Distinct roles of Broad isoforms in regulation of the 20-hydroxyecdysone effector gene, *Vitellogenin,* in the mosquito *Aedes aegypti*

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Abstract

We investigated the role of the mosquito *broad* (*br*) gene in regulating the 20-hydroxyecdysone (20E) effector *Vitellogenin* (*Vg*) gene. Injection of double-stranded RNA corresponding to the BR isoform Z2 led to a significant decrease in expression of the *Vg* gene at 8 and 24 h post-blood meal. Knockdown of Z1 or Z4 resulted in enhanced *Vg* expression beyond its normal expression time. *In vitro* studies suggested that the effects of BR require its direct binding to the *Vg* promoter, as well as protein-protein interaction between BR and the ecdysone receptor complex. The BR isoforms are therefore essential for a proper stage-specific biological response to 20E in the adult female mosquito. In particular, the isoform Z2 is required for 20E-mediated activation of *Vg*, while isoforms Z1 and Z4 serve as repressors to ensure appropriate termination of *Vg* expression.

Keywords

Steroid hormone; Transcriptional regulator; C2H2 type zinc-finger; RNA interference; Mosquito

1. Introduction

The insect steroid hormone, 20-hydroxyecdysone (20E), serves as a pivotal temporal cue and directs many critical events in embryogenesis, larval molting, metamorphosis and, in some insects, adult reproduction (Riddiford, 1993;Nijhout, 1994;Raikhel et al., 2005). The functional receptor for 20E is a heterodimer of two nuclear receptors—the ecdysone receptor (EcR) and the homologue of the retinoid X receptor, Ultraspiracle (USP) (Riddiford et al., 2000). The receptor complex acts as a transcription factor recognizing specific DNA sequences (ecdysone response elements) of their target promoters. Binding of 20E and agonist ligands to EcR/USP triggers a conformational change that attracts transcriptional coactivators, leading to transcriptional activation of the target genes (Palli et al., 2005). In response to the 20E titers during *Drosophila* metamorphosis, EcR/USP directly activates a group of primary-response target genes, including a small set of so-called early genes, such as *E74, E75* and *Broad* (*br*), which encode intermediate transcriptional regulators (Thummel, 2001). The proteins of these early genes in turn regulate transcription of a larger group of secondary-response genes that ultimately lead to altered cell functions.

A similar 20E regulatory pathway is utilized in the mosquito *Aedes aegypti* during egg maturation. An increase in the 20E concentration takes place shortly after blood feeding

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(Hagedorn, 1989). Yolk protein precursors (YPPs) are synthesized in the fat body, a metabolic tissue functionally analogous to the vertebrate liver, under the control of 20E; they are eventually deposited in the developing oocytes. The major YPP gene, *Vitellogenin* (*Vg*), is induced about 10,000-fold at its peak expression 20-24 h post-blood meal (PBM), but declines to a background level by 36 h PBM. Sequence analysis of the 5′ regulatory region of *Vg* reveals the presence of binding sites for EcR/USP, E74, E75 and BR (Kokoza et al., 2001;Chen et al., 2004). Genes encoding these transcriptional factors are upregulated after blood feeding, albeit with distinct patterns, suggesting the direct participation of both the EcR complex and early gene products in regulation of *Vg* (Pierceall et al., 1999;Wang et al., 2000,2002;Sun et al., 2002;Chen et al., 2004). Indeed, we have demonstrated that the EcR/USP dimer binds to the *Vg* promoter shortly after blood feeding and acts synergistically with E74 to activate the *Vg* expression (Zhu et al., 2003a;Sun et al., 2005).

