Differential responsiveness of *BmBR-C* promoters to ecdysone signals

Yoshinori Nishita* and Shigeharu Takiya

Section of Functional Biology, Department of Biological Sciences, and Research Center for Genome Dynamics, Faculty of Science, Hokkaido University, Sapporo 060-0810, Hokkaido, Japan

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The *Bombyx mori* homolog of the Broad-Complex gene (*BmBR-C*) is transcribed from two different promoters separated by 101 kbp. In our previous report where we used semi-quantitative RT-PCR to amplify the total RNA prepared from tissues at different stages, the distal (P\text{dist}) and the proximal (P\text{prox}) promoters appeared to respond differentially to ecdysone signals. To explore the expression control mechanism of *BmBR-C* in response to ecdysone, transfection assays using *Bombyx* BM-N cells were performed with luciferase reporter genes under the control of various P\text{dist} or P\text{prox} fragments. In these experiments, the promoter regions located between 3 and 5 kbp upstream from the proximal transcription start site (TSS), and between 2 and 5 kbp upstream from the distal TSS, appeared to function in the down-regulation of P\text{prox} and the up-regulation of P\text{dist}, respectively, in response to 20-hydroxyecdysone (20E). In electrophoretic mobility shift assays (EMSAs), proteins bound to the probe containing the candidate ecdysone-responsive element (cEcRE), located 3 kbp upstream from the distal TSS, were detected predominantly in the nuclear extract prepared from 20E-treated cells; however oligonucleotides containing the typical EcRE (that of the *hsp27* or *fbp1* genes of *Drosophila*) could not compete with probe for binding to these proteins. Furthermore, the level of responsiveness of P\text{dist} to 20E declined, but did not disappear, after deletion of the cEcRE. These results suggest that the functional ecdysone receptor (EcR) may bind to elements other than the cEcRE, or that transcription from P\text{dist} is activated by an ecdysone-responsive factor, such as βFTZ-F1, rather than a functional EcR.

**Keywords:** *Bombyx mori*, Broad-Complex, differential transcription, dual-promoter, ecdysone, steroid hormone receptor

**INTRODUCTION**

In many insects, the complex developmental processes of molting, larval ecdysis, and metamorphosis are driven by oscillations in the titers of the steroid hormone, ecdysone, which promotes metamorphic changes, and the antagonistically acting sesquiterpenoid juvenile hormone (JH). A rise in ecdysone concentration during development initiates rapid changes in stage- and tissue-specific gene expression through a genetic hierarchy of ecdysone-responsive genes (Berendes and Ashburner, 1978; Andres and Thummel, 1992; Riddiford, 1995; Riddiford et al., 2003; Warren et al., 2006).

At the top of the hierarchy, ecdysone activates transcription of a limited number of genes referred to as “early genes,” some of which encode transcriptional regulatory factors such as: E74 (Burtis et al., 1990), E75 (Segraves and Hogness, 1990), and Broad-Complex (BR-C; DiBello et al., 1991). The primary role of these early genes is to coordinate the temporal activation of appropriate sets of developmental genes referred to as “late genes” (Berendes and Ashburner, 1978).

In *Drosophila melanogaster*, BR-C, one of the early-gene products, plays key roles both in coordinating the ecdysone response among tissues and in selective control of the expression of downstream effector genes (Thummel, 2002). The N-terminal region of BR-C is comprised of a highly conserved common core region that includes a BTB (bric-a-brac, tramtrack, and Broad-Complex) domain [also known as a pox viruses and zinc fingers (POZ) domain; Bardwell and Treisman, 1994; Zollman et al., 1994] that serves in protein-protein interactions. The BTB/POZ domain is characteristic of the GAGA factor and other chromatin-altering factors (Albagli et al., 1995), and is known to interact with the silencing mediator of retinoid and thyroid (SMRT), a co-repressor of unliganded retinoic acid and thyroid receptors (Dhordain et al., 1997), suggesting a possible mechanism for the alteration of the chromatin structure through SMRT and histone deacetylase. The BTB/POZ region is fused to an isoform-specific C-terminal region containing one of four C\text{\textsubscript{2}H\textsubscript{2}} zinc-finger pair motifs designated Z1, Z2, Z3, and Z4, respectively. Accordingly, alternative mRNA splicing generates four main types of BR-C protein isoforms (DiBello et al., 1991; Bayer et al., 1996).

