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Identification of a DNA sequence critical for ecdysone receptor binding to the distal promoter of the *Bombyx Broad-Complex* gene

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The *Bombyx mori* homologue of the Broad-Complex gene, *BmBR-C*, is composed of 13 exons, and is transcribed from two promoters—a distal promoter, Pdist, and a proximal promoter, Pprox—separated by a distance of ~101 kbp. A highly homologous sequence (referred to as the cEcRE) that contains known binding sites for a functional ecdysone receptor (the canonical EcRE) was discovered on Pdist, however, surprisingly, the cEcRE does not bind to EcR/Usp. Further analyses indicated that two regions within Pdist, EcRE-D and EcRE-P, -4,950 bp and -3,480 bp upstream from the distal transcription start site, respectively, are important in the responsiveness of Pdist to 20-hydroxyecdysone. However, weak or non-detectable sequence similarities were found between the canonical EcRE and the EcRE-D and EcRE-P regions. Despite the fact that the sequence similarity to the canonical EcRE was less than that observed for the cEcRE that did not bind to EcR/Usp, the results obtained using electrophoretic mobility shift assays (EMSAs) strongly suggested that EcR/Usp could bind to the EcRE-D. In this study, the middle portion of EcRE-D, EcRE-Dbs3, containing an element exhibiting weak similarity to the canonical EcRE proved to be a key sequence mediating EcR/Usp binding to *BmBR-C* Pdist. Moreover, conserved residues, particularly the G and C residues positioned at -4, -2 and +4, between the EcRE-Dbs3 and the canonical EcRE were essential for EcR/Usp binding and for the ecdysone-driven transcriptional activation of *BmBR-C* Pdist.

Key words: *Bombyx mori*, *Broad-Complex*, ecdysone, ecdysone receptor

INTRODUCTION

A *Bombyx mori* homologue of the Broad-Complex gene (*BmBR-C*) is composed of 13 exons, and is transcribed from two promoters at a distance of about 101 kbp (Fig. 1; Nishita and Takiya, 2006). The transcriptional activity of the distal promoter, Pdist, but not the proximal promoter, Pprox, is activated by ecdysone (Nishita and Takiya, 2009). Sequence analysis revealed that a candidate ecdysone responsive element (referred to as the cEcRE), which is highly homologous to the canonical EcRE, exists on the Pdist at -2,976 bp from the distal transcription start site (TSS). However, removal of the cEcRE did not affect the responsiveness of the Pdist to ecdysone. A sequential deletion mutant of Pdist indicated that two regions, EcRE-D and EcRE-P, -4,950 bp and -3,480 bp upstream from the distal TSS, respectively, are important in the responsiveness of the Pdist to 20-hydroxyecdysone (20E; Nishita, 2014).

Ecdysone activates "ecdysone responsive" genes through binding to a nuclear receptor heterodimer comprising an ecdysone receptor (EcR; Koelle *et al*., 1991) and an ultraspiracle (Usp; Yao *et al*., 1992, BmCF1; Swevers *et al*., 1996). The presence of ecdysone responsive elements bound by an EcR/Usp (EcRE) have been observed on a variety of promoters, including those on *Drosophila hsp27, Fbp1, Eip28/29, Eip40,* and *Lsp-2* genes (Andres and Cherbas, 1992; Antoniewski *et al*., 1995). Together, these data allowed the consensus EcRE binding site for EcR/Usp to be determined as 5'-(A/G) G (G/T) T C A N T G A (C/A) C (C/T)-3' (Antoniewski *et al*., 1993; Cherbas *et al*., 1991). No significant sequence similarities have been noted between the canonical EcRE and the EcREs on the distal promoter of *BmBR-C,* however, both EcRE-D and -P can sequence-specifically bind *Bombyx mori* protein(s) (Nishita, 2014). Interestingly, those bound proteins may not include EcR/Usp, implying that *BmBR-C* transcription can be indirectly activated by ecdysone through the EcRE-P. Meanwhile, the mid-region of EcRE-D, EcRE-Db, might bind a functional *Bombyx* ecdysone receptor that activates the expression of *BmBR-C* from Pdist. Accordingly, it has been demonstrated that the expression of *BR-C* can be induced not only by juvenile hormone (JH; Zhou and Riddiford, 2002; Konopova and Jindra, 2008; Minakuchi *et al*., 2011), but also directly by ecdysone. Together, these results experimentally validate at a molecular level the fact that *BmBR-C* is classified as an ecdysone-induced gene.

In this study, we demonstrated that mutations of bases that are conserved between the canonical EcRE and the EcRE-D of *BmBR-C* Pdist reduced the binding activity of EcR/Usp to EcRE-D, and led to a functional decline in ecdysone responsive transcription from *BmBR-C* Pdist.