Br encodes a family of C2H2-type, zinc-finger, DNA-binding proteins. In *Drosophila*, four BR isoforms (Z1-Z4) share a common N-terminal Bric-a-brac-Tramtrack-Broad (BTB) domain, an evolutionarily conserved protein-protein interaction module (Zollman et al., 1994). Alternative splicing results in isoform-specific C-termini containing one of four pairs of C2H2 zinc fingers that recognize different DNA sequences (DiBello et al., 1991;Bayer et al., 1996). Transcripts of four BR isoforms are present in the fat body of *A. aegypti* females (Chen et al., 2004). Their expression is elevated after blood feeding, corresponding to the peak of 20E titers. In cell transfection assays, BR isoforms by themselves have no marked effects on the *Vg* promoter. However, Z1 and Z4 suppress the EcR/USP-mediated 20E activation of *Vg*, while Z2 enhances such activation. Z3 appears to have no effect in the same experiment (Chen et al., 2004). To explore the functions of BR in adult mosquitoes *in vivo*, we used isoform-specific RNA interference (RNAi) to knock down *Br* expression. Here, we show that BR isoforms play differential roles to ensure the appropriate temporal pattern of the *Vg* expression in the fat body after a blood meal. Additionally, *in vitro* studies suggest that the effects of BR require their direct binding to the *Vg* promoter. Our results also imply that BR proteins regulate *Vg* transcription via protein-protein interaction with the EcR/USP complex.

2. Materials and methods

2.1. Animals

Mosquitoes, *A. aegypti*, were raised as described by Hays and Raikhel (1990). Larvae were fed a standard diet (Lea, 1964) and adults were fed on a 10% sucrose solution continuously by wick. Vitellogenesis was initiated by feeding 3-5-day-old (post-emergence) females on white laboratory rats. All dissections were performed in *Aedes* physiological saline (Hagedorn et al., 1977) at room temperature.

2.2. RNA interference assay

Synthesis of double-stranded RNAs (dsRNAs) and microinjection were performed as described previously (Zhu et al., 2003b). dsRNAs corresponding to the BR isoform-specific region (Z1, nt 1466-1796; Z2, nt 1504-1717; Z3, nt 1540-1957; Z4, nt 1453-1953), where no other significant homology was found by Blast search (NCBI), were prepared using the HiScribe RNAi transcription Kit (New England Biolabs). The integrity of the dsRNAs was assessed by means of gel electrophoresis, and concentration was determined by spectrophotometry. One day after emergence, mosquito females were each microinjected intra-thoracically with approximately 0.5 μg of dsRNA. The injected mosquitoes were allowed a period of 5 days for recovery and then were fed blood. The transcription of *br* and *Vg* was analyzed using real-time PCR as described previously (Chen et al., 2004). Each sample was analyzed in triplicate and normalized to the internal control, β-*actin* mRNA.

2.3. Cell culture and transient transfection assay

The EcR-deficient *Drosophila melanogaster* L57-3-11 cell line, provided generously by Dr. Lucy Cherbas (Indiana University), was transfected according to the instructions of Hu et al. (2003). The coding region sequences of the four *BR-C* isoforms were inserted into the pAc5.1/V5/HisA (Invitrogen) vector and fused to a V5 and a His tag (Chen et al., 2004). The construction of plasmids pVg2.1-luc, pAc5-AaEcR and pAc5-AaUSP has been described elsewhere (Wang et al., 1998).

Western-blot analyses were carried out with a fraction of the transfected L57-3-11 cells. A 20-µl aliquot of each sample was separated by means of SDS-PAGE and tested independently with antibodies against AaEcR, AaUSP and V5 tag (Invitrogen).

2.4. Electrophoretic mobility shift assay (EMSA)

EMSAs for BR were performed as described previously (Chen et al., 2004). For competition reactions, unlabelled oligonucleotides were added in excess of 50-fold.

2.5. Immunoprecipitation

Transfected cells were harvested 40 h after transfection in ice-cold NP-40 lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.1% NP-40) supplemented with protease inhibitors. Assays were performed using the Immunoprecipitation Kit (Roche) following the manufacturer's instructions. Antibodies against AaEcR were used for this analysis. The resulting immune complexes were precipitated by addition of protein G-agarose beads. After extensive washing, the complexes were separated by means of SDS-PAGE and analyzed by means of Western blotting with anti-V5 antibody (Invitrogen).

3. Results

3.1. BR isoforms play important roles in controlling temporal expression of the Vg gene

The increase of *Br* transcripts after blood feeding, induced by a rise in the 20E concentration, suggests that BR isoforms are required for the 20E-activated production of YPP (Chen et al., 2004). To test this hypothesis, we synthesized dsRNAs corresponding to the isoform-specific regions of BR and injected them independently into newly emerged female mosquitoes. dsRNA corresponding to a bacterial *malE* gene was injected in a similar manner to act as a negative control. Five days after injection, these mosquitoes were given a blood meal. mRNA levels of *br* decreased considerably, both before and after blood feeding, in the fat body of female mosquitoes injected with *br* dsRNAs, but not in the control mosquitoes (Fig. 1).

