Biochimica et Biophysica Acta 1840 (2014) 2674–2684

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbagen

The Broad Complex isoform 2 (BrC-Z2) transcriptional factor plays a critical role in vitellogenin transcription in the silkworm *Bombyx mori*

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ARTICLE INFO

Article history:
Received 25 February 2014
Received in revised form 22 April 2014
Accepted 16 May 2014
Available online 23 May 2014

Keywords:
Ecdysone
Broad Complex
Vitellogenin
Bombyx mori

ABSTRACT

Background: Vitellogenin (Vg) is synthesized in the fat body of the female silkworm *Bombyx mori* and transported to the oocyte as a source of nutrition for embryo development. It is well known that ecdysone regulates physiological, developmental and behavioral events in silkworm. However, it is still not clear how the ecdysone regulates *B. mori Vg* (*BmVg*) transcription.

Methods: Electrophoretic mobility shift assay (EMSA) and cell transfection assay were used to reveal whether BmBrC-Z2 is involved in regulating *BmVg* transcription. RNAi was employed to illustrate the function of BmBrC-Z2 in the silkworm egg formation and development.

Results: (1) The transcription of *BmVg* can be induced by ecdysone in the female fat body. (2) Three putative BrC-Z2 *cis*-response elements were mapped to regions flanking the *BmVg* gene. (3) BmBrC-Z2 required direct binding to the *cis*-response elements on the *BmVg* promoter. (4) Over-expression of three BmBrC isoforms in the cell line showed that only BmBrC-Z2 could induce the *BmVg* promoter activity. (5) RNA interference (RNAi) of *BmBrC-Z2* in female remarkably reduced BmVg synthesis and led to destructive affection on egg formation. The dsRNA of BmBrC-Z2 treated moths laid fewer and whiter eggs compared to the control.

Conclusions: BmBrC-Z2 transported the ecdysone signal then regulated *BmVg* transcription directly to control vitellogenesis and egg formation in the silkworm.

General significance: The results of this study revealed that BmBrC-Z2 as a key factor to mediate ecdysone regulates reproduction in the silkworm.

1. Introduction

The development of insect eggs is dependent on the availability of vitellogenin (Vg), the precursor of vitellin (Vn), which is synthesized and secreted by the fat body [1,2]; and the mechanism underlying *Vg* transcription regulation is concerned. In the mosquito *Aedes aegypti*, a member of the Diptera with feeding in the adult, Vg is expressed specifically in the adult female and only when it has fed on the blood. The stringent control of Vg gene expression in mosquitoes by the blood meal provides an outstanding model for elucidating hormonal and tissue-specific regulation in the context of complex physiological events surrounding reproduction [3–5]. There are three yolk protein (Yp) genes, *yp1*, *yp2* and *yp3*, in the fruit fly *Drosophila melanogaster*. *Yp1* and *Yp3* are expressed in the female fat body and Yp2 is expressed in ovarian follicle cells [6,7]. The specific transcription is regulated by a tissue-specific enhancer, 20HE and juvenile hormone (JH) [8]. *Bombyx mori*, a holometabolous insect without feeding in pupa or adult, has been used as a model of lepidopteran insects. By contrast, Vg is expressed at the larva to pupa metamorphosis stage of the female silkworm [9]. The gene structure and expression feature were identified in 1994 [10]. Very little is known, however, about the transcriptional regulation mechanism of *BmVg*.

In insects, ecdysone, juvenile hormones and a class of neuropeptide hormones together regulate physiological, developmental and behavioral events [11]. Ecdysone activates a heterodimeric nuclear hormone receptor complex comprised of ecdysone receptor (EcR) and Ultraspiracle (USP), a homolog of the retinoid X receptor [12]. This heterodimer regulates a cascade of primary (early genes) and secondary response genes (late genes) [13].

One of the early genes, Broad-Complex (BrC), is a key regulator of the ecdysone cascade. BrC encodes a family of C2H2-type zinc finger, DNA-binding proteins. In *Drosophila*, four BrC isoforms (Z1–Z4) have the common N-terminal Bric-a-brac-Tramtrack-Broad (BTB) domain, an evolutionarily conserved protein–protein interaction module [14]. The BTB domain and the zinc-finger DNA-binding domain are very conservative in *B. mori*, *D. melanogaster* and *Manduca sexta* [15]. Four alternative splicing isoforms (Z1, Z2, Z3 and Z4) of BrC can be distinguished by C2H2 zinc finger domains in *D. melanogaster* [16]. *B. mori* BrC (BmBrC) isoforms Z1, Z2 and Z4 are conserved in *Drosophila* [15,17].

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http://dx.doi.org/10.1016/j.bbagen.2014.05.013
0304-4165/

Several other BmBrC isoforms without a zinc finger domain are reported [18]. Earlier studies found numerous downstream effects of genes that are regulated by BrC [19–23]. In *D. melanogaster*, BrC mutants prevent metamorphosis, including the morphogenesis of imaginal disks [24,25]. RNA interference (RNAi) with BrC mRNA has been done in *Oncopeltus fasciatus* [26], *Tribolium castaneum* [27,28], *Chrysopa perla* [28] and *B. mori* [29]. The results suggested that BrC regulated gradual morphogenesis through the successive molts and impaired pupal differentiation. The BrC complex participates in the regulation of reproduction in the cockroach *Blattella germanica* [30] and the mosquito *A. aegypti* [31]. Little is known, however, about whether or how BrC participates in the regulation of silkworm vitellogenesis and reproduction. This study showed the BmBrC-Z2 isoform is a key factor with unique roles in the starting temporal pattern of the *BmVg* transcription after the wandering stage. To explore the functions of BrC-Z2 in silkworm in vivo, we used isoform-specific RNAi to knock down BrC expression. Our results laid the foundation for study of the role of the silkworm BmBrC factor in reproduction as well as other insects.

2. Materials and methods

2.1. Animal strains

Dazao, the wild type strain of the silkworm, was obtained from the Silkworm Gene Bank in the Southwest University of China. Larvae were bred on fresh mulberry leaves (*Morus* sp.) and began wandering on day 8 after entering the fifth instar stage and pupated on day 3 of the wandering stage in a natural environment. The wandering stage was started from stopping feeding, and the pupa stage was started when spinning was finished. Fat body samples from day 5 of the fifth instar larval stage to day 3 of pupation were collected for isolation of total RNA and transcription analysis of *BmBrC isoforms*.

2.2. Bioinformatics analysis

The potential *cis*-response elements (CREs) in the *BmVg* gene promoter were predicted by the MatInspector program (http://www.genomatix.de/). The upstream 1.5 kb regulatory region of the *BmVg* gene was obtained from SILKDB (http://www.silkdb.org/silkdb/) [32]. The sequences of BmBrC-Z1, Z2 and Z4 were obtained from the National Center for Biology Information (NCBI) with accession numbers NM_001111334.1, NM_001111333.1 and NM_001043511.1, respectively.

2.3. Fat body cultured in vitro and hormone treatment

The method of fat body culture in vitro was a well-established methodology [33]. According to the *BmVg* transcriptional profile, the fat body from a female silkworm at day 1 of the wandering stage was treated by 20-hydroxyecdysone (20E), which is the active form of ecdysone (Sigma, USA). The fat body from a female silkworm at day 3 of the wandering stage was treated with juvenile hormone III (JHIII; Sigma, USA). The fat body was dissected, cleaned, sterilized in 0.85% (m/v) physiological saline solution divided into several sections and incubated in Grace insect medium culture (Gibco®, Invitrogen™) containing penethamate and streptomycin. As required, the cultural medium was supplemented with various concentrations of ecdysone and JH III in 0.1% (v/v) ethanol. As a control, the fat body was incubated with the same volume of 0.1% ethanol. The tissues were treated with different doses and for different lengths of time. After the treatment, samples were cooled immediately in liquid nitrogen and stored at −80 °C.