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MATERIALS AND METHODS

Preparation of nuclear extracts

Nuclear extracts of BM-N cells cultured in TC-100 (Sigma) supplemented with 10% fetal bovine serum ± 1 μM 20E (Sigma) were prepared as previously described (Dignam *et al*., 1983). Briefly, exponentially growing BM-N cells (1.5×10^6) were collected by centrifugation at 2,000 g for 1 min at 4°C. The pellet was washed with ice cold PBS, and then suspended in ice cold buffer A [10 mM HEPES-KOH pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol (DTT), 0.4 μM phenylmethylsulfonyl fluoride (PMSF)], allowed to swell on ice for 20 min, homogenized, and centrifuged. The resulting nuclear pellet was resuspended in ice cold buffer C [20 mM HEPES-KOH pH 7.9, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA pH 8.0, 25% glycerol, 0.5 mM DTT, 0.4 μM PMSF], and shaken at 4°C for 20 min. The extract was dialyzed against buffer D [20 mM HEPES-KOH pH 7.9, 20 mM KCl, 2 mM $MgCl_2$, 0.2 mM EDTA, 20% glycerol, 0.5 mM DTT, 0.4 μM PMSF]. Aliquots of the nuclear extract were stored at −80°C until use.

Electrophoretic mobility shift assay (EMSA)

The oligonucleotide sequences used for electrophoretic mobility shift assays are shown in Figs. 1A, 2A, and 3A. The annealed oligonucleotides used as the probes were labeled with biotin using the DIG Gel Shift Kit, 2nd Generation (Roche). For the chemiluminescence-detected gel shifts, binding reactions were performed as described by Yao *et al*. (1992) with slight modifications. Nuclear extracts were incubated with binding buffer (100 mM KCl, 7.5% glycerol, 20 mM HEPES pH 7.5, 2 mM DTT, and 0.1% NP-40) on ice for 20 min in the presence of 1 μg of nonspecific competitor poly [d(I-C)] or other oligonucleotide competitors as indicated in the text. The labeled probe (15.4 fmol) was added to the reaction and incubated at room temperature for 20 min. An anti-ecdysone receptor antibody (Abnova, MAB9070; Immunogen: native *Manduca* ecdysone receptor B1; Nishita, 2014) was added 10 min into the incubation. Then, the reaction was loaded into a 5% non-denaturing polyacrylamide gel in 0.5% TBE running buffer. After electrophoresis, the gel was blotted onto a positively charged Nylon membrane (Hybond N+; Amersham Biosciences). Membranes were developed using the DIG Gel Shift Kit, 2nd Generation (Roche) according to the manufacturer's protocol.

Preparation of promoter-containing reporter plasmids

To construct luciferase reporter plasmids driven by the distal promoter of *BmBR-C* harboring a single base mutation within EcRE-D, we used PCR to amplify sequences from a PGV-BmBR-C Pdist (-5k) luciferase reporter plasmid containing a -5,124 to +52 bp region of the *BmBR-C* distal promoter (Nishita and Takiya, 2009). Reporter plasmids were amplified using a combination of one of the oligonucleotides shown in Fig. 4A as a forward primer and the reverse primer, 5'- CCCAAGGTTACAAATTC ATAGCTCCATTAGAATG-3'.

Transfections and luciferase reporter assays

BM-N cells were seeded in 24-well plates the day before transfection at a density of 1.25×10^5 cells in TC-100 supplemented with 10% fetal bovine serum at 25°C. The cells were transiently transfected using FuGENE HD Transfection Reagent (Promega) with 0.5 μg of the test plasmid and 0.05 μg of the control plasmid, pRL-BmA3, which was used to normalize for transfection efficiency. This control plasmid (Nishita and Takiya, 2009) contained the 128 bp *Bombyx mori* A3 gene promoter (Mangé *et al*., 1997) upstream of the *Renilla* luciferase reporter gene in the pRL-null vector (Promega). After transfection, the cells were cultured ± 1 μM 20E (Sigma).

After 48 h in culture, the cells were harvested and assayed for luciferase activity using the Dual-Luciferase Assay System (Promega) according to the manufacturer's instructions with a Luminescencer-PSN (AB-2200; ATTO). The firefly luciferase activity of the reporter plasmid was normalized to the *Renilla* luciferase activity of the control plasmid. The results were expressed as the normalized firefly luciferase activity ± 20E. Data represent the mean ± 95% confidence intervals (CI; n = 6).

RESULTS AND DISCUSSION

Identification of a key EcR/Usp binding element within EcRE-D on *BmBR-C* Pdist.