We previously isolated cDNAs from the silkworm, *Bombyx mori*, encoding homologs of all the main BR-C (*BmBR-C*) isoforms except Z3 (Nishita and Takiya, 2004). Comparison of the cDNA and genomic DNA sequences yielded the structure of the entire genomic DNA sequence of *BmBR-C* with its 5’ flanking region. *BmBR-C* spans a
distance of about 158 kilobase-pairs (kbp) including 13 exons. The 5’ ends of the cDNAs were mapped to two different positions, indicating the presence of dual promoters, a distal promoter \( P_{\text{dist}} \) and a proximal promoter \( P_{\text{prox}} \), separated by 101 kbp. Semi-quantitative RT-PCR results indicated that these promoters are used differently. For example, at the onset of pupation, transcription from \( P_{\text{prox}} \) was suppressed in all tissues examined; whereas, transcription from \( P_{\text{dist}} \) was increased in some tissues, such as the carcass and the anterior silk gland. A combination of two promoters differing in responsiveness to a signal (such as ecdysone) might allow for complex regulation of downstream genes by a simple hormonal signal (Nishita and Takiya, 2006).

Up-regulation of ecdysone-responsive genes is mediated by the binding of ecdysone to a receptor complex comprising of the ecdysone receptor (EcR) and ultraspiracle (Usp) proteins (Yao et al., 1993). The first ecdysone-response element (EcRE) identified was a 13-bp fragment \( 5’-\text{GGTTCA A TGCACT}-3’ \) of the \textit{Drosophila hsp27} gene promoter (Riddihough and Pelham, 1987, Martinez et al., 1991), and upon further analysis, the consensus sequence for EcRE was revealed to be \( 5’-(A/G)G(G/T)\text{TCA N TGA (C/A)C(C/T)}-3’ \) (Cherbas et al., 1991). The gene encoding a \textit{Drosophila} EcR (DmEcR) was cloned and reported to be a member of the nuclear receptor superfamily (Koelle et al., 1991). In addition, \textit{Bombyx} genes encoding homologs of DmEcR (BmEcR) and DmUsp (BmCF1) have been cloned, but the deduced amino acid sequences of the homologous sequences are divergent (Swevers et al., 1995, Tzertzinis et al., 1994). However, upon dimerization with BmCF1, BmEcR binds 20-hydroxyecdysone (20E) with avidity similar to that of the DmEcR/DmUsp dimer. The BmEcR/BmCF1 dimer forms a specific complex with an EcRE derived from the \textit{Drosophila} hsp27 gene promoter, strongly suggesting that BmEcR and BmCF1 are the functional counterparts of DmEcR and DmUSP (Swevers et al., 1996) in \textit{Bombyx}.

In the present study, we investigated the molecular mechanism of \textit{BmBR-C} expression, which is induced by ecdysone \textit{in vivo} and antagonized by JH. We evaluated the transcriptional activity of reporter genes driven by a series of truncated \( P_{\text{dist}} \) or \( P_{\text{prox}} \) sequences in transfected \textit{Bombyx} BM-N cells when exposed or not exposed to 20E, and in the presence or absence of the JH analog (JHA) methoprene. In response to 20E, transcription from \( P_{\text{dist}} \) was up-regulated. This effect was blocked by the concurrent addition of JHA. On the other hand, transcription from \( P_{\text{prox}} \) was down-regulated. A candidate EcRE (cEcRE) was identified 3 kbp upstream from the distal transcription start site (TSS) of \( P_{\text{dist}} \). We also examined the participation of the cEcRE in the up-regulation of \textit{BmBR-C} expression in response to ecdysone.

