with respect to the transcription initiation site) was amplified by PCR using *B. mori* P50 strains genomic DNA. The 2160bp and 319bp fragments containing the upstream region of *CPR28* were amplified by 2160 forward primer *(*5'-CGCGCTAGCAAGTTGCAAATGTGTATGAAAATAGG- 3') and 319 forward primer (5'- C G C G C T A G C G T C A A T G C A A A A G A G A C A A C A T T A C C - 3') respectively combined with reverse primer (5'- C G G C T C G A G G A T T G G A T T T C G T A G C T T G T T T G A C- 3') according to Ali et al., (2013). The restriction enzymes sites are underlined.The PCR products were digested with *NheI* and *XhoI* and then subcloned into the pGL3-basic vector (Promega). A *Renilla* luciferase reporter (*PhRG-hsp)* driven by the *Drosophila* heat-shock protein70 promoter was used as a normalization control.

Mutation of *CPR28* promoter

Mutagenesis of EcRE was performed with the Quick-ChangeTM Site-Directed Mutagenesis Kit (Stratagene) using the *CPR28-319* plasmid as the template. Twenty nanograms of plasmid DNA were amplified with *Pfu* DNA polymerase followed by digestion of the parental plasmid by *DpnI*. The mutated plasmid was transformed into XL1-Blue super competent cells. The mutagenic reactions were performed according to the manufacturer's instructions. The introduction of each mutation was confirmed by sequencing. The oligonucleotide primers used to generate the mutation are shown as follows.

(-212/-208) EcRE mut: 5'- C G G C G A C T T A A T G G A T C G T T G C T A G C T T G T T C A A A T T A T T A T T C G-3' and: 5'- C G A A T A A T A A T T T G A A C A A G C T A G C A A C G A T C C A T T A A G T C G C C G -3'. The underlined bases are mutated

Transient expression of the reporter constructs

Transient expression of the reporter constructs in wing discs was performed according to Ali et al. (2013). Twelve point five mg of gold particles (diameter: 1.0μm) were coated with plasmid DNA (50μg

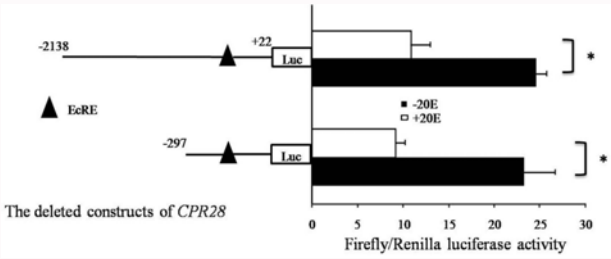

Figure 3: Promoter activity of *CPR28*.Transient expression analysis of the *CPR28* promoter in wing discs. The ecdysone responsiveness of the upstream sequences of *CPR28* was examined. The promoter activity was observed after bombarded wing discs were cultured for 48h at 25°C with or without 2μg/ml 20E. The left side of the graph shows different constructs and putative EcREs existing in the upstream region of *CPR28*. The horizontal axis indicates the relative luciferase activity in arbitrary units. Filled boxes: wing discs were cultured without 20E; empty boxes: wing discs were cultured with 20E. All experiments were performed at least three times. The results are expressed as the mean ± S. E. M., and significance was p<0.01 (*) by the student's t-test. The result of different lengths of constructs introduced into wing discs.

each of pGL3-derived vectors and 5μg phRG-hsp) according to the manufacturer's instructions. The reporter constructs were introduced into wing discs using a particle gun (Bio-rad). Bombardment proceeded under helium pressure of 150psi (pound per square inch). After bombardment, wing discs were cultured for 48h at 25°C in Grace's medium (Invitrogen) with or without 2 μg/ml of 20E (Sigma) according to Ali et al., [14].