2.4. RT-PCR and quantitative RT-PCR

Total RNAs were extracted using TRIzol® (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Corresponding cDNA was synthesized by reverse transcription using M-MLV reverse transcriptase (Promega, Madison, WI).

For quantitative RT-PCR (qPCR), a SYBR Green Kit was used according to the manufacturer's instructions (TaKaRa Biotech, Japan). The relative levels of the mRNA of target genes were calculated with the $2^{-\Delta\Delta CT}$ method [34], and *BmTIF4A* gene C_t values were calculated according to the manufacturer's instructions. Three independent replicates were used for each data set. The primers for RT-PCR and qPCR are given in Table S1.

2.5. Vector construction

The upstream regulatory region (from −1432 to +42 bp) of the *BmVg* gene was amplified from the silkworm genome, the primer with XhoI and HindIII restriction enzyme sites, and cloned into pMD-19T simple vectors (TaKaRa Co.) for sequencing. The fragments were digested by *XhoI* and *HindIII* and transferred to the luciferase reporter plasmid pGL_3-basic vector (Promega, USA). The reporter vector was named pGL_3-VgP1.5K Luc.

Some mutation vectors with the BmBrC-Z2 CRE broken were constructed by site-specific mutagenesis, on the basis of pGL3-BmVg1.5K-Luc. The core site of BrC-Z2 cis-response elements “AA/TTA” was changed into “GGGG”. The whole vector was amplified by the special primer, which contains four nucleic acids mutated in the target CRE site, and the production was digested by the restriction enzyme *DpnI* to digest the template vector and the mutation vector was transformed into *Escherichia coli* competent cells. The three BrC-Z2 *cis*-response elements were broken as different forms in the mutation vectors.

The open reading frame (ORF) of *BmBrC-Z1*, *Z2* and *Z4* was amplified from the cDNA of the day 1 pupa fat body and cloned into pal 1180-Hrs-A4 SV40 expression vector (stored in our laboratory) for over-expression assays. The primers for *BmVg* promoter, mutation vector and amplification of different *BmBrC* isoforms are given in Table S1.

2.6. Cell culture and transfection assay

B. mori embryonic cell line BmE-SWU1, which was originally developed from embryo tissues (stored in our laboratory), was maintained at 27 °C in Grace medium (GIBCO, Invitrogen) supplemented with 10% (v/v) fetal bovine serum (FBS; Thermo Scientific™ HyClone™). Cell transfection and co-transfection assay used Lipofectamine 2000 (Invitrogen), as described [35]. The pRL-SV40 vector (Promega, USA) was transferred as an inner control [36]. The vector ratio (w/w) of pGL3-VgP1.5K Luc and pRL-SV40 was 1:0.1. The ratio (w/w) of reporter vector with *BmVg* promoter and psl 1180-A4-BmBRC-Z SV40 was 1:1.

The transfection assay was done with the Dual-Luciferase Reporter Assay System (Promega, USA) in the GloMax-Multi Detection System Photometer (Promega, USA). Assays were done three times ($n = 3$) independently and the average expression levels of the target genes were presented as mean ± SE. The measurement of luciferase activity was as described [37]. The promoter activity was represented as *x*-fold greater compared to the control.

2.7. RNA interference (RNAi) assay

Synthesis of double-stranded RNAs (dsRNAs) and microinjection were done as described [9]. An optimum target position of *BmBRC-Z2* (amino acid positions 307–426), was selected to synthesize dsRNA, which was located in the BmBrC-Z2 C terminus and contained the specific ZnFn-C2H2 domain. The 358 bp nucleic acid fragment with the bacteriophage T7 promoter sequence was obtained by PCR and cloned into the pMD19-T simple vector. The dsRNAs were generated by the T7 RiboMAXTM Express RNAi System (Promega, Madison, WI). EGFP

gene fragment dsRNA was synthesized as a negative control. The primers are given in Table S1.

Considering the *BmBrC-Z2* and *BmVg* transcription profile, the female silkworm at day 2 of the wandering stage, when spinning had just finished, was prepared for injection of 15 μg of dsRNA and 5 μg into day 1 and day 4 pupae using a micro-injector (laboratory-made from capillary glass tubing). The fat bodies harvested from day 2 pupae were used for the *BmBrC-Z* and *BmVg* gene transcript and protein synthesis by immunofluorescence histochemistry. After eclosion, moths injected with dsRNA were mated with untreated males and subsequently laid eggs. Observation of egg formation and statistical analysis were done.

2.8. Electrophoretic Mobility-Shift Assay (EMSA)

EMSA was used to examine whether BmBrC-Z2 could bind to the putative BRC-Z2 CRE of the *BmVg* promoter as described [38]. The 5′ terminus of the probe was labeled with biotin (synthesized by Sangon Biotech, Shanghai). The recombination BmBrC-Z2 protein with a SUMO tag was expressed in *E. coli*, purified and stored in our laboratory. The EMSA experiment was done with a LightShift Chemiluminescent EMSA Kit (Thermo pierce). The DNA–protein binding reactions were done according to the manufacturer's protocol. After incubation for 20 min at 27 °C, reaction mixtures were loaded onto 9% (w/v) native polyacrylamide gel and submitted to electrophoresis in TBE buffer (45 mM Tris–borate, 1 mM EDTA, pH 8.3). The proteins were transferred electrophoretically to a nylon membrane (Roche, Indianapolis, IN). The bound horseradish peroxidase (HRP)-conjugated antibodies were displayed by the ECL enhanced chemiluminescence system (Thermo, USA) and photographed using a Clinx ChemiScope 3400 Mini (Science Instrument, China).

2.9. Immunofluorescence histochemistry

The female silkworm fat body from larvae just entering the wandering stage, day 3 of the wandering stage, day 1 of pupation and from day 2 after dsRNA-treated pupae were fixed overnight at 4 °C in 4% (v/v) formaldehyde. Part of a section was treated with the primary antibody anti-BmBrC-Z2 and secondary antibody, Alexa Fluor® 555 Donkey Anti-Rabbit IgG (H + L) (Invitrogen, Carlsbad, CA) for 1 h, respectively, to determine the expression pattern of BmBrC-Z2 in the fat body; another part was treated with the primary antibody, anti-BmVg and then with the secondary antibody, Alexa Fluor® 488 Goat Anti-Rabbit IgG (H + L) (Invitrogen, Carlsbad, CA) for 1 h, to determine the expression pattern of BmVg in the fat body. The primary and secondary antibodies were diluted 1:200 in PBST (PBS with 0.2% (v/v) Tween-20) and washed with PBST three times after each operation. DAPI was used to dye the cell nucleus: the tissue was fixed onto the glass slide with Antifade Mounting Medium (Beyotime, China) and the sections were examined under a fluorescence microscope (DMI4000B; Leica).

2.10. Statistical analysis

Independent samples *t*-test or one way analysis of variance (ANOVA) followed by a Duncan new's multiple range test were applied for gene expression analysis and promoter activity assays using SPSS 13.0. Results were considered significance if $P < 005$.

