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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

-1363
*ACGGCCATCGTGTTGCTA*GACGAGTCTGATAAAATGCTCGAGCGCCTCCTAGCGGCCCGCTTTTTCAGC
TATTCCAGCTACACCCGGACGAGTAGGTGAGCTCACGGACTCAATATGAATGGATTTGCTAACACTAG
CCCTAGCAAGAGCAGTGCTTCGCAGAATCTACCACCGGATAGAATTTGCAATCACAAATCTACAATAC
AAATACAGAATCCCTTTTTCCTTTTCCCAGGGTCCCTTTTTTAAACCAAAATCCATCTTGGAAGAGGAC
AAATCAATAACAAGTCTGACGCCCGTTTGAAGCGTTTACACTGCACACGTCATCAATGTTGTTAACCG
TTTGTGCAGCATTGCTATCGCGACGGAAATAGTATTCCATAATTACTCTTATTTTTATCAGCCATACAT
ACATTACCTACGTTTTAAATGGCTATTATTGTAAAGCGACAACTTTGTAAGTAAAGGTTCACTAATATT
TAATTTTTTTATTTTAAATTCCTCCTTTTGGAATGAAACAAAGTTAATTTAACTGAAATTTTAATCAAA
EcRE3 EcRE2
TTAAATAAAATGGGATGAAACGAGACTACATGGAATAAAGTTACAGTAAAATTGTGAGAATTAACTG
TCTTCGGTGGACTTCTTGGAAAATCCTTAAAAGCCACATCCAGCAACTTTTAATAGTTTCCTACATTTA
TTCAGTTTCACAAATGTTTAAGACTTATCAACCAGTGTTATACGTTTAAAACTTTTTTTTTGTTAACGCT
GGGGAATCCATTTACGGATACCCCGTCGAAGGGGGTAACGGACCGGGCTATGTCGGACTCCGGCGTC
TTCAGAGAGGCAAGGGAGACGAGGATGGCCAAGGTCCTCTACCTCTCCTTCCTACCTACCTTCCTCGC
AGAGACTGCCCTGCGCGCCTCGCAAAACCGACTACCAACTAAGCTCCATCGAGCTCCACCACACCGAC
TACGTTTAAATAATAGGTTTAAACCAGAAGAAAATCTAAATTAAGAAATAAAACAATTCATACAGTT
TCGTACCTCGCGTTTCCGTAAAAAAATCTGTTTCCATTATCATGCAAACCAGCTTGCAACTCAACTCAT
CTAATGCAGTCTGTAACTCAAATGAAAAAGTCAATGTCACTTAGCAGTTGTCTATTGTTGTGATTTTCA
AATTTTTCAATGATGTCCGGCGATTTGTGACATAATTTAATCTGTTTTTACCATAGAGTTACTATTGTTT
TTACTACTTACTGCGTTGTTTTTCGATTGACAAGACGTTACGGTCACAGTGTTCTATTTTAATAAATCA
EcRE1
ATAAATAACAATAGTCAATTTCTCATAAGCAGTACGTTGAAATAGAAATCCGTTCTTGCCTAAATCCA
GAATGTTTTTTTTTATTAAAAACTTGTCGCAATAAATATTTAATTCATTTCGTGTGTTGCAGAGTCGCC
TAGTTGCGGCTTCTATG

Fig. 1. Promoter region sequences of *BmCatD* and putative EcREs. Underlined and italicized letters indicate PCR primers. The predicted EcREs are designated EcRE1, EcRE2, and EcRE3. The putative TATA box, transcription start site, and translation start site are indicated in red. The first nucleotide of this sequence is located 1363 bp upstream from the transcription start site. (For interpretation of color references in this figure legend, the reader is referred to the web version of this article.)