Dual-luciferase assay

Wing discs were harvested and washed twice in PBS after 48h of culture. The tissues were suspended in a 25μl 1x reporter Passive Lysis Buffer (Promega) and frozen/thawed for 5 cycles in liquid nitrogen before the supernatant was equilibrated at 4°C for 1h. The supernatant was collected by centrifugation at 12,000g for 2min at 4°C. The Luciferase reporter assay was carried out using a Dual-Luciferase reporter assay system (Promega) in a luminometer (Perkin Elmer) according to the manufacturer's protocol. The luciferase activity was normalized to the level of Renilla luciferase activity. All experiments were performed at least three times. The results are expressed as the mean ± S. E. M., and significance was set at $p < 0.05$.

Quantitative *RT-PCR*

The wing discs were collected and washed three times in phosphate-buffered saline (PBS) buffer and then frozen and stored at -80°C. Total RNA was isolated from wing discs using ISOGEN (Nippongene, Japan). First-strand cDNA was synthesized from 1μg total RNA in a 20μl reaction mixture ReverTraAce (Toyobo, Japan). RT-qPCR was conducted on an ABI7500 real-time PCR machine (Applied Biosystems) using FastStart Universal SYBR Green Master (Roche) according to the manufacturer's protocol. Each amplification reaction was performed in a 25μl qPCR reaction under the following conditions: denaturation at 95°C for 10min followed by 40 cycles of treatment at 95°C for 10sec and at 60°C for 1min. Ribosomal protein S4 (*Bmrpl*: GenBank accession no. NM_001043792) was used as a control gene. The data were normalized by determination of the amount of *Bmrpl* in each sample to eliminate variations in mRNA and cDNA quality and quantity. The transcript abundance value of each individual was the mean of three replicates.

Each pair of primers was designed to contain an intron in the genomic sequence using Primer3 software (http://frodo.wi.mit.edu/). The oligonucleotide primer sets used for RT-qPCR were 5'- G T G G G A A T A G T T G C C G C T T C -3' and 5'- C T G C T C A A A T A C G G T C C C A T C -3' for *CPR28;*

Gene Bank accession No. of the gene is follows; *CPR28*: BR000529

Results

Developmental changes of *CPR28* expression

Expression of *CPR28* increased from V1 and peaked at V3-V4, then decreased after V5 when ecdysone begins to be secreted in the hemolymph (Figure 1). The result was similar to that of *BMWCP11* [20]. The expression of cuticular proteins at later stages was regulated by ecdysone responsive transcription factors [10,11,14], so we examined the effect of ecdysone to the expression of *CPR28*.

Inhibition of expression by ecdysone

To examine the effect of ecdysone, we cultured wing discs of V4 stage with and without 20E. The expression of *CPR28* decreased in all the case during the culture (Figure 2). Rapid decrease was observed in wing discs cultured with 20E or cycloheximide. The addition of 20E or cycloheximide inhibited the transcription of *CPR28* in wing discs. The addition of 20E and cycloheximide completely inhibited expression of *CPR28*. The results indicate that 20E and 20E-inducible factors inhibit *CPR28* transcription, and *CPR28* transcription needs factors that are produced in the absence of 20E.

Promoter activity showed the inhibition by the addition of 20E

We applied our transient reporter assay system on this analysis to clarify the effect of ecdysone on the promoter activity of *CPR28*. Significant difference was observed in the promoter activity of *CPR28* in wing discs cultured with and without 20E (Figure 3). The promoter activity of *CPR28* decreased in the presence of 20E, which revealed that 20E inhibited the promoter activity.

Genomic data and mutagenesis brought about surprising result

The promoter activity of *CPR28* did not change after deletion of the upstream region of *CPR28* (Figure 3). In the CPR28-297, putative EcRE exist, so it is suggested that this EcRE functioned for the decrease of promoter activity. Then, we mutated the putative EcRE. Surprisingly, the promoter activity of CPR28-297 decreased after mutation in wing discs cultured without 20E (Figure 4).

Discussion

Three different expression patterns of cuticular protein genes were found in wing discs in the fifth larval instar, and the regulation of cuticular protein genes of *B. mori* was clarified through the genomic database. Two of them, were clarified by concerning ecdysone responsive transcription factors. The promoter of different cuticular protein genes were bound and activated by different ecdysone responsive transcription factor, ßFTZ-F1, EcR, BR-C Z2 and BR-C Z4, resulted in the different expression pattern. These studies succeeded by using genomic information and transient reporter assay in wing discs. The third one is the group of cuticular protein genes expressed in the feeding stage. The present study was operated to clarify the mechanism of this type of cuticular protein genes. *CPR28* belongs to this group and was examined its expression to clarify its developmental fluctuation during the fifth larval instar.