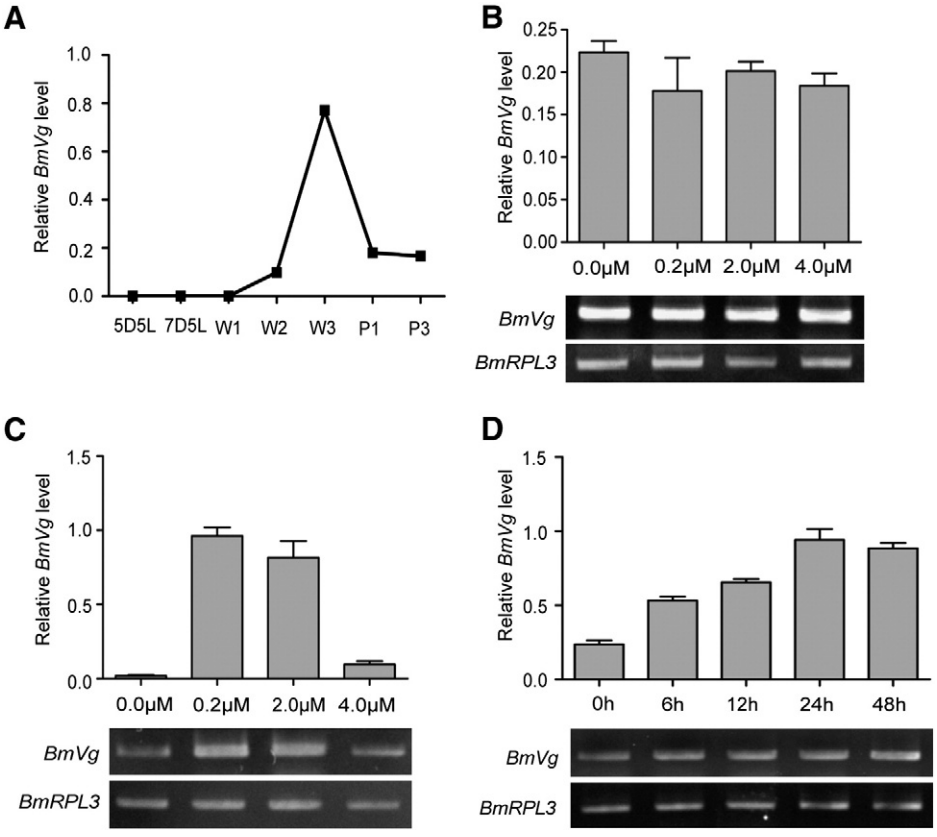


Fig. 1. *BmVg* transcript response to hormones in fat body cultured in vitro. (A) *BmVg* expression tendency in the fat body from larva to pupa in females (5D5L, 7D5L: days 5 and 7 of the 5th instar larva; W1, W2 and W3: days 1, 2 and 3 of wandering; P1 and P3: days 1 and 3 of pupation). (B) *BmVg* transcription without obvious change in treatment with different concentrations of JHIII ($P = 0.138$). (C, D) *BmVg* transcription induced by ecdysone is related to concentration and time ($P < 0.001$ and $P < 0.001$ respectively). (ANOVA and Duncan's new multiple range tests).

Table 1
The predicted response elements of BmVg promoter.

Gene	Detailed information	DNA site	Link	Position		Strand	Ration	Sequence
				From	To			
Drosophila chorion factor 1	Homologous to retinoid × receptor	DCF1	I$CF1.01	−16	−25	(+)	0.87	gGGGTgacc
Hairy	*Drosophila* transcriptional repressor	Hairy	I$HAIRY.01	−54	−39	(−)	0.91	ggtaCACGcgtccca
Drosophila chorion factor 1	Homologous to retinoid × receptor	DCF1	I$CF1.01	−67	−58	(+)	0.87	aGGGTcaca
Drosophila doublesex (DSX)	Involved in sex determination	DSX	I$DSX.01	−99	−86	(−)	0.99	aatACAAtgtaac
Giant	*Drosophila* giant transcription factor	DGT	I$GT.01	−105	−90	(+)	0.94	tgattcGTTAcattg
Onecut transcription factor	*Drosophila* homeobox transcription factor with CUT domain	ONECUT	I$ONECUT.01	−172	295	(+)	0.99	cttGATTta
Heat shock factor	*Drosophila* heat shock factor	HSF	I$HSF.01	−194	−169	(−)	0.85	aagcgCGAAtattcgcttttaataa
Abdominal B	*Drosophila* Abd-B group	ABDB	I$ABDB.01	−197	−186	(−)	0.92	tttaATAAaat
Antennapedia	*Drosophila* homeoproteins	ANTP	I$ANTP.01	−199	−184	(−)	0.96	ctttTAATaaaataa
POU	Ventral veins lacking, transcription factors with POU-domain-N-terminal to homeobox domain	VVL	I$VVL.01	−199	−186	(+)	0.96	ttattttATTAaa
Antennapedia	*Drosophila* homeoproteins	ANTP	I$ANTP.01	−203	−188	(+)	0.95	atttTTATtttatta
Hunchback	*Drosophila* gap gene hunchback, early maternal and zygotic zinc finger gene, activated by bicoid	DHB	I$HB.02	−205	266	(−)	0.98	aaaatAAAAatta
Paired	Paired homeodomain factor, *Drosophila* homeoproteins	DPRD	I$PRD.01	−209	−188	(−)	0.95	taataaaataaaaATTAgaac
Broad-Complex Z2	*Drosophila* Broad-Complex for ecdysone steroid response	DBRCZ2	I$BRCZ2.01	−212	−193	(−)	0.94	aaataaaaATTAgaacgcg
Heat shock factor	*Drosophila* heat shock factor	DHSF	I$HSF.01	−230	253	(−)	0.79	gaacgCGAAagatcgcgtaccatcc
Achintya	TGIF (TG-interacting factor)-Exd (extradenticle) group	ACHI	I$ACHI.01	181	187	(+)	1	TGTCata
Drosophila T-cell factor	Homolog of TCF/LEF, interacts with Armadillo (b-Catenin homolog)	DTCF	I$DTCF.01	−295	−282	(+)	0.87	tcgtTTGAagaag
Zeste	Zeste transvection gene product	ZESTE	I$ZESTE.01	−383	−372	(+)	0.94	tggtGAGTggt
Snail	*Drosophila* snail zinc finger protein, involved in dorso-ventral patterning	DSN	I$SN.02	−392	−381	(+)	0.97	cccACCTgatg
Hunchback	Early maternal and zygotic zinc finger gene, activated by bicoid	DHB	I$HB.01	−429	−416	(−)	1	gcaatAAAAaaaa
Grainyhead transcription factor	*Drosophila* neuronal cis element binding factor	DGRH	I$GRH.01	−559	−543	(+)	0.88	cactccGGTTtgaacgc
Heat shock factor	*Drosophila* heat shock factors	DHSF	I$HSF.01	−650	−626	(−)	0.85	cacctAGAAgatccggcaagaaact
Fushi tarazu	involved in body segmentation of the *Drosophila* embryo	DFTZ	I$FTZ.02	−682	−668	(−)	0.91	gggtTAATtcgctcg
Iroquois-complex caupolican	Iroquois group of transcription factors	CAUP	I$CAUP.01	−760	−752	(+)	0.98	ccattAACA
Ovo/shavenbaby	*Drosophila* OVO transcription factor	DOVO	I$OVO.01	−764	−748	(−)	0.88	gcactGTTAatggatcg
Brain-specific homeoprotein	*Drosophila* Brain-specific homeoprotein	DBSH	I$BSH.01	−765	−751	(−)	0.99	ctgtTAATggatcgg
Paired	*Drosophila* homeoproteins	DPRD	I$PRD_HD.01	−768	−748	(−)	0.75	gcactgttaatgGATCgggag
Buttonless	*Drosophila* homeoproteins	DBTN	I$BTN.01	−785	−771	(−)	0.99	accgTAATgacgtgt
DREF	DNA replication-related element factor	DREF	I$DREF.01	−846	−836	(+)	0.85	tcaCGATacat
BEAF	Boundary element-associated factor of 32 kD	BEAF	I$BEAF32.01	−849	−837	(+)	0.93	acatcaCGATaca
DREF	DNA replication-related element factor	DREF	I$DREF.01	−861	−851	(+)	0.9	aagCGATacac
BEAF	Boundary element-associated factor of 32 kD	BEAF	I$BEAF32.01	−864	−852	(+)	0.89	cgtaagCGATaca
DREF	DNA replication-related element factor	DREF	I$DREF.01	−884	−874	(+)	0.83	tccCGATacag