Previous work suggests that the mid-region of EcRE-D, termed EcRE-Db, might be bound by a functional *Bombyx* ecdysone receptor and contributes to the induction of *BmBR-C* expression from Pdist (Nishita, 2014). To elucidate the key functional elements of EcRE-Db (Fig. 1), four overlapping oligonucleotides (EcRE-Dbs1-4), shown in Figure 2A were synthesized, DIG-labeled, and used as probes in electrophoretic mobility shift assays (EMSAs). Each probe was incubated with nuclear extracts obtained from cultures of the *Bombyx mori* cell line, BM-N. As shown in Figure 2B, an appreciable band representing a DNA-protein complex was observed only when EcRE-Dbs3 was used as a probe (Lane 11). Since the addition of a 50- or 100-fold excess of unlabeled probe successfully inhibited probe-protein binding (Lanes 12 and 13), the bound factor(s) appeared to interact in a sequence-specific manner with the EcRE-Dbs3 probe. Furthermore, the binding of the EcRE-Dbs3 probe to the factor(s) was com-

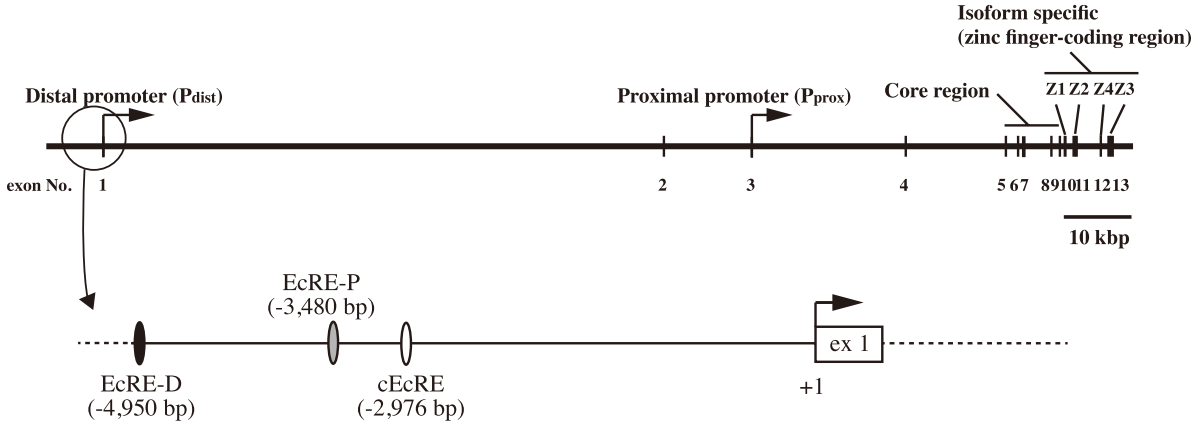

Fig. 1. The genomic structure of *BmBR-C* is shown diagrammatically. Exons are numbered from the 5'-end of the gene and depicted by black boxes. The introns and the flanking regions are depicted by interconnecting solid lines. Nucleotides are numbered relative to the distal transcription start site (+1). The position of the candidate ecdysone-responsive element (cEcRE; open oval), EcRE-P (gray oval) and EcRE-D (black oval) is shown.

pletely inhibited by the addition of the *Drosophila hsp27* EcRE oligonucleotide (Riddihough and Pelham, 1987). These results demonstrated that EcR/Usp may interact with the EcRE-Dbs3 sequence within EcRE-D of the *BmBR-C* Pdist. To obtain additional evidence that the protein(s) that bound to the EcRE-Dbs3 represented EcR/Usp, we tested the ability of an anti-EcR antibody (Abnova, MAB9070) to interfere with EcRE-Dbs3 probe-protein complex formation. We noted that the signal intensity resulting from the EcRE-Dbs3 probe-protein(s) complex (arrowhead) decreased in the presence of increasing concentrations of the anti-EcR antibody (Fig. 2C).

These observations suggested that an EcR formed part of the protein-probe complex, and that 5'- TGGAGCGGT CATTCACACCGTG-3', a sequence within EcRE-Dbs3 containing an element exhibiting weak similarity to the canonical EcRE (underlined), was the target of EcR/Usp binding. Shirai *et al*. (2012) advocated that a 14 bp consensus motif is conserved among EcREs for three *Bombyx mori* nuclear receptor genes (*Bm-EcRB1, BmE75A, BHR3B*). However, the EcRE-Db3 EcR/Usp binding sequence bears a higher sequence similarity to the canonical EcRE than does the 14 bp consensus motif (Fig. 2A).

Importance of the residue at position -2 of cEcRE for EcR/Usp binding.

Visual inspection indicated improved matching of homologous sequences at 2,976 bp upstream from the distal transcription start site (TSS) within *BmBR-C* Pdist, referred to as the candidate EcRE (cEcRE; Fig. 1), which differed from the canonical EcRE by only a single base pair (Nishita and Takiya, 2009). A comparison of the canonical EcRE sequence with the cEcRE showed that the cEcRE has one mutation at the -2 position within the Usp-binding site (Fig. 3A; Grad *et al*., 2001). It has been suggested that as an EcR/Usp heterodimer binding to EcRE, Usp may rigidly recognize a specific DNA sequence, while EcR alone may be more promiscuous in its binding (Devarakonda *et al*., 2003). To test the importance of the nucleotide at the -2 position within the Usp-binding site, an oligonucleotide containing an A to C base substitution at the -2 position of the cEcRE was synthesized and used as a probe for EMSA (Fig. 3B; Lanes 6–10). Binding of the cEcRE -2 (A→C) probe to the undefined factor(s) was observed (Lane 6); this binding was completely inhibited by the addition of either a 50- or 100-fold excess of the *Drosophila hsp*27 EcRE oligonucleotide (Lanes 9–10) or unlabeled probe (Lanes 7–8). To confirm that the protein(s) bound to the cEcRE -2 (A→C) probe represented EcR/Usp, we tested the ability of an anti-EcR antibody to interfere with the probe-protein complex formation (Nishita, 2014). We noted that the signal intensity resulting from the cEcRE -2 (A→C) probe-protein complex (arrowhead) decreased in response to increasing concentrations of the anti-EcR antibody (Fig. 3C).