After confirmation of the knockdown of *br* transcripts, we then looked into their effects on the expression of the *Vg* gene. In control mosquitoes, the *Vg* mRNA levels started to rise after blood feeding, peaked at about 24 h PBM, declined thereafter and returned to the background levels by 36 h PBM (Fig. 2A). The knockdown of Z2 was associated with a significant decrease in the *Vg* mRNA levels at 8 h ($P < 0.05$) and 24 h PBM. The reduction of Z3 resulted in a substantial decrease in the *Vg* mRNA levels at 24 h PBM. Conversely, injection of dsRNA corresponding to Z1 or Z4 not only markedly augmented the *Vg* transcription at 24 h PBM, but also led to a considerable increase in *Vg* expression at 36 h PBM (Fig. 2A). Importantly, enhanced *Vg* transcription was detected even at 48 h PBM in the fat body of mosquitoes with reduced Z1 and Z4 expression (Fig. 2B). Injection of a combination of dsRNA corresponding to both Z1 and Z4 caused a slightly stronger transcription of *Vg* at 24 h PBM than injection of Z1 or Z4 dsRNA alone, suggesting that these isoforms act cooperatively in temporal regulation of the *Vg* expression (Fig. 2B). Together, these data indicate that the *br* gene is required for the proper expression pattern of *Vg*. Moreover, isoforms Z2 and Z3 likely function as transcriptional activators of the *Vg* gene, and Z1 and Z4 as its repressors.

3.2. BR isoforms are implicated in regulation of the usp gene

Binding sites for BR isoforms are also found in regulatory regions of the *usp* gene (Wang, Zhu and Raikhel, unpublished data), encoding an obligatory component of the EcR complex, which is involved in the primary and secondary control of the *Vg* gene. The two mosquito USP isoforms, USP-A and USP-B, are differentially expressed and regulated by the 20E ligand (Wang et al., 2000). *usp-a* is inhibited by 20E and is thus expressed in postvitellogenic fat bodies, while the *usp-b* transcript levels increase 5-8-fold during the vitellogenic stage and are activated by 20E. Injection of dsRNA corresponding to the Z1 isoform resulted in modestly enhanced transcription of *usp-a* at 24 h and its dramatic activation at 36 h PBM, suggesting that Z1 is a stage-specific repressor of *usp-a* expression (Fig. 3A). Downregulation of Z2 and Z3 led to a substantial decrease in the *usp-a* expression at 36 h PBM, indicating their possible roles in stage-specific activation of *usp-a*. Lessening of Z4 had no marked effect on the *usp-a* expression (Fig. 3A). Injection of dsRNA corresponding to the Z1 isoform resulted in enhanced transcription of *usp-b* at 24 and 36 h PBM, suggesting that Z1 is a stage-specific repressor of *usp-b* expression (Fig. 3B). Reduction of Z4 prolonged the induced *usp-b* transcription to 36 h PBM, at a level comparable to its normal peak at 24 h PBM, implying that Z4 could serve as an additional stage-specific regulator of *usp-b* expression (Fig. 3B). Downregulation of Z2 and Z3 had no obvious effects on the *usp-b* promoter. These results thus indicate that the *br* gene has a profound role in modulating the stage-specific 20E response.

3.3. Regulatory functions of BR require their binding to the Vg promoter

Consistent with results of the RNAi experiment, potential binding sites of the BR isoforms have been identified in the *Vg* 5′ upstream regulatory region. Furthermore, cell transfection assays have demonstrated that the Z1, Z2 and Z4 isoforms modulate transactivation of the *Vg* promoter by the EcR/USP dimer (Chen et al., 2004). To elucidate the mechanism underlying the regulation by BR, we systematically removed the BR binding sites on the *Vg* promoter by means of site-directed mutagenesis and exploited the derivative pVg-luc reporter plasmids in transfection assays. Since the Z3 isoform only marginally increased the effect of EcR/USP in the presence of 20E in our previous study (Chen et al., 2004), we did not test the Z3 binding sites in this experiment. When AaEcR/AaUSP and the Z1 isoform were co-expressed in L57 cells, Z1 significantly dampened activation of the *Vg* promoter by AaEcR/AaUSP in the presence of 20E (Fig. 4A). In contrast, the dose-dependent repression by Z1 was mostly relieved when the pVg-luc reporter construct was deprived of the only Z1 binding site on the *Vg* promoter.