### MATERIALS AND METHODS

#### Semi-quantitative RT-PCR

Total RNA was extracted from BM-N cells treated for 48 h with or without 1.0 μM (500 ng/ml) 20E. Fifty nanograms of total RNA per RT-PCR were reverse transcribed using the PrimeScript® 1st strand cDNA Synthesis Kit (Takara Bio) according to the manufacturer’s instructions. \textit{BmBR-C} mRNA expression levels were normalized against the ribosomal protein Rp49. The primer sequences used for the amplification of partial cDNA sequences from the \( P_{\text{dist}} \) transcript, \( P_{\text{prox}} \) transcript, common “core region”, or Rp49 are shown in Table 1. These cDNA samples were amplified by PCR at a volume of 50 μl. The amplification was performed for 1 cycle at 94°C for 5 min; then 22 to 35 cycles at 94°C for 30 s, 64°C (Rp49: 54.5°C) for 30 s, and 72°C for 30 s; and finally, 1 cycle at 72°C for 10 min. The number of cycles and conditions were optimized to produce a linear relationship between the amount of template used and the amount of PCR product. The RT-PCR products were run on a 2% agarose gel, stained with ethidium bromide, destained, and photographed with the AE-6910 gel imaging system (ATTO).

#### Construction of luciferase reporter genes

The distal and proximal promoter regions of \textit{BmBR-C}
Ecdysone-responsiveness of *BmBR-C* promoters were amplified by PCR, and the amplified fragments were subcloned into the PGV-B firefly luciferase reporter vector (TOYO B-Net; Fig. 1). The minimal promoter of the *Bombyx A3* cytoplasmic actin gene (the region from −128 to +28) was also amplified (Mangé *et al.*, 1997). This fragment was subcloned into the pRL-null *Renilla* luciferase vector (Promega) to create pRL-BmA3 (Fig. 1), which was used as a control for transfection. The primers used for PCR are shown in Table 2. The sequences of all constructs were confirmed by restriction enzyme digestion and DNA sequencing.
Transfection and luciferase activity assay

All transfection assays were carried out in at least triplicate in Eppendorf tubes. BM-N cells (2 × 10^5 cells) were transiently transfected using Cellfectin Reagent (Invitrogen) according to the manufacturer’s instructions. Two micrograms of the luciferase reporter constructs described in Figure 1, along with mock (promoter-less) plasmid (PGV-B), were co-transfected with 0.2 μg of pRL-BmA3. The cells were incubated in the transfection mixture for 5 h and then cultured for 48 h in the presence or absence of 1.0 μM (500 ng/ml) 20E with or without 3.7 μM (1000 ng/ml) JHA.

After harvesting, the cells were assayed for luciferase activity using the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer’s instructions. Briefly, the cell lysates were prepared in 20 μl Passive Lysis Buffer, mixed with Luciferase Assay Substrate and Stop & Glo substrate, and measured for luminescence using a Luminescencer-PSN (AB-2200; Atto, Tokyo, Japan). The firefly luciferase activity of the reporter plasmids was normalized to the *Renilla* luciferase activity of the cotransfected control plasmid (Fig. 1). Non-transfected and mock-transfected cells were used as negative controls. Firefly luciferase activity was normalized against that of *Renilla* luciferase, and the relative luciferase activity (firefly/Renilla; *P* < 0.01) was compared amongst the samples. Data represent the mean ± the standard error of the mean (SEM) from at least four experiments.