Although the results strongly suggested that EcR/Usp could bind to EcRE-Dbs3, the latter's sequence similarity to the canonical EcRE (Fig. 2A) was less than that observed for the cEcRE that did not bind to EcR/Usp (Figs. 3A and B; Lanes 1–5). Compared to the canonical EcRE sequence, the cEcRE has one mutation in the Usp-binding site, but EcRE-Dbs3 is not mutated at this site (compare Figs. 2A and 3A). These results demonstrated that the C residue located at -2 position within an Usp-binding site may be one of the most important residues for the binding of EcR/Usp to the EcRE-D of *BmBR-C* Pdist.

Sequence specificity of EcR/Usp binding to the EcRE-D

Although the sequence similarity was weak, a known binding site for EcR/Usp was discovered within EcRE-

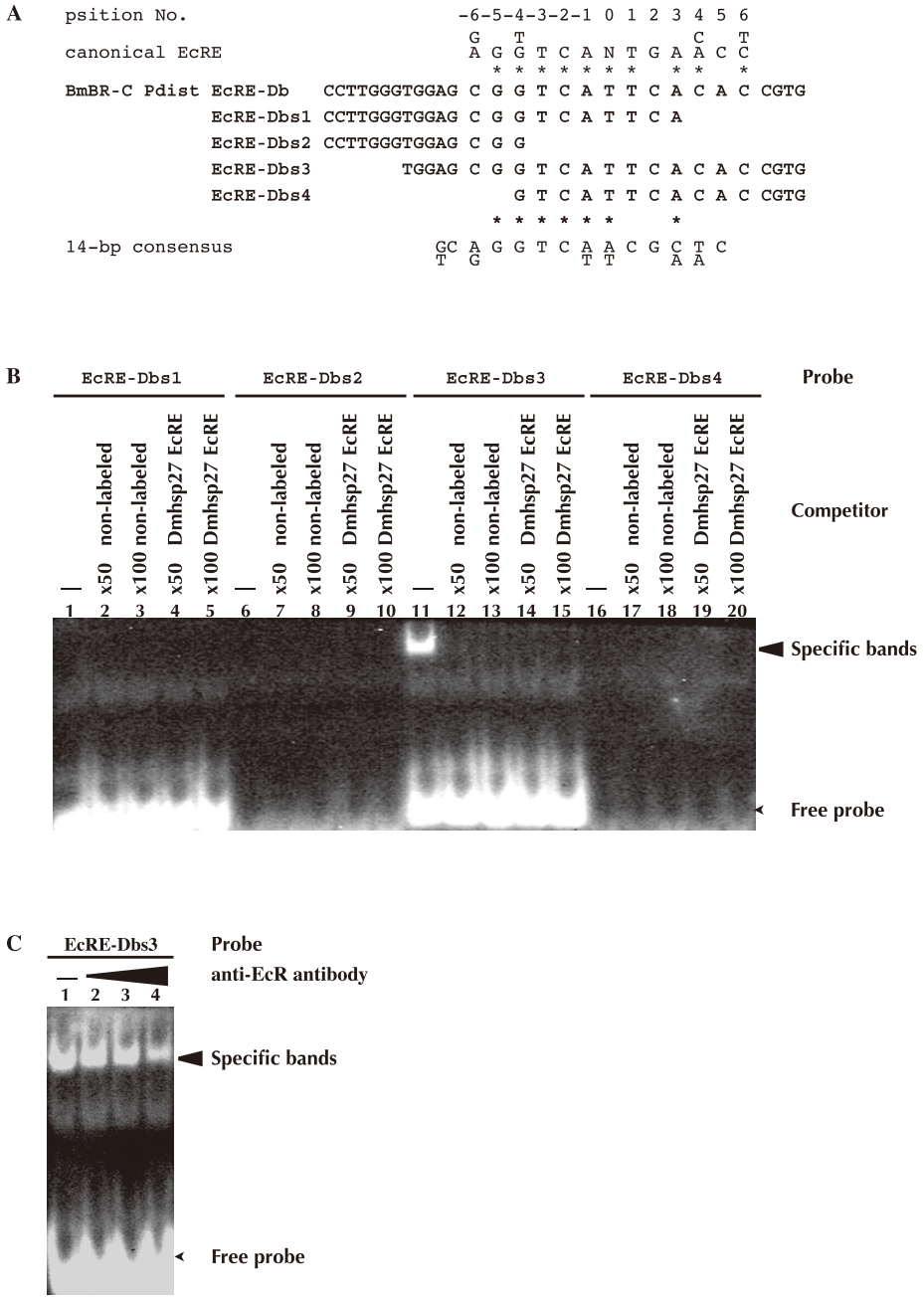

Fig. 2. A core element of the EcR/Usp binding site is observed within EcRE-Db. (A) Alignment of the (+) nucleotide sequences containing the canonical EcRE (Cherbas et al, 1991), 14-bp consensus (*Bombyx* EcRE; Shirai et al., 2012), EcRE-Db, and the deletion mutants within EcRE-Db (EcRE-Dbs1–4). (B) EMSA using EcRE-Dbs1, 2, 3, or 4 as a probe. Sequence specific probe-protein bindin is inhibited by the addition of a 50- or 100-fold excess of either unlabeled probe or a *Drosophila hsp27* EcRE-containing oligonucleotide (Riddihough and Pelham, 1987). (C) Addition of an anti-EcR antibody (MAB9070, Abnova) confirms the involvement of an EcR with the Pdist EcRE-Dbs3. Varying amounts of an anti-EcR antibody were added to BM-N cell nuclear extracts before incubation with the probe (Lane 2: 0.5 μg, Lane 3: 1 μg, Lane 4: 2 μg).

Dbs3 (Fig. 2B). To further dissect the exact sequence responsible for EcR/Usp binding, we synthesized a series of EcRE-Dbs3 oligonucleotides differing by a single nucleotide mutation from the canonical EcRE within the putative EcR/Usp binding element (Fig. 4A). Then, we tested the ability of these oligonucleotides to compete EcR/Usp-probe complex formation as detected by EMSA. As shown in Figure 4B, the addition of excess amounts of the cold EcR/Usp-probe or *Drosophila hsp27 EcRE* abolished the shifted band representing the DNA-protein complex (Lanes 2–5). Oligonucleotides containing a mutation at either position -3 or -1 within the Usp-binding half site were also effective competitors of complex formation (Lanes 10, 11, 14, and 15). By contrast, oligonucleotides containing a