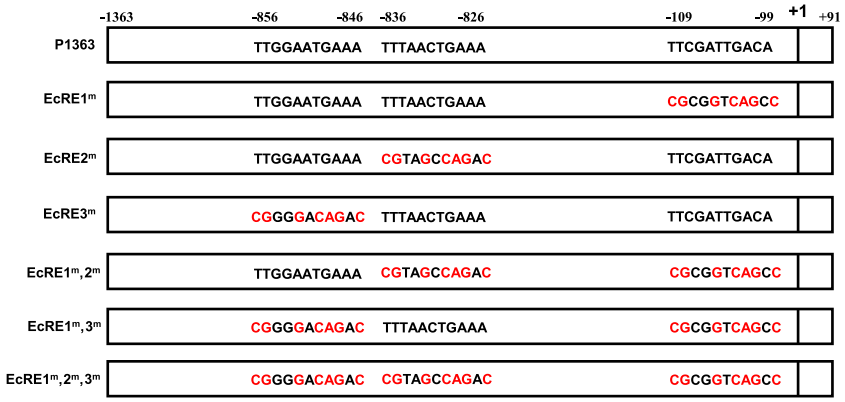

Fig. 2. The *BmCatD* EcRE sequences and mutations. The mutated residues are denoted in red.

TCACGAC–3′) primers were used to determine the purity of the recombinant bacmid. This process is shown schematicaly in Fig. 3.

Cellfectin reagent was used to transfect each bacmid DNA construct into Sf9 cells in 6-well plates (10^6 cells per well in 2 ml media). The generation of recombinant baculovirus and the large-scale harvest of budded virus (BV) were performed in the Sf9 cell line according to the Bac-to-Bac Baculovirus Expression System manual (Invitrogen).

2.7. Characterization of BV production and injection of silkworm larvae

Viruses were collected by centrifugation at 35,000*g* for 60 min. The viruses were resuspended in PBS (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, and 1.5 mM KH_2PO_4, pH 7.4–7.6) supplemented with 1% (v/v) fetal bovine serum and stored at −70 °C. The virus titer was determined by the tissue culture infectious dose 50% ($TCID_{50}$) method. Silkworm larvae in early fifth instar were infected by recombinant viruses by injecting 10 μl of the viral solution (1×10^6 PFU/larva) into the hemocoel. Control larvae were injected with EGT-null AcMNPV (1×10^6 PFU/larva) [19].

2.8. Dual-luciferase assay

Five days after injection, the fat body was collected, washed twice in ice-cold PBS and homogenized in 500 μl Passive Lysis Buffer (Promega) with liquid nitrogen. The supernatant was collected after centrifugation at 12,000*g* for 10 min at 4 °C. The luciferase reporter assay was performed with the Dual-Luciferase Reporter Assay Kit (Promega) in a Turner Biosystems 20/20n luminometer (Turner BioSystems Inc., USA) according to the manufacturer's protocol. Promoter activity in the silkworm fat body lysate was

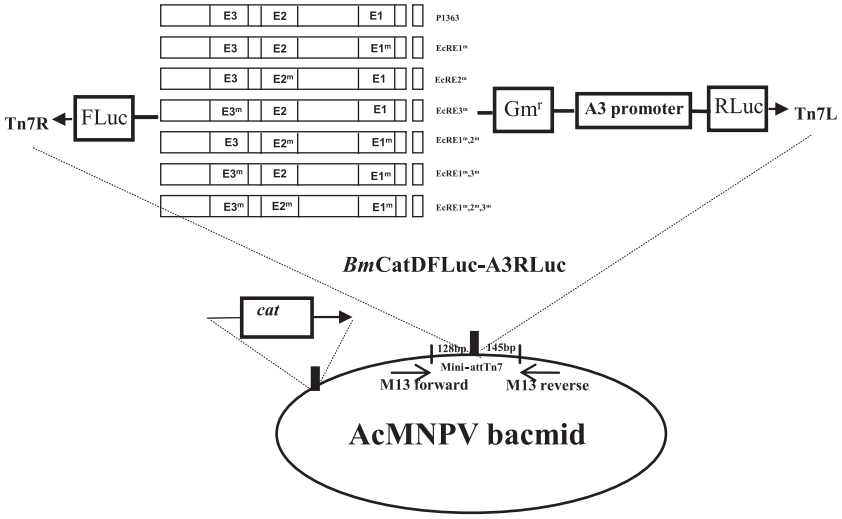

Fig. 3. Schematic representation of the recombinant bacmid procedure. The EGFP cassette was cloned into the multiple cloning site (MCS) of pFFa2 to form the recombinant donor plasmid. When the recombinant plasmid was transfected into *E. coli DH10BacDEGT*, the EGFP cassette was transposed into the polyhedrin site of the AcMNPV bacmid by Tn7-based transposition.

quantified by calculating the ratio of luciferase photon counts per minute (c.p.m.) to the copy number of the luciferase gene [20]. All experiments were performed at least three times. The results are expressed as the mean ± S.E.M. (significance, $p < 0.05$).