(continued on next page)

Table 1 (*continued*)

Gene	Detailed information	DNA site	Link	Position		Strand	Ration	Sequence
				From	To			
BEAF	Boundary element-associated factor of 32 kD	BEAF	I$BEAF32.01	−887	−875	(+)	0.93	cattccCGATaca
Krueppel	Zinc finger protein, involved in body segmentation of the *Drosophila* embryo	DKR	I$KR.01	−899	−889	(+)	0.9	attGGCTtacg
Tramtrack 69K zinc finger protein	Binding sites in eve gene, *Drosophila* Tramtrack protein	DTTK	I$TTK.01	−912	−900	(−)	0.97	acggTCCTgccta
Brachyenteron	*Drosophila* T-box transcription factors	DBYN	I$BYN.01	−926	−912	(−)	0.87	aattttgCCACgtga
Slow border cells	*Drosophila* C/EBP like bZIP transcription factors	DSLB	I$SLBO.01	−973	−960	(+)	0.93	tATTGgtgaaacc
Caudal	*Drosophila* homeodomain protein, vertebrate homolog cdx	DCAD	I$CAD.01	−995	−984	(+)	0.99	atttTTTAtaa
Tailless	*Drosophila* gap gene tailless, involved in embryonic segmentation	DTLL	I$TLL.01	−998	−989	(−)	0.95	aaaaaTCAA
Onecut transcription factor	*Drosophila* homeobox transcription factor with CUT domain	DONECUT	I$ONECUT.01	−999	−990	(+)	0.98	attGATTtt
Onecut transcription factor	*Drosophila* homeobox transcription factor with CUT domain	DONECUT	I$ONECUT.01	−1006	−997	(−)	0.98	attGATTac
Ventral veins lacking	Transcription factors with POU-domain — N-terminal to homeobox domain	DVVL	I$VVL.01	−1007	−994	(−)	0.95	tcaattgATTAct
Paired	*Drosophila* homeodomain	DPRD	I$PRD_HD.01	−1008	−987	(+)	0.73	tagtaatcaattGATTtttta
Paired	*Drosophila* homeodomain	DPRD	I$PRD_HD.01	−1009	−988	(−)	0.8	aaaaaatcaattGATTactaa
Deformed	Homeotic gene in *Drosophila* development, *Drosophila* homeoproteins	DFD	I$DFD.01	−1009	−994	(+)	1	ttagTAATcaattga
Hunchback	*Drosophila* gap gene hunchback, early maternal and zygotic zinc finger gene, activated by bicoid	DHB	I$HB.02	−1020	−1007	(−)	0.99	aaaacAAAAatgt
Drosophila Elf-1 (NTF-1)	Vertebrate homolog CP2 (human, mouse), *Drosophila* neuronal cis element binding factor	DELF	I$ELF1.01	−1078	−1061	(+)	0.93	tagaaagGTTTtgtgaa
Broad-Complex Z2	*Drosophila* Broad-Complex for ecdysone steroid response	DBRCZ2	I$BRCZ2.01	−1088	−1069	(+)	0.93	gatcacgaATTAgaaaggt
Abdominal B	*Drosophila* Abd-B group	ABDB	I$ABDB.01	−1099	−1088	(+)	0.92	gatgATAAata
Broad-Complex Z2	*Drosophila* broad-complex for ecdysone steroid response	DBRCZ2	I$BRCZ2.01	−1100	−1081	(+)	0.91	agatgataAATAgatcacg
DREF	DNA replication-related element factor	DREF	I$DREF.01	−1122	−1111	(−)	0.8	aatCGACaatc
Onecut transcription factor	*Drosophila* homeobox transcription factor with CUT domain	DONECUT	I$ONECUT.01	−1125	−1116	(+)	0.97	attGATTgt
Paired	*Drosophila* paired homeodomain	DPRD	I$PRD_HD.01	−1127	−1106	(+)	0.71	ttattgattgtcGATTctaa
DREF	DNA replication-related element factor	DREF	I$DREF.01	−1129	−1118	(−)	0.83	aatCAATaata
Mirror	Iroquois group of transcription factors	MIRR	I$MIRR.01	−1132	−1123	(−)	1	ataatAACA
Paired	*Drosophila* paired homeodomain factor	DPRD	I$PRD_HD.01	−1134	−1113	(+)	0.78	aatgttattattGATTgtcga
Extra-extra	*Drosophila* homeoproteins	DEXEX	I$EXEX.01	−1134	−1119	(+)	0.88	aatgttATTAttgat
Antennapedia	*Drosophila* homeoproteins	DANTP	I$ANTP.01	−1135	−1120	(−)	0.96	tcaaTAATaacattt
Paired	*Drosophila* PAX6 P3 homeodomain binding site, Paired homeodomain factors	DPAX6	I$PAX6_HD.01	−1139	−1118	(−)	0.8	aatcaataataacATTTtcgc
Dorsal	Protein for dorso-ventral axis formation, homologous to vertebrate c-rel	DL	I$DL.02	−1140	−1129	(−)	0.94	acatTTTCgct
E74	E74A early ecdysone-inducible gene in onset of *Drosophila* metamorphosis	DE74A	I$E74A.01	−1156	−1141	(+)	0.89	ctaaaacGGAAaatg
Giant	*Drosophila* giant transcription factor	DGT	I$GT.01	−1158	−1143	(−)	0.88	ttttccGTTTtagtc
Hairless	*Drosophila* suppressor of Hairless, linked to notch pathway	DSUH	I$SUH.01	−1193	−1180	(−)	0.84	cttGTGGaaaaca
Achintya	TGIF (TG-interacting factor)-Exd (extradenticle) group	ACHI	I$ACHI.01	−1272	−1265	(−)	1	TGTCaga
Brain-specific homeobox	*Drosophila* homeoproteins	DBSH	I$BSH.01	−1316	−1301	(+)	0.99	ttatTAATggatttc
Paired	*Drosophila* paired homeodomain factor	DPRD	I$PRD_HD.01	−1319	−1298	(+)	0.81	cggttattaatgGATTtcaga
Extra-extra	*Drosophila* homeoproteins	DEXEX	I$EXEX.01	−1320	−1305	(+)	0.87	ccggttATTAatgga
Abdominal A	*Drosophila* homeoproteins	DABDA	I$ABDA.01	−1321	−1306	(−)	0.94	ccatTAATaaccggt
Ovo/shavenbaby	*Drosophila* OVO transcription factor	OVO	I$OVO.01	−1322	−1305	(+)	0.89	caccgGTTAttaatgga
Grainyhead transcription factor	*Drosophila* neuronal *cis*-element binding factor	DGRH	I$GRH.01	−1329	−1312	(−)	0.87	ataaccGGTGgaatgtc
Vismay	TGIF (TG-interacting factor)-Exd (extradenticle) group	VIS	I$VIS.01	−1332	−1325	(−)	1	TGTCaac
Vismay	TGIF (TG-interacting factor)-Exd (extradenticle) group	VIS	I$VIS.01	−1344	−1337	(−)	1	TGTCaat
Deformed	*Drosophila* homeoproteins, homeotic gene in *Drosophila* development	DFD	I$DFD.01	−1402	−1387	(+)	0.99	gcagTAATgcgtttc

3. Results

3.1. Ecdysone induced BmVg transcription

To investigate the effect of ecdysone and juvenile hormones on *BmVg* transcription, we first detected the transcription profile of *BmVg* by qRT-PCR during female silkworm metamorphosis. *BmVg* was expressed in the female silkworm from day 2 of the wandering stage with the highest peak on day 3 of the wandering stage and a high level of expression was sustained throughout the pupal stage (Fig. 1A).