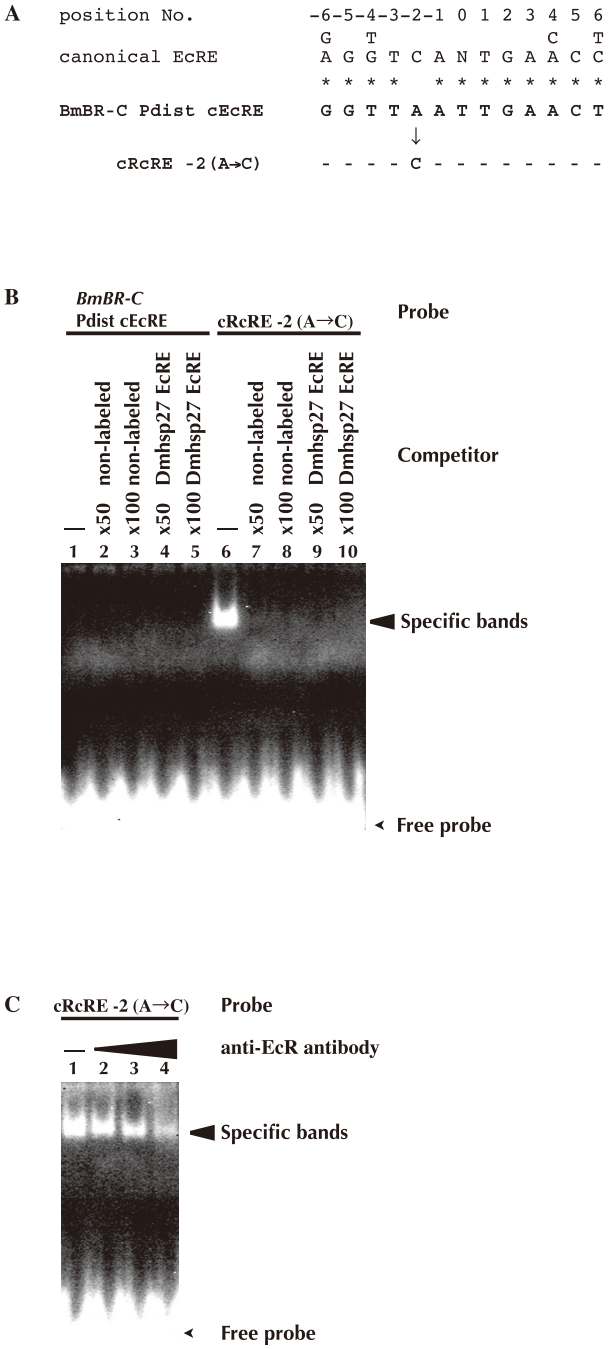

Fig. 3. Mutation at the -2 position in cEcRE acquired EcR/Usp binding ability. (A) Alignment of oligonucleotides containing the canonical EcRE, the *BmBR-C* Pdist cEcRE (Nishita and Takiya, 2009), and the 14-bp consensus sequence. The mutation within the cEcRE [cEcRE -2 (A→C)] is indicated. (B) EMSA using wild type or -2 (A→C) mutated cEcRE probes. Each probe was incubated with nuclear extracts obtained from cultures of the *Bombyx mori* cell line, BM-N. (C) Addition of an anti-EcR antibody (MAB9070, Abnova) to an EMSA reaction mixture (Lane 2: 0.5 μg, Lane 3: 1 μg, Lane 4: 2 μg).

mutation at either position -5, -4, or -2 (with the most striking effects observed at -4 or -2) did not abrogate DNA-protein complex formation (Lanes 6, 7, 8, 9, 12, and 13). In addition, an oligonucleotide bearing a mutation at +3 within the EcR-binding half site effectively abrogated probe-protein complex formation (Fig. 4C; Lanes 8, and 