**Preparation of nuclear extracts**

The procedure described by Takiya *et al.* (2004) was used to perform large-scale isolations of nuclear extracts from the tissues of *Bombbyx* larvae or whole embryos. Nuclear extracts of BM-N cells (~ 1 × 10^7 cells) were prepared on a small scale from exponentially growing cells cultured in TC-100 insect medium supplemented with 10% heat-inactivated fetal bovine serum in the presence or absence of 1.0 μM (500 ng/ml) 20E. Briefly, the cells were suspended in cold buffer A [10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 1 mM dithiothreitol (DTT), 0.5 mM phenylmethylsulfonyl fluoride (PMSF)], allowed to swell on ice for 15 min, homogenized, and centrifuged. The resulting nuclear pellet was re-suspended in ice-cold buffer C [20 mM HEPES (pH 7.9), 0.4 M NaCl, 1 mM

<p>| Table 2. Sequences of primers used in constructing luciferase reporter plasmids |</p>
<table>
<thead>
<tr>
<th>Name</th>
<th>Primer Sequence</th>
<th>Tm (°C)</th>
</tr>
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<tbody>
<tr>
<td>Distal promoter region forward primers</td>
<td></td>
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<tr>
<td>-5k</td>
<td>CGACCTCCTGGACTGTACCAGTTGGTGCGATGG</td>
<td>70.5</td>
</tr>
<tr>
<td>-4k</td>
<td>CCGAGTICAAAAGTTTCTATGTGGTGGCC</td>
<td>60.5</td>
</tr>
<tr>
<td>-3k</td>
<td>GAACCTCAATCTCTCACCACATACAAAC</td>
<td>60.5</td>
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<tr>
<td>-1k</td>
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</tr>
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<td>0k</td>
<td>GAGATGCAGAAAAATATTTGCAATGCG</td>
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<td>Distal promoter region common reverse primer</td>
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<td>67.6</td>
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<td>Proximal promoter region forward primers</td>
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<td></td>
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<tr>
<td>-5k</td>
<td>CCGGAGAACCACAAAACATGACAGAAAACGTACCTTG</td>
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<td>-4k</td>
<td>CTCGAACATTATGATTACAGCTGTCGG</td>
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<td>Proximal promoter region common reverse primer</td>
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<td>60.5</td>
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<tr>
<td>Control (BmA3) forward primer</td>
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<tr>
<td>Control (BmA3) reverse primer</td>
<td>gagctgacctaaacctagctggccggcg</td>
<td>60.4</td>
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</table>

*Lowercase letters indicate nucleotides added to create restriction sites (Eco RI or Xho I).*
EDTA, 1 mM DTT, 1 mM PMSF], and shaken at 4°C for 15 min. The extract was dialyzed against a buffer consisting of 50 mM Tris-Cl (pH 7.9), 12.5 mM MgCl₂, 100 mM NaCl, 20% glycerol, and 0.1% Nonidet P-40. Aliquots of the nuclear extract were stored at -80°C.

Electrophoretic mobility-shift assays

Electrophoretic mobility-shift assays (EMSAs) were performed as described in Takiya et al. (2004) using synthetic oligonucleotides containing an EcRE or a mutant EcRE sequence. The sequences of the upper strands are shown in Figure 2. To make double-stranded DNA probes, complementary strands were mixed together, heated at 95°C for 5 min, and annealed at room temperature for 1 h. The annealed DNAs were labeled with [α-32P]dATP and Klenow fragment.

In binding reactions, 0.04 ng of the labeled probe was incubated with 12 μg of the crude nuclear extract for 15 min at 4°C in a 10-μl reaction mixture containing 10 mM Tris-Cl (pH 7.9), 7.5 mM MgCl₂, 60 mM NaCl, 1 μg poly(dI-dC), 20% glycerol, and 0.6% Nonidet P-40. In competition assays, the reaction mixture also contained 2 ng of unlabeled double-stranded competitor DNA. Reaction mixtures were electrophoresed on 5%-polyacrylamide (acrylamide:bisacrylamide ratio, 29:1) gels in TBE buffer (25 mM Tris, 26.5 mM borate, 0.5 mM EDTA) at 4°C. After the gel was dried, shifted bands were detected with a Bio-Image Analyzer (Fujifilm).