The female fat body harvested at day 1 of the wandering stage was treated with ecdysone, and the female fat body of day 3 of the wandering stage was treated with JH. RT-PCR and qRT-PCR showed that JH could not affect the transcription of *BmVg* markedly at any concentration tested (Fig. 1B), but ecdysone could induce *BmVg* transcription dramatically. The induction effect depends on the hormone dose and time. Treatment with 0.2 μM ecdysone for 24 h is the best way to induce *BmVg* transcription (Fig. 1C and D).

3.2. Cis-Response elements (CREs) in the BmVg gene promoter

To analyze the role of ecdysone-regulated *BmVg* transcription, the upstream 1474 nucleotide sequence of *BmVg* was predicted by the MatInspector software (http://www.genomatix.de/). In total, 73 *cis*-response elements (CREs) were detected on the *BmVg* promoter (Table 1). The *Drosophila* sex determination transcription factor doublesex motif has the ability to regulate *BmVg* transcription specifically in the female silkworm [39]; DBRC-Z2 and DE74 are the elements

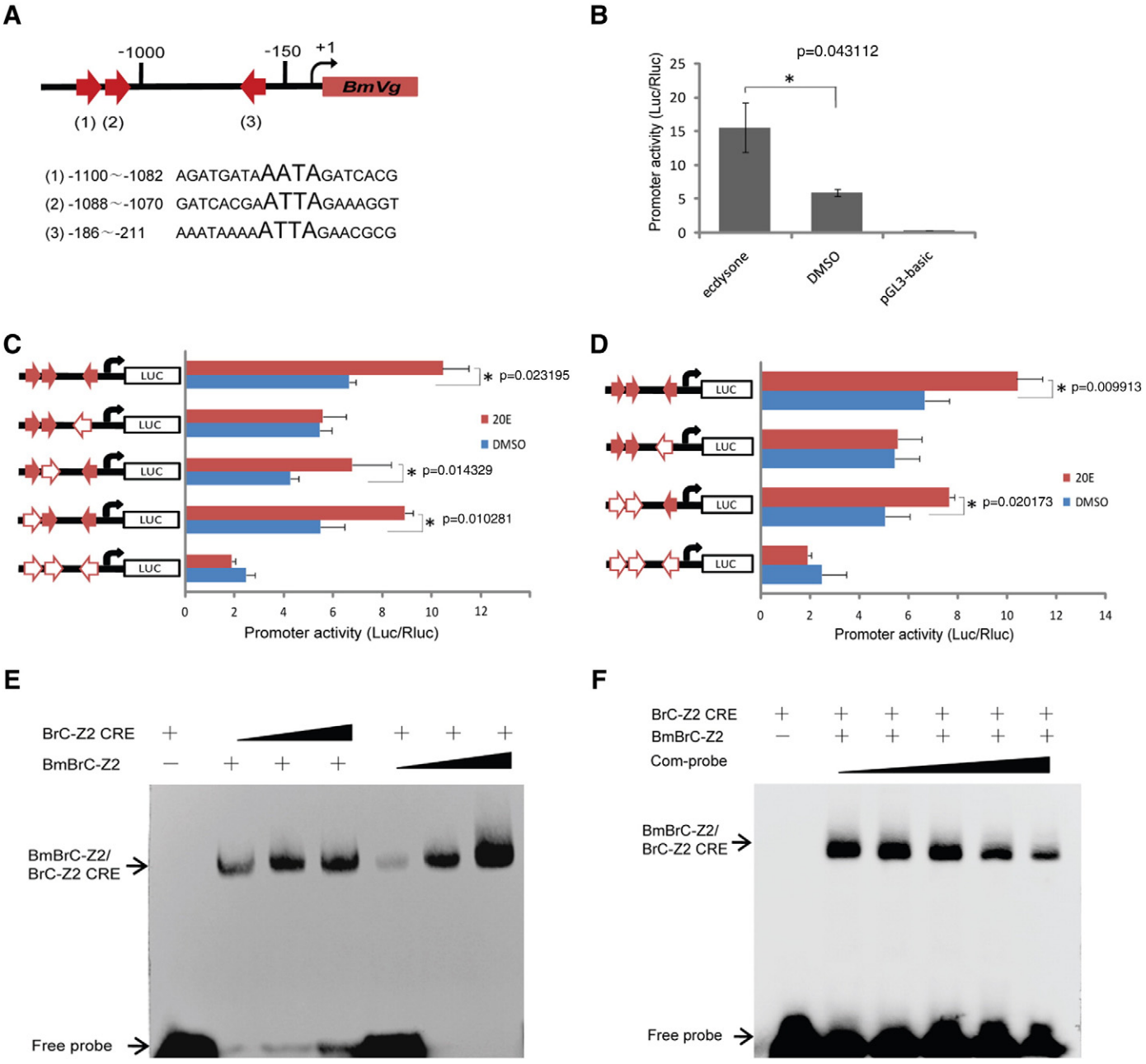


Fig. 2. Ecdysone-regulated *BmVg* transcription by BmBrC-Z2. (A) The response element of BrC-Z2 of the *BmVg* promoter predicted by MATINSPECTOR software (http//www.genomatrix.de). Upper: schematic structure of predicting three BrC-Z2 CREs in the regulatory region of *BmVg*; lower: the putative BrC-Z2 conserved sequences. (B) Ecdysone induced the *BmVg* promoter to drive luciferase expression in the BmE-SWU1 cell line. (C, D) The effect of ecdysone toward *BmVg* promoter activity with different formations of mutated BrC-Z2 CRE. (E) Binding assays of the BrC-Z2 CRE to the recombination BmBrC-Z2 protein. Probe concentrations, 0.1, 0.5 and 1 pM; protein quantities, 0.2, 0.4 and 0.8 μg. (F) The binding assays of the BrC-Z2 CRE to the recombination BmBrC-Z2 protein with 1-, 5-, 10-, 25- and 50-fold excess cold probes. (20E treatment vs. DMSO treatment control; *t*-test).