9). However, oligonucleotides containing mutations at positions +1, +4, or +6 (with the weakest effect observed at +4) did not affect complex formation (Lanes 6, 7, 10, 11, 12, and 13).

Together, these results suggested that the conserved residues between EcRE-Dbs3 and the canonical EcRE are essential for EcR/Usp binding. In particular, the G and C residues at -4, -2 and +4 were critically essential for EcR/

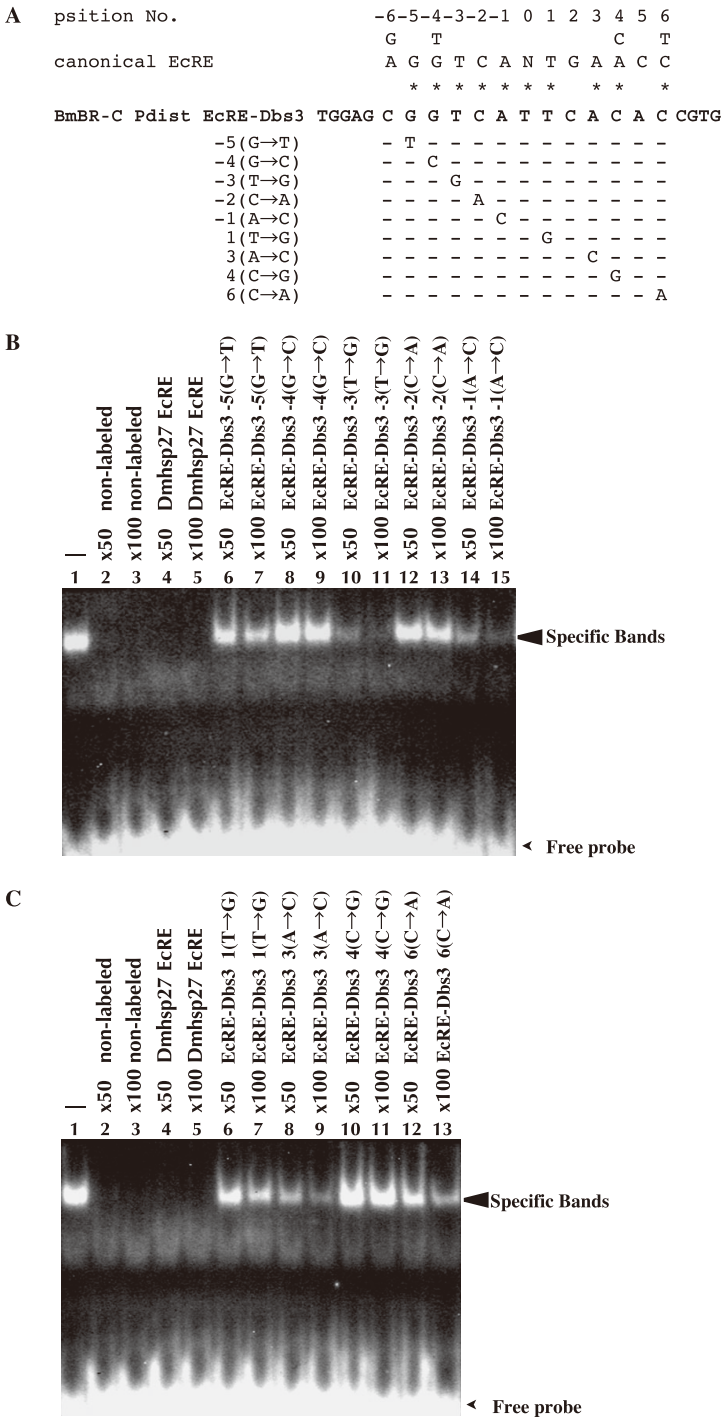

Fig. 4. The sequence specificity governing EcR/Usp binding to EcRE-D. (A) The wild type EcRE-Dbs3 sequence is shown above the mutant oligonucleotides used as competitors of binding. (B, C) EcRE-Dbs3 was used as a probe for EMSA and was incubated with BM-N cell nuclear extracts. In order to verify the sequence specificity of EcR/Usp binding to EcRE-Dbs3, oligonucleotides corresponding to the wild type and mutated EcRE-Dbs3 sequences shown in Panel A were added at 50- or 100-fold excess of the labeled oligonucleotide in a competition assay.