The organs used that are stage specifically developed and contain stage specific factors in the nuclei, which function for reporter assay

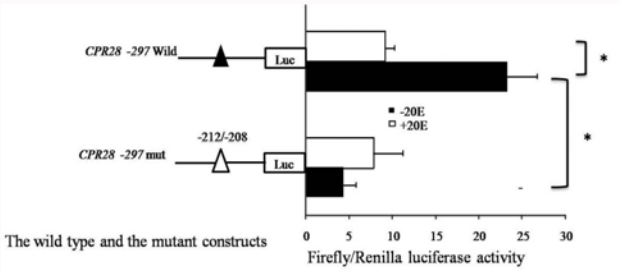

Figure 4: Promoter activity of *CPR28*.Transient expression analysis of the *CPR28* promoter in wing discs. The ecdysone responsiveness of the upstream sequences of *CPR28* was examined. The promoter activity was observed after bombarded wing discs were cultured for 48h at 25°C with or without 2µg/ml 20E. The left side of the graph shows different constructs and putative EcREs existing in the upstream region of *CPR28*. The horizontal axis indicates the relative luciferase activity in arbitrary units. Filled boxes: wing discs were cultured without 20E; empty boxes: wing discs were cultured with 20E. All experiments were performed at least three times. The results are expressed as the mean ± S. E. M., and significance was p<0.01 (*) by the student's t-test. Effect of site-directed mutagenesis of the putative -212/208 EcRE sites of the *CPR28* promoter. The closed triangles indicate the wild-type sequence, and the open triangle indicates the mutated sequences.

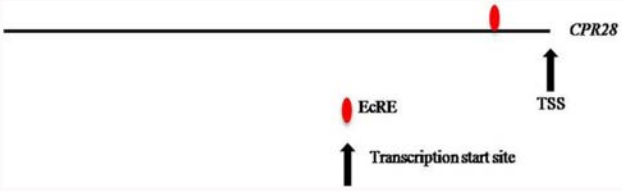

Figure 5: Schematic representation of the putative EcRE binding sites located on the upstream of cuticular protein gene *CPR28*. Bars indicate 2 kb upstream region from the transcription start site. TSS indicates the place of the putative transcription start site. Symbol shows EcRE binding site position.

system. Therefore the mechanism could demonstrate, regulating the stage specific expression of cuticular protein genes. This method can connect the genomic information with the expression profiles of cuticular protein genes. Sometimes this method will bring about surprising new findings as observed in this study.

Expression of *CPR28* decreased in the presence of ecdysone, and also promoter activity decreased in the presence of ecdysone. These results suggest that ecdysone inhibits the transcription of *CPR28*, which reflects *in vivo* expression of *CPR28*. It decreases after V5 when ecdysteroid in the hemolymph starts to be secreted, which is different from the regulation of other types of cuticular protein genes whose promoter was activated by the ecdysone responsive transcription factors. The mechanism of down-regulation by ecdysone has not been clarified. Therefore, the present study showed the possibility that ecdysone depressed the target gene promoter activity by replacing the locus of activating transcription factor. The present results indicate that the putative binding site that was mutated is for EcR/USP (Figure 5). It was used for EcR after secreted ecdysone, inhibiting the promoter activity of *CPR28*. From this, we concluded that the transcription of *CPR28* is inhibited by ecdysone.

Conclusion

Through the genomic database, we have become able to obtain the information of regulatory region of genes. By this we have clarified the regulation of the expression of a cuticular protein *CPR28* whose peak of transcripts are observed in wing discs in the feeding stage of the fifth larval instar of *B. mori*. Through the mutagenesis in the reporter assay, we identified EcRE is one of the factor related with the regulation of *CPR28*. There may have another factor functioned together with EcRE that could be involved for the regulation of *CPR28* gene in the feeding stage of *B. mori*. We have to clarify it in future.

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