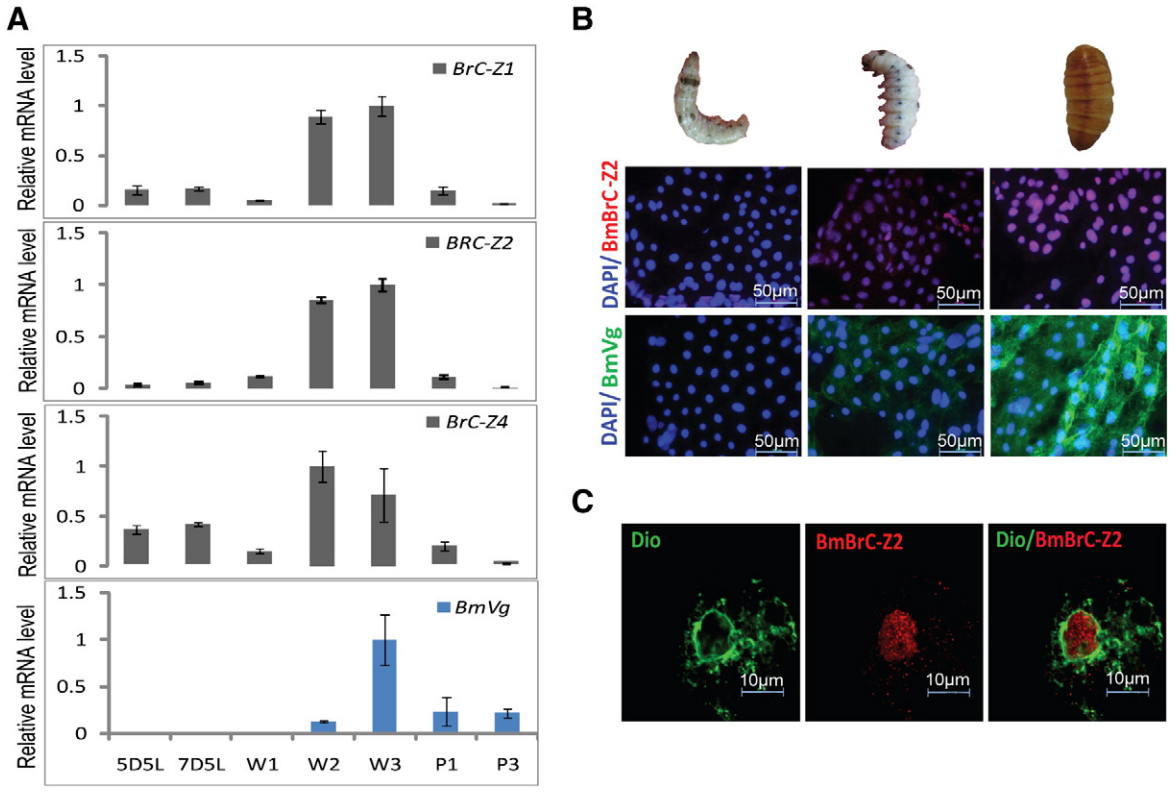


Fig. 3. Transcription and synthesis of BmBrC-Z2 corresponded with that of *BmVg*. (A) qRT-PCR analysis of the transcription of *BmBrC* isoforms and *BmVg* at the metamorphosis of the female silkworm. (B) The synthesis of BmBrC-Z2 and BmVg in the female silkworm fat body at metamorphosis was determined by immunofluorescence histochemistry. (C) The subcellular localization of BmBrC-Z2 (green, cell membrane; red, BmBrC-Z2).

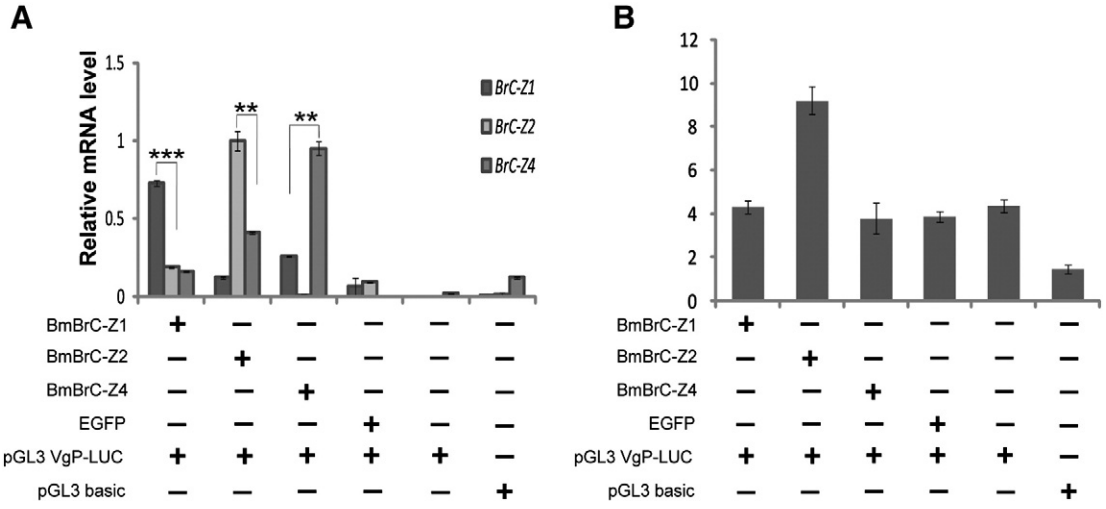
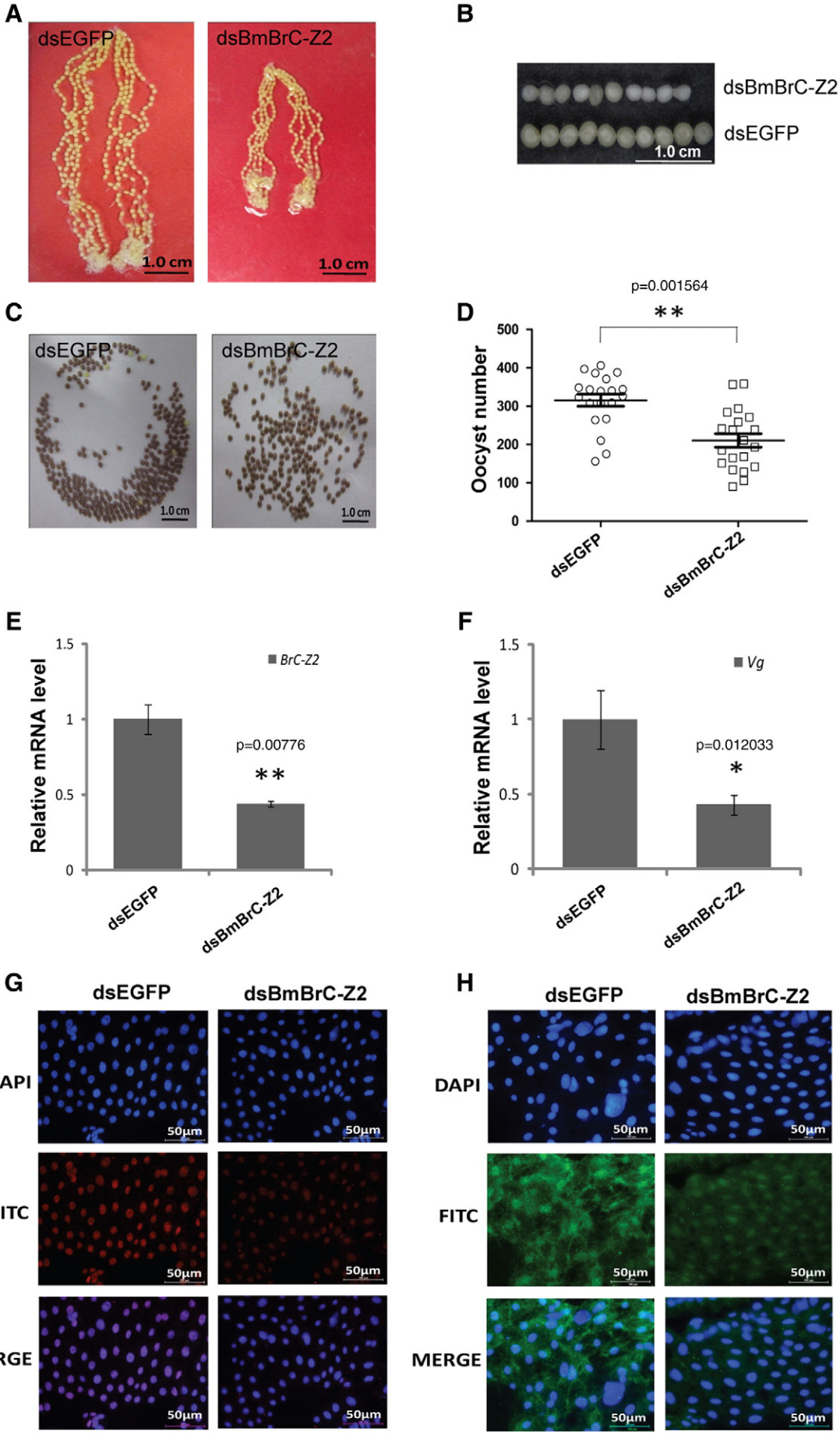