Usp binding to EcRE-Dbs3.

The sequence corresponding to the canonical EcRE within EcRE-D is essential for *BmBR-C* Pdist responsiveness to 20E

To confirm the effect of the mutations in the key sequence within EcRE-Dbs3 on the responsiveness of *BmBR-C* Pdist to ecdysone, single base mutations corresponding to the nucleotides shown in Figure 4A were introduced into the luciferase reporter plasmid PGV-BmBR-C Pdist -5k (Nishita and Takiya, 2009), in which luciferase expression is driven by a 5 kb region of the distal promot-

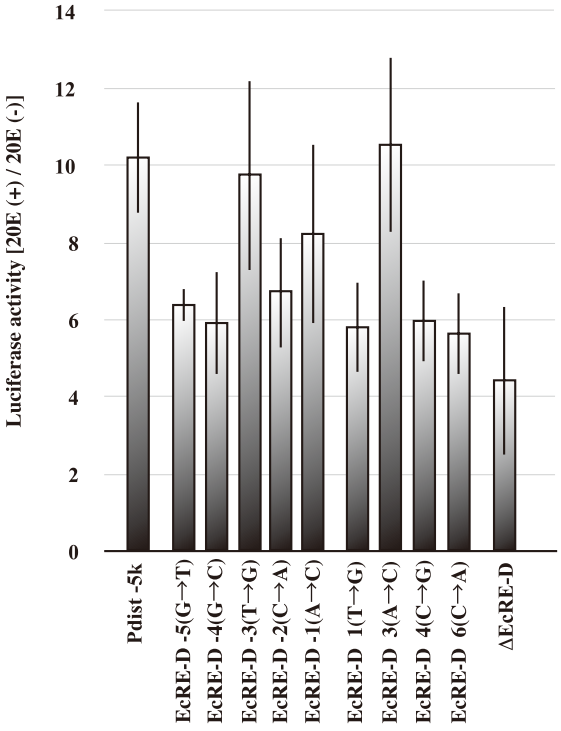

Fig. 5. The effect of single base mutations in the EcRE-D of *BmBR-C* Pdist on EcR/Usp binding to EcRE-Dbs3 is replicated using a reporter system that reflects ecdysone responsive transcription. Reporter plasmids bearing a single-base mutation within EcRE-D corresponding to the residues shown in Fig. 4A were prepared and transfected into BM-N cells. After 48 h in culture ± 1 μM 20E, luciferase activity was measured. Data represent the mean ± 95% CI (n = 6).

er of *BmBR-C*. Changes in 20E responsiveness were measured by a luciferase assay (Fig. 5). Reporter plasmids were transiently transfected into BM-N cells. After 48 h in culture ± 1 μM 20E, luciferase activity was measured. In accord with the results shown in Fig. 4B, point mutations at -5, -4, or -2 within the Usp-binding half site or at +1, +4, or +6 within the EcR-binding half site of EcRE-D reduced 20E responsiveness to almost the same level as a reporter plasmid bearing a 75 bp deletion of the EcRE-D sequence (ΔEcRE-D).

In general, a good correlation was observed between DNA binding affinity and transactivation levels. Together, the results identified a sequence, 5'- TGGAGCGGTCAT TCACACCGTG-3', found in the mid-region of EcRE-D (termed EcRE-Dbs3) on the *BmBR-C* distal promoter that bears a weak sequence similarity to the canonical EcRE (underlined). Furthermore, this identified sequence supported the binding of EcR/Usp. Conserved residues between the EcREs, at positions -5, -4, -2, +1, +4, and +6, were important for EcR/Usp binding. In particular, the G and C residues positioned at -4, -2 and +4 were critically essential for both EcR/Usp binding to EcRE-D and the subsequent ecdysone-dependent transcriptional activation of *BmBR-C* from Pdist. *BR-C* has long been known as a gene induced by ecdysone (Kiss *et al*., 1988), although the molecular mechanism of this induction, and especially the location of the EcRE, has been unclear. It has been suggested that as an EcR/Usp heterodimer binding to EcRE, Usp may rigidly recognize a specific DNA sequence [5'-TC (A/T)-3'; Devarakonda *et al*., 2003, Grad *et al*., 2001], while EcR may be more promiscuous in its binding. DNA sequence diversity in the EcR-binding half site of EcRE-D (-Dbs3) may make it difficult to identify the EcRE bound by EcR/Usp on the *BmBR-C* promoter by homology to the canonical EcRE sequence.