3. Results

3.1. The BmCatD promoter contains potential EcREs

To investigate functional sequences of the *BmCatD* promoter, we analyzed the upstream region of *BmCatD* using the Gene Quest Module of DNASTAR. The transcription start site of *BmCatD* was determined by 5′-Full RACE-PCR analysis. DNA sequencing of a RACE-PCR product identified an ending position 90 bp upstream from the translation start site. A search of this upstream region of *BmCatD* showed a typical TATA box at −67 bp. To screen for putative binding sites for the ecdysone receptor on the *BmCatD* promoter, we compared the nucleotide sequence between −1363 and +91 bp to the contigs of the GenBank *B. mori* genome using the Gene Quest Module of DNASTAR. Three putative EcRE consensus sequences were identified and listed in Table 2 (Fig. 1).

3.2. Mutation of EcREs in BmCatD promoter using overlap extension PCR

Site-directed mutagenesis using overlap extension PCR was performed on EcREs in the *BmCatD* promoter. The six mutant promoter sequences of EcRE1^m, EcRE2^m, EcRE3^m, EcRE1^m,2^m, EcRE1^m,3^m and EcRE1^m,2^m,3^m are listed in Fig. 2. Unfortunately, the EcRE2^m,3^m promoter mutant could not be successfully created due to the short distance (10 bp) between EcRE2 and EcRE3.

By overlapping mutation of the EcRE regions, seven recombinant AcMNPVs were constructed: AcP1363, AcEcRE1^m, AcEcRE2^m, AcEcRE3^m, AcEcRE1^m,3^m, AcEcRE1^m,2^m and AcEcRE1^m,2^m,3^m. The actin 3 (A3) control donor plasmid, pFA3Luc-A3RL2, was recombined with bacmid Ac*DH10BacDEGT* and named Ac*DH10BacDEGT* A3 (Fig. 3).

3.3. Dual-luciferase activity analysis of the EcREs in the BmCatD promoter

Luciferase activity was quantitated in larval fat body extracts 5 days post injection of the recombinant AcMNPVs by normalization of firefly luciferase to *Renilla* luciferase activity (Fig. 4). The luciferase activity of the mutated promoters was significantly reduced compared to control promoter P1363. The EcRE1^m mutant resulted in the largest reduction in promoter activity. EcRE1^m had 0.99% luciferase activity when compared to the control promoter P1363. The luciferase activities of the EcRE2^m and EcRE3^m promoters were 15.0% and 2.21% of P1363, respectively. The triple mutant, EcRE1^m,2^m,3^m, had 1.22% the luciferase activity of the P1363. These results indicate that all of these EcREs are responsible for suppressing expression of *BmCatD* in the fat body, and EcRE1, closest to the transcription start site, plays the most important role in *BmCatD* gene expression. These data suggest that the EcRE located in the −109 to −99 bp region upstream from the transcription start site of the *BmCatD* promoter confers the highest repression of *BmCatD* expression in the fat body.

4. Discussion

The silkworm *B. mori* has been studied for its susceptibility to wild-type *A. californica* multiple nucleopolyhedrovirus (AcMNPV) and is recognized as being nonpermissive to AcMNPV infection either through oral ingestion or by intrahemocoelical injection [21–23]. Recent studies demonstrated that the intrahemocoelical

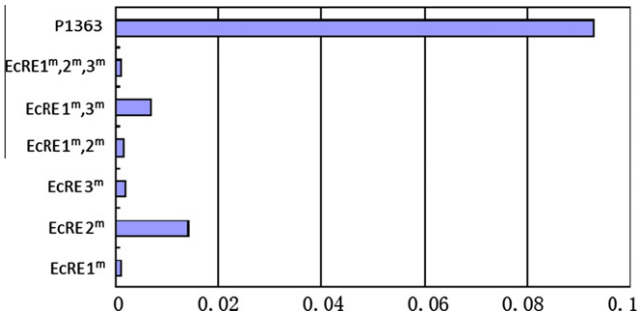

Fig. 4. Effect of site-directed mutagenesis of EcRE sequences on the transactivation of the *BmCatD* promoter. Fat body extracts were assayed for luciferase activity 5 days after injection of the recombinant AcMNPVs. Firefly luciferase activity was normalized to *Renilla* luciferase activity. Luciferase assays were performed in quadruplicate, and the results represent mean (Firefly/*Renilla*)/(FA3/RA3) ratios ± S.E.M.

infection of AcMNPV to the silkworm is not a rare and isolated phenomenon [24]; and some strains of silkworm are permissive to recombinant *A. californica* nucleopolyhedrovirus (rAcNPV) [19]. In this study, strain 54A of *B. mori*, which are permissive to rAcMNPV, were selected and used.