Fig. 4. Effect of over-expression of BmBrC isoforms on *BmVg* promoter activity in the BmE-SWU1 cell line. (A) qRT-PCR analysis of the transcription of *BmBrC* isoforms. (B) The effect of the *BmVg* promoter on the over-expression of BmBrC isoforms ($P < 0.001$). (ANOVA and Duncan's new multiple range tests).

of *Drosophila* ecdysone steroid early response genes Broad-complex Z2 and E74 [40,41]; Fushi tarazu (FTZ) is involved in body segmentation of the *Drosophila* embryo; BEAF is a boundary element-associated factor; and DREF is a DNA replication-related element factor. In addition, some transcriptional elements have the ability to bind with various homeodomain transcriptional factors, including the *Drosophila* homeobox transcription factor with the CUT domain, the POU domain, the Abd-B group, paired homeodomain factors etc. The homeodomain

Fig. 5. Effect of RNAi *BmBrC-Z2* in the female silkworm. (A) Differentiation of wild type and dsRNA-treated silkworm ovaries. (B) Eggs from dsBrC-Z2-treated adults were smaller and whiter compared to dsEGFP-treated adults. (C) Eggs from wild type and dsBmBrC-Z2-treated female adults. (D) Number of eggs from dsBmBrC-Z2-treated adults after fertilization. Each group had 9 samples. (E) qRT-PCR analysis of BmBrC-Z2 transcription in dsRNA-treated female pupal fat body at 2 days after treatment with dsRNA. (F) *BmVg* transcription in dsRNA-treated female pupal fat body at 2 days after treatment with dsRNA. (G) Protein synthesis of BmBrC-Z2 in treatment with dsRNA of the female pupal fat body at 2 days after treatment with dsRNA detected by immunofluorescence histochemistry. (H) Protein synthesis of BmVg in dsRNA-treated female pupal fat body at 2 days after treatment of female pupae with dsRNA detected by immunofluorescence histochemistry. (dsBmBrC-Z2 treatment vs. dsEGFP treatment control; *t*-test).


A
dsEGFP
dsBmBrC-Z2
1.0 cm
1.0 cm
B
dsBmBrC-Z2
dsEGFP
1.0 cm
C
dsEGFP
dsBmBrC-Z2
1.0 cm
1.0 cm
D
p=0.001564
**
Oocyst number
500
400
300
200
100
0
dsEGFP
dsBmBrC-Z2
E
Relative mRNA level
1.5
1
0.5
0
BrC-Z2
p=0.00776
**
dsEGFP
dsBmBrC-Z2
F
Relative mRNA level
1.5
1
0.5
0
Vg
p=0.012033
*
dsEGFP
dsBmBrC-Z2
G
dsEGFP
dsBmBrC-Z2
DAPI
FITC
MERGE
50μm
H
dsEGFP
dsBmBrC-Z2
DAPI
FITC
MERGE
50μm

transcriptional factors were widely reported in differentiation of the hindbrain and axial skeleton [42]. A recent report showed that BmPOU-M2 and Abd-B transcription factors participate in regulating the transcription of cuticular proteins in silkworm metamorphosis [37].

Among these CREs, BrCZ2 and E74A elements are able to bind with ecdysone response early transcription factors DBrC and DE74 in *Drosophila* [40,41]. Furthermore, there are three elements of BrC-Z2 in the *BmVg* promoter. Two of them on the distal elements are in the reverse direction and the proximal element is in the forward direction (Table 1 and Fig. 2A), suggesting that these putative BmBrC-Z2 *cis*-response elements might bind with the BmBrC-Z2 transcriptional factor for response ecdysone, so we examined the BmBrC-Z2 regulation of *BmVg* transcription.

3.3. BmBrC-Z2 regulated BmVg transcription by binding to the promoter motif

To understand the mechanism underlying the regulation of *BmVg* expression by ecdysone, the 5′-end upstream regulatory region was cloned into the pGL3 basic vector with the luciferase reporter gene. The vector was transfected into the BmE-SWU1 cell line with ecdysone treatment and the effect of luciferase activity on the promoter activity was assessed. The results showed that *BmVg* promoter activity is induced markedly by ecdysone (Fig. 2B), which means that the system is suitable for the analysis of how ecdysone responds to *BmVg*.

From the result given in Section 3.2, above, three putative BmBrC-Z2 *cis*-response elements were predicted on the *BmVg* promoter. The first and second of the BmBrC-Z2 putative CREs on the distant region of the promoter are close to each other and the third is near the transcriptional start site of the promoter with a reverse direction. The core sequences of these BmBrC-Z2 CREs are AA/TTA (Fig. 2A). To analyze the activity of these three binding sites in response to ecdysone, mutation vectors were made on the basis of the pGL3-Vg1.5K Luc vector. Three BrC-Z2 binding sites on the *BmVg* promoter were mutated one by one. A transfection assay showed that the promoter cannot respond to ecdysone when the third BmBrC-Z2 CRE was broken. However, the promoter activity could be induced, in part, by ecdysone on each of the other broken BmBrC-Z2 CREs (Fig. 2C), indicating that the BmBrC-Z2 CRE located close to the transcript start site might be key for the promoter response to ecdysone. So, the other two sites were mutated together, and the transfection assay showed only when the third CRE was not mutated, the *BmVg* promoter activity toward ecdysone is not obvious and the other two sites might enhance the response of ecdysone toward *BmVg* transcription (Fig. 2D).

To determine whether BrC-Z2 could bind to the putative BrC-Z2 CRE of the *BmVg* promoter, we synthesized the probe with biotin-labeled and recombinant BmBrC-Z2 protein using the sumo tag. EMSA showed that an obvious lag band was formed, which became stronger when greater amounts of probe and protein were added (Fig. 2E). The band became weaker when more of the competitive probe was added (Fig. 2F). The results showed that BmBrC-Z2 binding onto the *BmVg* promoter regulation sites will participate in *BmVg* transcriptional regulation.

3.4. The synthesis of BmBrC-Z2 corresponds with BmVg in the female silkworm

Then, we investigated by qRT-PCR transcription of the *BmBrC* isoforms Z1, Z2 and Z4 in metamorphosis of the fat body in the female silkworm. The transcript profile of *BmBrC-Z1* and *BmBrC-Z4* had a small peak in the late stages of the 5th instar larvae and had a high peak at day 2 of the wandering stage during metamorphosis of the female silkworm (Fig. 3A). Isoform Z2 was quite different from the Z1 and Z4 isoforms, however, it had only one peak at day 2 of the wandering stage (Fig. 3A) and immunofluorescence histochemistry showed that the synthesis of BmBrC-Z2 corresponded to *BmVg* (Fig. 3B). The BmBrC-Z2 is localized in the cytoplasm and it also translocated into the nucleus of the cells (Fig. 3C).