REFERENCES

Andres, A.J. and Cherbas, P. (1992) Tissue-specific ecdysone responses: regulation of the *Drosophila* genes *Eip28/29* and *Eip40* during larval development. *Development* 116, 865-876.

Antoniewski, C., Laval, M. and Lepesant J.A. (1993) Structural features critical to the activity of an ecdysone receptor binding site. *Insect Biochem. Molec. Biol.* **23**, 105-114.

Antoniewski, C., O'Grady, M.S., Edmondson, R.G., Lassicur, S.M. and Benes, H. (1995) Characterization of an EcR/USP heterodimer target site that mediated ecdysone responsiveness of the *Drosophila Lsp-2* gene. *Mol. Gen. Genet.* **249**, 545-556.

Cherbas, L., Lee, K. and Cherbas, P. (1991) Identification of ecdysone response elements by analysis of the *Drosophila Eip28/29* gene. *Genes Dev* **5**, 120-131.

Devarakonda, S., Harp, J.M., Kim, Y., Ozyhar, A. and Rastinejad, F (2003) Structure of the heterodimeric ecdysone receptor DNA-binding complex. *EMBO J.* **22**, 5827-5840.

Dignam, J.D., Leboviz, R.M. and Roeder, R.G. (1983) Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. *Nucl. Acids. Res.* **11**, 1475-1489.

Grad, I., Niedziela-Majka, A., Kochman, M. and Oayhar, A. (2001) Analysis of Usp DNA binding domain targeting reveals critical determinants of the ecdysoen receptor complex interaction with the response element. *Eur. J. Biochem.* **268**, 3751-3758.

Koelle, M.R., Talbot, W.S., Segraves, W.A., Bender, M.T., Cherbas, P. and Hogness, D.S. (1991) The *Drosophila* EcR gene encodes an ecdysone receptor, a new member of the steroid receptor superfamily. *Cell* **67**, 59-77.

Kiss, I., Beason, A.H., Tardiff, J., Fristrom, D. and Fristrom, J. W. (1988) Interactions and developmental effects of mutations in the *Broad-Complex* of *Drosophila melanogaster*. *Genetics* **118**, 247-259.

Konopova, B. and Jindra, M. (2008) Broad-Complex acts downstream of Met in juvenile hormone signaling to coordinate primitive holometabolan metamorphosis. *Development* **135**, 559-568.

Mangé, A., Julien, E. ,Prudhomme, J.C. and Couble, P. (1997) A strong inhibitory element down-regulates SRE-stimulated transcription of the A3 cytoplasmic actin gene of *Bombyx mori*. *J Mol Biol* **265**, 266-274.

Minakuchi, C., Tanaka, M., Miura, K. and Tanaka, T. (2011) Developmental profile and hormonal regulation of the tran-

scription factors broad and *Krüppel homolog 1* in hemimetabolous thrips. *Insect Biochem. Mol. Biol.* **41**, 125-134.

Nishita, Y. (2014) Ecdysone response elements in the distal promoter of the *Bombyx Broad-Complex* gene, *BmBR-C*. *Insect Mol. Biol. in press*.

Nishita, Y. and Takiya, S. (2006) Differential usage of two promoters of the *Broad-Complex* gene in the silkworm, *Bombyx mori*. *Insect Biochem. Mol. Biol.* **36**, 779-788.

Nishita, Y. and Takiya, S. (2009) Differential responsiveness of *BmBR-C* promoters to ecdysone signals. *Insect Biotech. Sericology* **78**, 127-138.

Riddihough, G. and Pelham, R.B. (1987) An ecdysone response element in the *Drosophila hsp27* promoter. *EMBO J.* **6**, 3729-3734.

Shirai, H., Kamimura, M., Yamaguchi, J., Imanishi, S., Kojima, T. and Fujiwara, H. (2012) Two adjacent cis-regulatory elements are required for ecdysone response of *ecdysone receptor (EcR) B1* transcription. *PLoS One* **7**, e49348.

Swevers, L., Cherbas, L., Cherbas, P. and Iatrou, K. (1996) *Bombyx* EcR (BmEcR) and *Bombyx* USP (BmCF1) combine to form a functional ecdysone receptor. *Insect Biochem. Mol. Biol.* **26**, 217-221.

Yao, T-P., Segraves, W.A., Oro, A.E., McKeown, M. and Evans, R.M. (1992) *Drosophila* ultraspiracle modulates ecdysone receptor function via heterodimer formation. *Cell* **71**, 63-72.

Zhou, X. and Riddiford, L.M. (2002) Broad specified pupal development and mediates the 'status quo' action of juvenile hormone on the pupal-adult transformation in *Drosophila* and *Manduca*. *Development* **129**, 2259-2269.