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Identification of ecdysone response elements (EcREs) in the *Bombyx mori* cathepsin D promoter

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ABSTRACT

Bombyx mori Cathepsin D (*BmCatD*) is specifically expressed in the fat body, and plays a critical role for the programmed cell death of the larval fat body and pupal gut during metamorphosis. To better understand the transcriptional control of *BmCatD* expression, we conducted this study to identify the ecdysone response elements (EcREs) in the *BmCatD* promoter and clarify their regulational functions. We inserted EcREs into a recombinant AcMNPV (*Autographa californica* multiple nucleopolyhedrovirus) vector and performed luciferase assay with a dual-luciferase quantitative assay system. Three putative EcREs were located at positions −109 to −99, −836 to −826 and −856 to −846 relative to the transcription start site. Overlapping deletion studies of this EcRE region showed that the three EcREs could suppress the ectopic expression of the *BmCatD* promoter. EcRE mutations resulted in the loss of the fat body-specific expression of the *BmCatD* gene. These results suggest that the EcREs are vital for activation of the promoter by 20-hydroxyecdysone (20E) in the larval fat body and further support the crucial role of ecdysone signaling to control cathepsin D gene transcription. It may suggest that the heterodimeric complex EcR/USP mediates the activation of ecdysone-dependent *BmCatD* transcription in the larval fat body of *B. mori*.

1. Introduction

Insects possess many different proteases; the majority use serine proteases as the primary digestive protease [1] as well as cysteine and aspartate proteases (cathepsins B, D, H, L) as intracellular lysosomal enzymes [2]. Cathepsin D is a proteolytic enzyme of lysosomes. As most of the lysosomal enzymes, it is usually a glycoprotein. This protease is optimally active at an acidic pH, between 3 and 5, and relies on two aspartic acid residues for catalysis. In insects, cathepsin D may be involved in physiological processes such as: vitellogenin production and degradation in mosquitoes [3], fat body histolysis in *Ceratitis capitata* [4], and bloodmeal digestion in ectoparasitic mites and ticks [5–6]. Our previous studies have established that the expression of the *Bombyx mori* Cathepsin D (*BmCatD*) gene is ecdysone-dependent and strictly tissue- and stage-specific. Transcription of its unique mRNA takes place exclusively in the larval fat body and pupal gut. *BmCatD* plays a critical role during metamorphosis for programmed cell death (PCD) of the larval fat body and pupal gut [7].

Insect life cycles include metamorphosis, which is characterized by drastic changes in morphology and physiology, and occurs with the programmed disruption and/or differentiation of internal structures [8]. Ecdysteroid plays important roles during metamorphosis initiation and serves as a major signaling molecule in the life cycle. Ecdysteroid, 20-hydroxyecdysone (20E) activates a number of ecdysone-regulated genes through a heterodimeric receptor complex composed of two members of the nuclear receptor superfamily: the ecdysone receptor (EcR) and Ultraspiracle (USP), an ortholog of the vertebrate retinoid X receptor (RXR), to form a functional ecdysone receptor [9]. The EcR/USP complex binds to ecdysone response elements (EcREs), specific sequences near ecdysone-responsive target genes, to regulate transcription. A number of ecdysone-responsive genes are expressed in various larval and imaginal tissues and in cultured cell lines [10–11]. Currently, there are very few high-resolution maps of *cis*-regulatory EcRE DNA sequences. Riddihough and Pelham [12] identified the first functional EcR binding site in a 23 bp region of the *hsp27* promoter. Deletion mapping of the *Fbp1 cis*-acting regulatory sequences by germ line transformation identified a 70 bp element located in the −138 to −68 region of the promoter that is required for expression of the strictly stage- and fat body-specific gene [13]. EcREs have been identified upstream of the *Drosophila Eip28/29* and *Eip40* and the *larval serum protein 2* (*Lsp-2*) gene promoters, and the EcR/USP

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complex has been shown to interact with *Eip28/29* and *Eip40* EcREs [14–15]. Broad-Complex (BR-C) is another key regulator gene in the morphogenesis of *Drosophila* that controls ecdysone-responsive gene expression [16].

It has been reported that 20E indirectly affects PCD of the larval fat body of *B. mori*, most likely through the head and/or thorax [17]. Although the regulatory interactions between 20E and its responsive transcription factors have been well characterized, the mechanism of EcRE regulation of *BmCatD* gene expression remains unclear.

Dual-luciferase assay system generates both firefly and *renilla* luciferase luminescence signals from cells, and allows quantitation of stable luminescence from two reporter genes in a single sample. Firefly and *renilla* luciferases are monomeric and neither requires post-translational processing, so they can function as genetic reporters immediately upon translation. This system has been widely used as co-reporters for these normalized studies because both assays are quick, easy and sensitive.

The present study investigates the putative *cis*-acting EcREs that determine tissue-specific expression of the *BmCatD* gene *in vivo*. Mutations of conserved bases in EcREs of the *BmCatD* promoter by successive deletions were inserted into an recombinant AcMNPV (*A. californica* multiple nucleopolyhedrovirus) vector with a dual-luciferase quantitative assay systerm. We took firefly luciferase gene as the reporter gene (*BmCatD* promoter), while introducing *renilla* luciferase gene as reference gene in reason, to record the intensity of the reporter gene normalized by reference gene. The recombinant virus was injected into the hemocoele of the newly ecdysed fifth instar larvae of the silkworm. The mutants were ectopically expressed in the larval fat body of silkworms and assayed for *BmCatD* gene expression by activity of luciferase and normalized by the copy number of recombinant virus.