RESULTS

Hormonal effects on BmBR-C promoters

Because P_dist and P_prox of BmBR-C are regulated differently in various tissues (Nishita and Takiya, 2006), we expected these promoters to respond differently to ecdysone. Using semi-quantitative PCR and total RNA prepared from BM-N cells cultured with or without 1 μM (500 ng/ml) 20E as a template, we examined the expression of BmBR-C from endogenous P_dist and P_prox in response to 20E (Fig. 3). Three primer sets were used: a specific primer set for the P_dist transcript, a specific primer set for the P_prox transcript, and a third primer set for the common core region (Fig. 3A, arrows; Table 1). Exposure of the cells to 20E caused an increase in the endogenous P_dist transcript compared to non-treated cells. However, the endogenous P_prox transcript could not be detected, regardless of 20E treatment (Fig. 3B). Similar to previous studies examining BmBR-C expression in the carcass and anterior silk gland (ASG; Nishita et al., 2006), BmBR-C was predominantly transcribed from the endogenous P_dist in BM-N cells in response to 20E.

To analyze the regulatory mechanism controlling BmBR-C expression, we present the EcRE consensus sequence located at the start of the P_dist promoter in BM-N cells in response to 20E.

<table>
<thead>
<tr>
<th>Oligonucleotide Probes</th>
<th>Binding Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>BmBR-C candidate EcRE</td>
<td>:GTT TAT ATT GGT ATT GGT TAA T TGAAC T AAT G</td>
</tr>
<tr>
<td>Dmhs27 EcRE</td>
<td>:aatt GAC AAG GGTCA A TGCAC T TGCCAC T AAT G</td>
</tr>
<tr>
<td>Dmfbp1 EcRE</td>
<td>:aatt TCC CGA TTG GGTCA A TGAAT TT GCG T</td>
</tr>
<tr>
<td>hHNF4 binding sequence</td>
<td>(aatt GCC TGAAC T CCA A GTTCA GTC CCT TCG)</td>
</tr>
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</table>

Fig. 2. Oligonucleotide probe and competitor DNAs used in electrophoretic mobility shift assays (EMSAs). The upper strands of double-stranded oligonucleotides containing the EcRE consensus sequence from Bombyx BR-C or Drosophila hsp27 or Fbp1 or containing the human HNF-4 binding sequence are shown. The EcRE is indicated by arrows. Mutated oligonucleotides were used as competitors, and the mutations are shown directly under the individual wild-type sequences.
expression at the molecular level, a transient transfection assay was performed using BM-N culture cells. Cells were transfected with firefly luciferase reporter constructs containing the 5-kbp 5' upstream region of P \textit{dist} or P \textit{prox} [PGV-P \textit{dist}(-5k) or PGV-P \textit{prox}(-5k); Fig. 1] and assayed for luciferase activity after exposure to 20E, alone or in combination with JHA. Firefly luciferase activity derived from the reporter plasmid was normalized to the \textit{Renilla} luciferase activity derived from the co-transfected control plasmid pRL-BmA3 (Fig. 1).

The 5-kbp P \textit{dist} and P \textit{prox} fragments were able to promote transcription in BM-N cells (Fig. 4). When the cells were exposed to 1 μM (500 ng/ml) 20E, the promoter activity of P \textit{dist} increased (20E +/- = 22.0). Exposure of the cells to both 1 μM 20E and 3.7 μM (1000 ng/ml) JHA essentially eliminated the 20E-induced increase in P \textit{dist} activity. On the other hand, exposure of the cells to 20E caused the promoter activity of P \textit{prox} to decrease (20E +/- = 0.75), and concurrent exposure to JHA enhanced this effect (Fig. 4). Therefore, the ecdysone signal up-regulated the expression of \textit{BmBR-C} from P \textit{dist}, but down-regulated its expression from P \textit{prox}.