3.5. Over-expression of BmBrC-Z2 enhanced BmVg promoter activity

We asked whether only the BmBrC-Z2 isoform can induce transcription of *BmVg*. The over-expression vectors of the BmBrC isoforms were constructed (psl1180 A4-BmBrC-Z1, Z2 and Z4 plasmids) and cotransfected with the pGL3-Vg1.5K Luc vector in the cell line BmE-SWU1. From qPCR, the isoforms of BmBrC-Z1, BmBrC-Z2 and BmBrC-Z4 were over-expressed in the BmE-SWU1 cell line (Fig. 4A) and the *BmVg* promoter activity was increased only in Z2 over-expressed (Fig. 4B), showing that over-expression of BmBrC-Z2 induced *BmVg* promoter activity specially. However, over-expression of Z1 and Z4 isoforms cannot induce the *BmVg* promoter activity.

3.6. RNAi of BmBrC-Z2 affected egg formation and embryonic development

To analyze the role of BmBrC-Z2 in the regulation of *BmVg* and the effects on egg formation and embryo development, dsRNAs of *BmBrC-Z2* and *EGFP* were synthesized and injected into female silkworms. Only 20% of dsBmBrC-Z2-treated pupae died or were deformed. Differentiation of the ovary in the surviving pupae was incomplete and the egg ducts were shorter and contained fewer eggs compared to dsEGFP-treated pupae (Fig. 5A and B). Eggs were counted after eclosion and mating with wild type male moths. We found significantly fewer eggs in dsBmBrC-Z2-treated moths compared to the dsEGFP-treated insects (Fig. 5C and D).

The transcript of *BmBrC-Z* and *BmVg* was analyzed by qRT-PCR. We found that treatment with dsRNAs reduced the transcript of *BmBrC-Z2*, which is specific to the fat body in the female pupa and *BmVg* transcript was reduced (Fig. 5E). Immunohistochemistry showed that BmBrC-Z2 synthesis was reduced significantly by treatment with dsRNA and this reduction caused a reduced synthesis of BmVg (Fig. 5F), suggesting that the reduction of BmBrC-Z2 affects the *BmVg* transcription and egg formation.

4. Discussion

From our results, many transcription factor CREs were detected in the *BmVg* promoter, including *Drosophila* heat shock factors, vismay, hunchback and C/EBP factors etc. Unexpectedly, we found a large class of homeodomain transcription factor CREs. The homeodomain transcriptional factor determined segment morphology and appendage number in arthropods. These genes regulate vertebra morphology as well as limb and central nervous system patterning in vertebrates [43,44]. A recent study found that the homeodomain transcription factors BmPOUM2 and Abd-A take part in the regulation of silkworm metamorphosis [37]. However, elucidation of the role of the homeodomain transcriptional factor in *BmVg* synthesis and reproduction needs more data. As described, the stage-specific expression of Vg is controlled stringently by a blood meal in female adult mosquitos [3–5]. A class of transcription factors participated in the regulation, including EcR/USP, BrC, E74, E75, Forkhead box (Fox) and GATA [45–50]. As in *D. melanogaster*, yolk protein genes *Yp1* and *Yp2* were expressed in the female fat body and *Yp3* was expressed in ovarian follicle cells. Our results showed that ecdysone induction of *BmVg* synthesis in the female silkworm fat body was dose and time-dependent. As described elsewhere, ecdysone participates in Vg regulation in lepidopteran insects [51]. In our data, however, treatment of the silkworm fat body with JH did not affect *BmVg* transcription significantly. Whether the synthesis of BmVg needs additional hormones as described earlier needs confirmation [51]. On the basis of these results, we indicated that the transcription regulation of *BmVg* in the silkworm is a complex regulation mechanism with transcriptional factor participation.

BrC is one of the key ecdysone responsible genes, which has an important role in insect metamorphosis and development [22,29]. In this study, three putative BrC-Z2 CREs were identified on the *BmVg* promoter. And the results from cell transfection showed that BrC-Z2 CREs were important to respond to an ecdysone signal for regulating *BmVg* expression. EMSA showed that the transcriptional factor BmBrC-Z2 participated in regulating *BmVg* transcription by direct binding with the BrC-Z2 CRE. Similar to the report for the mosquito, BrC-Z2 participated in Vg gene expression as an enhancer to Vg promoter activity. Z2, Z1 and Z4 as a repressor act on Vg regulation [49]. We found that the difference of transcription pattern between *BmBrC-Z2* and *BmBrC-Z1*, *Z4* was that *BmBrC-Z2* was only one peak at day 2 of wandering in the female fat body. Meanwhile, the transcription of *BmVg* is also induced rapidly into a high level. Then, we over-expressed BmBRC-Z1, Z2 and Z4 in the BmE-SWU1 cell and found that the *BmVg* promoter activity was enhanced significantly, only when BmBrC-Z2 was over-expressed, indicating that BmBrC-Z2 had an important role in the regulation of *BmVg* transcription in the silkworm. The effects of BmBrC-Z1 and BmBrC-Z4 were not clear.

The results of this study confirmed that the transcription of *BmVg* can be induced by ecdysone. However, without any complete ecdysone receptor, a response element was predicted on the *BmVg* promoter and the cell transfection assay showed that BmBrC-Z2 CRE was essential to the response of ecdysone, especially near the transcriptional start site of the *BmVg* promoter because the activity of the *BmVg* promoter was not induced. By contrast, the EcR/USP complex in the mosquito was a switch to respond to ecdysone [45]. Early response transcript factors E74, E75 and Hr3 participated in *Vg* regulation by enhancing or inhibiting the ecdysone signal [3,4]. The cell transfection assay showed that the switch of transcriptional regulation of *BmVg* by ecdysone depends on the BrC-Z2 CRE near the promoter transcriptional start site. Unfortunately, we cannot explain how to realize the switch function. Interestingly, the homeodomain transcription factor POU CRE near the BmBrC-Z2 CRE on the BmVg promoter was predicted. More data are needed to determine whether homeodomain transcriptional factor POU participates in this. The result indicated that the regulation mechanism of *BmVg* by BmBrC in the silkworm is quite different compared to the mosquito.

RNAi of BmBrC-Z2 showed that egg formation was affected with whiter and smaller eggs compared to the control, indicating that the synthesis of BmVg was blocked. The phenotypes were similar with regard to down-regulation compared to BmVg (unpublished data). By contrast, an oogenesis mutation called scanty vitellin (*vit*) results in smaller and whiter eggs compared to the wild type, and the mutation is embryonically lethal because BmVg cannot be transported into the ovary by the defective vitellogenin receptor (BmVgR) [9,52,53]. The *D. melanogaster* BR-C mutation made the ovary follicle cells at the anterior of the oocyte failling to migrate correctly in an anterior direction [54]. By the systemic interference of BmBrC isoforms by RNAi, the failure of the silkworm to complete the larva–pupa transition or by later morphogenetic defects, including differentiation of adult compound eyes, legs and wings from their larval progenitors. It also perturbed apoptosis of the larval silk gland [29]. These results suggested that BmBrC-Z2 has an important role in metamorphosis and in vitellogenesis in the silkworm.

5. Conclusion

We found that only BmBrC-Z2 responded to ecdysone and regulated *BmVg* transcription. Further, it participates in the regulation of egg formation. This showed a new mode of regulation by *Vg* responding to ecdysone and it helps us to understand how a hormone participates in the regulation of reproduction in an insect. This study provides the foundation for future design of silkworm pupa stage-specific expression cassettes with predicted stage and tissue specificity.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbagen.2014.05.013.

Acknowledgements

We thank Dr. Feng Wang for providing the vector and associate professor Daojun Chen for the BmBrC-Z2 antibody. This work was supported by the National Basic Research Program of China (No. 2012CB114600), the Hi-Tech Research and Development Program of China (No. 2011AA100306), and the National Natural Science Foundation of China (No. 31101768).

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