Hormonal signals are important regulators of insect development during molts and metamorphosis, especially the steroid 20-hydroxyecdysone (20E) [9,25]. 20E binds the ecdysone receptor (EcR) and directly activates the transcription of a small set of the early genes, that/which coordinate the subsequent transcription of tissue-specific late genes [26]. The *Drosophila* EcR is a member of the nuclear receptor superfamily and has three isoforms (A, B1, and B2). These isoforms contain common DNA- and ligand-binding domains but diverse N-terminal regions due to different promoters and alternative splicing [27–28]. Molecular analyses determined that various EcR isoform combinations are required for different metamorphic responses [28]. The *Bombyx* EcR (*Bm*EcR) has been isolated [29] and is a B1 isoform that can heterodimerize with *Bm*CF1, the *Bombyx* homologue of Ultraspiracle [30]. *Bm*EcR-B1 is highly expressed in the wing imaginal disc throughout the last larval instar, which differs from the expression pattern in *Drosophila* [29].

We have previously shown that the *BmCatD* expression pattern is controlled by *B. mori* nuclear polyhedrosis virus (*Bm*NPV), temperature and insect hormones, such as 20E and a juvenile hormone analog [31]. 20E upregulates *BmCatD* expression [7,31], which suggests that the *BmCatD* promoter is directly activated by 20E. Bioinformatic analysis identified three EcRE sequences in the upstream region of the *BmCatD* promoter (1363 bp): 5′–TTCGATTGACA–3′ (−109/−99), 5′–TTTAACTGAAA–3′ (−836/−826) and 5′–TTGGAATGAAA–3′ (−856/−846) (Fig. 1 and Table 1).

In this study, we determined the direct regulation of *BmCatD* by 20E through site-directed mutagenesis of EcRE sequences. We identified three functional EcREs of *BmCatD* that are necessary for the hormone-mediated activation of gene expression in the fat body (Fig. 4). Previous studies suggest EcRE1 in *BMWCP10* (a cuticle protein gene in *B. mori*) is necessary for 20E activation of it's promoter in the wing disc because the mutation of EcRE1 causes loss of responsiveness to 20E [32]. Mutation or deletion of the EcRE in the *VM32E* gene results in the loss of reporter gene expression in dorsal follicle cells. The data indicate that the EcR-B1/USP heterodimer activates *VM32E* expression in dorsal follicle cells by binding to the EcRE [33]. Our current study identified conserved EcRE sequences in Diptera and Lepidoptera. Similar binding sequences in *bFTZ-F1* [34], *BR-Z2* [35], *BR-CZ4* [36] and *BMWCP10* [32] upstream of cuticle protein genes were also conserved in Diptera and Lepidoptera. Bonneton et al. [37] demonstrated that transcription factors and their target-gene binding sites have co-evolved, with most of the transcription factors appearing before the arrival of the holometabolous insect in the evolutionary record. These ecdysone-responsive transcription factors and their binding sequences are conserved [32].

The insect fat body releases growth factors [38] and is homologous to mammalian livers as it is the primary organ for metabolism and synthesis of hemolymph components [39]. During metamorphosis, the fat body transforms from clusters into individual cells. As described previously, *BmCatD* is specifically expressed in the larval fat body and pupal gut, where it is implicated in important physiological processes such as PCD during the larval – pupal transformation in *B. mori*. Injection of 20E into larval *B. mori* fat bodies has been shown to induce PCD [7]. Moreover, recent results indicate that 20E might not directly affect PCD of the fat body, but rather through the head and/or thorax [17]. Our current study may instigate further examination of promoter regions through stage- and tissue-specific promoter assays.

Insect development is regulated by ecdysone signaling through ecdysone-responsive transcription factors and effector genes, but many questions remain unanswered. These results support the role for control of *BmCatD* gene expression by EcREs and provide a good model system for understanding the molecular mechanisms by which ecdysone exerts its effects on insect development.

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