Deletion analysis of the \textit{BmBR-C} promoters

Reporter constructs containing P \textit{prox} fragments of different lengths (Fig. 1) were transfected into BM-N cells in the presence or absence of 20E (Fig. 5). As described above (Fig. 4), the activity of the 5-kbp P \textit{prox} region [PGV-P \textit{prox}(-5k)] was inhibited by 20E. Luciferase activity with the 4-kbp P \textit{prox} region [PGV-P \textit{prox}(-4k)] was also down-regulated in response to 20E (20E +/- = 0.56; t\textsubscript{s} = 4.302 > 2.306, P < 0.05). Constructs containing even shorter P \textit{prox} fragments exhibited weaker promoter activity and lost most of their responsiveness to 20E. For example, inclusion of the PGV-P \textit{prox}(-3k) region resulted in t\textsubscript{s} = 0.4869 < 2.365 (P < 0.05), demonstrating that the 2-kbp region located 3 kbp upstream from the proximal TSS is involved in the down-regulation of P \textit{prox} in response to 20E. The shortest construct [PGV-P \textit{prox}(0k)], which contained only 114 bp of the \textit{BmBR-C} sequence including the initiator (Inr) sequence [5'-TCA(+1)GTC-3'], the consensus sequence [5'-TCA(G/T)T(T/C)-3'; Arkhipova, 1994], and the TSS (numbered +1), retained its promoter activity. Therefore, the 114-bp fragment including the proximal TSS was sufficient for basal expression.

The 5-kbp region of P \textit{dist} was similarly dissected (Fig. 1) to evaluate its responsiveness to 20E (Fig. 6A). The PGV-P \textit{dist}(-5k) fragment promoted transcription and was responsive to 20E and JHA (Figs. 4 and 6A). The shorter constructs PGV-P \textit{dist}(-4k) and PGV-P \textit{dist}(-3k) were less responsive to 20E, but 20E still increased the reporter activity (20E +/- = 6.6 and 1.7, respectively). The 20E-induced up-regulation of PGV-P \textit{dist}(-4k) and PGV-P \textit{dist}(-3k) was inhibited by concurrent exposure to JHA. The even shorter fragments, PGV-P \textit{dist}(-2k) and PGV-P \textit{dist}(-1k), retained basal promoter activity, but were non-responsive to 20E. The
Ecdysone-responsiveness of $BmBR-C$ promoters

The shortest fragment, PGV-P$_{\text{dist}}$(0k), which contained the shortest promoter sequence, retained almost no promoter activity or ecdysone responsiveness. Therefore, the promoter region located between 2 and 5 kbp upstream from the distal TSS appeared to be involved in the responsiveness of P$_{\text{dist}}$ to 20E. In particular, the 3-kbp region upstream of the distal TSS, which was included in the reporter plasmid PGV-P$_{\text{dist}}$(−3k), was 20E-responsive ($t_{10} = 4.377 > 2.228$, $P < 0.05$), but the shorter 2-kbp region, as in PGV-P$_{\text{dist}}$(−2k), did not respond to 20E ($t_{12} = 1.062 < 2.179$).

From the above results, it was anticipated that the promoter region located between 2 and 3 kbp upstream from the distal TSS might contain an element important in the ecdysone response. Indeed, a cEcRE (5′-GGTT$\Delta$A T TGAAC-3′; single base substitution toward the EcRE consensus sequence for $Drosophila$ is underlined) was identified 2,976 bp upstream from the distal TSS in P$_{\text{dist}}$. To assess the involvement of the cEcRE in the transcriptional response to ecdysone, novel luciferase assay reporters containing the cEcRE [PGV-P$_{\text{dist}}$(−3k EcRE+)] or lacking the cEcRE [PGV-P$_{\text{dist}}$(−3k EcRE−)] were constructed (Fig. 1). As shown in Figure 6B, the 20E-induced response was reduced for PGV-P$_{\text{dist}}$(−3k EcRE−) compared with that of P$_{\text{dist}}$(−3k EcRE+), but deletion around the cEcRE did not eliminate 20E responsiveness ($t_{8} = 8.535 > 2.306$; $P < 0.05$).