2. Materials and methods

2.1. Insects

B. mori (strain 54A, permissives to recombinant *A. californica* multiple nucleopolyhedrovirus (rAcMNPV)) silkworm larvae were supplied by the Sericultural Research Institute, Chinese Academy of Agricultural Science, China. The silkworms were reared on fresh mulberry leaves at 25 °C with 75 ± 5% relative humidity and a 12 h light: 12 h dark photoperiod.

2.2. Tissue collection and RNA extraction

The larval fat bodies were collected on ice at five day post-treatment and washed twice with phosphate-buffered saline (PBS) (140 mM NaCl, 27 mM KCl, 8 mM Na_2HPO_4 and 1.5 mM KH_2PO_4, pH 6.5). The homogenates were centrifuged at 10,000×*g* for 20 min and the supernatant was discarded. Total RNA was extracted from fat bodies using TRIzol (GENERAY, China) according to the manufacturer's instructions and resuspended in diethyl pyrocarbonate (DEPC)-treated water. RNase-free DNase I was used to digest the gemnomic DNA and the RNA concentration was measured using a UV-1601 spectrophotometer (Shimadzu, Kyoto, Japan). The RNA quality was analyzed on a 1.0% agarose gel.

2.3. 5′-Full RACE

The transcription initiation site was determined using the 5′-Full RACE kit (TaKaRa) according to the manufacturer's instruction. Briefly, 10 μg of total RNA was treated with calf intestine alkaline phosphatase (CIAP) to remove the 5′-terminal phosphates from non-capped mRNA. Cap structures were removed by tobacco acid pyrophosphatase (TAP) treatment to produce 5′ monophosphate on all full-length mRNAs. These mRNAs were ligated into an RNA adapter oligonucleotide using T4 RNA ligase. First-strand cDNA was synthesized from the adapter-ligated RNAs using random primers. The cDNA was amplified by a gene-specific outer primer, a gene-specific inner primer (listed in Table 1), and two nested primers corresponding to the 5′ RACE adapter sequence. The PCR products were separated on 1.5% agarose gels and then purified for sequencing.

Table 1
List of primers.

Name	Sequence (5′–3′)
Primers for 5′-RACE	
Outer	CATGGCTACATGCTGACAGCCTA
Inner	CGCGGATCCACAGCCTACTGATGATCAGTCGATG
Gene-specific outer	ACTTGCGGCTGTCGTACTTGTTGT
Gene-specific inner	AACAAGCGATGTTGGTGTAGTGGC
Primers for promoter cloning	
*Bm*CatD promoter F1	GTCGACACGGCCATCGTGTTGCTA
*Bm*CatD promoter R1	CCGCGGTAGAAGCCGCAACTAGGC
Primers for Over-lap PCR	
EcRE1^{m}F1	GTCTGGCTGACCGCGAAACAACGCAGTAAG
EcRE1^{m}R1	TTTCGCGGTCAGCCAGACGTTACGGTCAC
EcRE1^{m}F2	AAAGTCTGGCTACGTTAACTTTGTTTCAT
EcRE1^{m}R2	TTAACGTAGCCAGACTTTTAATCAAATTAA
EcRE1^{m}F3	TTGGTCTGTCCCCGAAGGAGGAATTTAAA
EcRE1^{m}R3	CTTCGGGGACAGACCAAAGTTAATTTAACT

2.4. Sequence cloning and bioinformatics analysis of the BmCatD promoter

Total DNA was isolated from silkworm pupa using the TaKaRa Genomic DNA Extraction Kit (TaKaRa) according to the manufacturer's instruction. The F1 and R1 primers were designed to amplify the *Bm*CatD promoter. To assemble the genomic DNA sequence, the *BmCatD* cDNA sequences were BLAST against the contigs of the *B. mori* genome on GenBank. An approximately 1400 bp region upstream of the *BmCatD* transcription start site was blasted using NCBI's BLAST (http://www.blast.ncbi.nlm.nih.gov/Blast.cgi) and analyzed by the Gene Quest Module of DNASTAR [18].

2.5. Mutagenesis of EcREs

EcRE mutagenesis was performed with overlap extension PCR. The oligonucleotides used for PCR are listed in Table 1. Mutagenesis was confirmed by DNA sequencing.

2.6. Construction and transfection of recombinant AcMNPV

Mutants of the p1363 promoter (EcRE1^{m}, EcRE2^{m}, EcRE3^{m}, EcRE1^{m},2^{m}, EcRE1^{m},3^{m} and EcRE1^{m},2^{m},3^{m}) were inserted into seven donor plasmids and transfected into *E. coli DH10BacDEGT* [19] cells to create recombinant baculoviruses (bacmids). Bacmid recombination was verified by PCR analysis using the EGFP-specific primer PEGFP-1 (5′–AAGCTTGTCGACAGATCTGCATGCATGGTGAGC–3′) and the M13 reverse primer (5′–CAGGAAACAGCTATGAC–3′). The M13 reverse and forward (5′–GTTTTCCCAG

Table 2
Putative EcREs in the 1363 bp upstream region.

Sequence (5′–3′)	Site name	Location
TTCGATTGACA	EcRE1	−109 bp/−99 bp
TTTAACTGAAA	EcRE2	−836 bp/−826 bp
TTGGAATGAAA	EcRE3	−856 bp/−846 bp