**Factors binding to the cEcRE region of P$_{\text{dist}}$ of BmBR-C**

EMSAs were performed using synthetic double-stranded probe and competitor DNAs (Fig. 2) to determine
whether a functional EcR complex would bind to the cEcRE located 3 kbp upstream from the distal TSS. The probe was incubated with crude nuclear extracts from several *Bombyx* tissues or from BM-N cells grown in the presence or absence of 20E, and then electrophoresed on a nondenaturing polyacrylamide gel. As shown in Figure 7, two major bands, one migrating more slowly (arrowhead) and the other migrating more quickly (arrow), were detected, along with some other minor bands. The intensity of the more slowly migrating band in the BM-N nuclear extract considerably increased as a response to 20E treatment (lanes 10 and 11).

Using the BM-N nuclear extracts, we performed a competition assay to determine whether the more slowly migrating band represented binding of the BmEcR/BmCF1 complex to the DNA. A 50-fold excess of unlabeled probe successfully competed with all bands formed in nuclear extracts regardless of 20E exposure (Fig. 8A, lane 2), but unexpectedly, mutant competitor DNA (BmBRC candidate EcRE M1 in Fig. 2) yielded a similar result (Fig. 8A, lanes 2 and 3). Similarly, when other competitor oligonucleotides containing the well-characterized EcREs of *Drosophila* *Fbp1* (D EcRE) and *hsp27* (hsp27 EcRE), as well as the mutated *hsp27* EcRE M1 (Antoniewski et al., 1993) and the human HNF4 binding sequence (Fig. 2; Costa et al., 1989; Paulson et al., 1990; Sladek et al., 1990), were used, neither the mutant *hsp27*, the HNF4 competitors, nor the *hsp27* and *Fbp1* competitors competed for binding of the above-mentioned protein to the probe (Fig. 8A, lanes 4-7). These results suggest that the band-shift was not a result of binding of BmEcR/BmCF1 to the cEcRE.

To confirm that the BmEcR/BmCF1 complex was present in the nuclear extracts, another EMSA was carried out using the *Drosophila* *hsp27* EcRE as a probe (Fig. 8B). The nuclear extracts from 20E-exposed cells yielded doublet (or triplet) bands (lane 1), and those from untreated cells yielded a single band migrating with almost the same mobility as the lower band indicated in lane 1 (lane

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**Fig. 6.** Functional deletion mapping of the *P*<sub>dist</sub> promoter of *BmBR-C*. A 2-μg aliquot of the series of *P*<sub>dist</sub> deletion constructs (A) or of *P*<sub>dist</sub> constructs with or without the cEcRE (B) (Fig. 1) was co-transfected into BM-N cells along with 0.2 μg of pRL-BmA3, and the cells were cultured in the absence or presence of 20E or JHA. Results shown are the mean values ± SEM for at least three separate transfections. t: two-sample sign tests indicated pairwise comparison.
regulated by exposure to 20-hydroxyecdysone (20E) (Fig. 4). The results of the present experiments indicate that the 2-kbp region located 3 kbp upstream from the proximal TSS is needed for 20E-induced down-regulation of \( P_{\text{prox}} \) (Fig. 5). However, the 20E-induced down-regulation of expression from \( P_{\text{prox}} \) was not affected by JHA, suggesting that 20E and JHA might each individually inactivate, or act as repressors of \( P_{\text{prox}} \) (Fig. 4), and that this effect was not directly mediated by a functional EcR.

Luciferase expression, driven by the 5-kbp region of \( P_{\text{dist}} \) [PGV-P\(_{\text{dist}}\)-(5k); Fig. 1], was up-regulated in response to exposure to 20E, and this up-regulation was blocked by concurrent exposure to JHA (Figs. 4 and 6). The deletion analysis (Figs. 1 and 6) indicated that the region between 3 and 5 kbp upstream from the distal TSS is needed for 20E responsiveness.

A cEcRE sequence with a single mismatch compared to the EcRE consensus sequence was found 2,976 bp upstream from the distal TSS, and binding of the protein(s) to the cEcRE-containing probe was specifically detected in nuclear extract prepared from 20E-exposed BM-N cells (Fig. 7). However, oligonucleotides containing the well-established EcRE sequences of *Drosophila hsp27* and *hfp1* (Fig. 2) did not compete for probe-protein binding (Fig. 8), raising the possibility that the bound protein(s) was not the BmEcR/BmCF1 complex. Additionally, deletion of the cEcRE reduced, but did not eliminate, the up-regulatory effect of 20E on \( P_{\text{dist}} \) activity (Fig. 6B). Taken together, strong transcriptional activation of \( P_{\text{dist}} \) in response to 20E may result from a combination of the 20E-responsive region between 2 and 3 kbp upstream from the distal TSS, except around the cEcRE, and the transcriptional enhancer function in a region farther upstream. The protein(s) detected via EMSA may function as one of the transactivators for up-regulation of \( P_{\text{dist}} \) in response to the ecdysone signal.

Here, we showed that \( P_{\text{dist}} \) is up-regulated and \( P_{\text{prox}} \) is down-regulated in response to 20E; however, as of now no conclusive evidence showing an interaction between the functional EcR heterodimer complex and *BmBR-C* promoter region has yet been obtained. The discrepancy between the generally accepted phenomenon that *BmBR-C*, as one of the early genes, is activated by ecdysone and our EMSA result suggesting that BmEcR/BmCF1 does not bind directly to the cEcRE in \( P_{\text{dist}} \) of *BmBR-C* (Fig. 8) can be explained in several different ways. First, the ecdysone signal pathway regulating \( P_{\text{dist}} \) of *BmBR-C* might be mediated by another nuclear receptor, such as *Drosophila* DHR38 or *Bombyx* BHR38, which forms a heterodimer with transactivated Usp (Sutherland *et al.*, 1995; Baker *et al.*, 2003).

Second, *BmBR-C* might not actually be an early gene, and an early gene product (other than BR-C) may control...
its expression directly or indirectly. For example, ectopic expression of an ecdysone-induced orphan receptor βFTZ-F1 leads to enhanced levels of BR-C in *Drosophila*, suggesting that the function of βFTZ-F1 is at least in part epistatic to that of BR-C in the ecdysone cascade (Woodard *et al.*, 1994; Broadus *et al.*, 1999).

Finally, a functional EcR may bind (directly or indirectly) to the P_{dist} region, independent of the cEcRE. The amino acid sequence of BmEcR is divergent from that of DmEcR (Tzertzinis *et al.*, 1994; Swevers *et al.*, 1995); therefore, a functional BmEcR may be able to bind to an element distinct from the typical EcRE in *Drosophila* (Woodard *et al.*, 1994; Broadus *et al.*, 1999).

Recently, studies on the molecular mechanism of BmBR-C transcriptional regulation have been conducted. One such study has shown that a putative JH receptor, Methoprene-tolerant (Met), regulates BR-C expression both positively and negatively in young pupae (Konopova and Jindra, 2008). However, we could not provide evidence that JHA affected the up-regulation of BmBR-C promoters in the presence (Figs. 4 and 6) or absence of 20E (data not shown). Further research using antibodies against BmEcR or BmCF1, such as in the supershift or chromatin immunoprecipitation (ChIP) assay, is needed to understand whether...
er ecdysone-induced BmBR-C expression depends directly on the binding of a functional EcR to the promoter region or whether it is mediated by other factors. Analyzing the precise expression control mechanism of BmBR-C will be helpful in understanding the molecular mechanisms of larval development and metamorphosis in insects.

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