A similar experiment was carried out to test the two binding sites of BR-Z2 (2A and 2B) separated apart in the *Vg* promoter. After the 2A sequence was mutated in the reporter plasmid, the EcR/USP-mediated activation of the *Vg* promoter became slightly stronger ($P < 0.01$). Nonetheless, co-expression of the Z2 protein enhanced the activation of the *Vg* promoter ($P < 0.01$) despite the loss of the 2A site. However, when 2B was destroyed, alone or in combination with 2A, Z2 lost its effect on the *Vg* promoter in the transfection assays (Fig. 4B). These results imply that the isoforms Z1 and Z2 directly bind to the regulatory region of *Vg* and modulate its transcription after a blood meal.

Seven binding sites of Z4 (4A-4G) are scattered in a 2.1 kb upstream region of *Vg*, exhibiting different binding affinity to Z4 (Chen et al., 2004). Starting from pVg2.1-luc, a series of mutations were introduced to remove potential Z4 binding sites, individually and in combination. Even when seven binding sites were destroyed altogether in the reporter plasmid, the repression by Z4 on *Vg* promoter was not completely relieved (Fig. 5A). Since the Z4 recognition sequences are rich in A/T base pairs, we could not rule out that additional Z4 binding sites exist on the *Vg* promoter. Instead, we constructed three BR-Z4 mutants, in which four cystidines (at positions of 495, 498, 525 and 528) in the two zinc-finger DNA binding

domains were changed into alanines (Fig. 5B). The first and second zinc fingers were disrupted individually in ZF-A and ZF-B, and collectively in ZF-AB. As expected, DNA binding affinity of the mutated Z4 proteins was reduced drastically in the EMSA experiment (Fig. 5C). The suppression on the EcR-mediated activation was severely reduced when Z4 was replaced by its mutants in the cell transfection assay, indicating that the intact DNA binding domain of Z4 is essential for its effect on the *Vg* promoter (Fig. 5D).

3.4. BR binds the EcR complex in vitro

The fact that BR modulates activity of the EcR complex on the *Vg* promoter prompted us to test whether a protein-protein interaction exists between BR and the EcR/USP dimer. Individual BR isoforms (as fusions to the V5 epitope tag) were co-expressed with AaEcR-B and AaUSP-B in *Drosophila* L57 cells. Whole cell extracts of these cells were then subjected to immunoprecipitation assays. The three BR isoforms we tested separately (Z1, Z2 and Z4) were precipitated using antibodies against mosquito EcR in a 20E-independent manner (Fig. 6). Together, these results suggest that BR regulates *Vg* expression via protein-protein association with EcR/USP on the *Vg* promoter.

4. Discussion

The role of *br* during metamorphosis is well understood in holometabolous insects, such as *Drosophila* and moths (Riddiford et al., 2003). Extensive genetic analyses in *Drosophila* have shown that *br* is a critical mediator of the ecdysone response during metamorphosis (Belyaeva et al., 1980;Kiss et al., 1988;Karim et al., 1993). The *br* mutants exhibit abnormal regulation of many intermolt, early and late gene activities (Karim et al., 1993;Zhou and Riddiford, 2002). More recently, it has been demonstrated that *br* is expressed during each nymphal molt of the direct-developing insect *Oncopeltus fasciatus* (Erezyilmaz et al., 2006).

Much less is known about the role of *br* in adult insects. In adult *Drosophila*, genetic analysis has shown that *br* is a key control gene during oogenesis (Deng and Bownes, 1997). The different expression patterns of BR isoforms regulate the number of eggs produced by determining whether the cells in each individual egg chamber become committed to apoptosis or to progress through normal development (Terashima and Bownes, 2004). Moreover, Z1 and Z3 overexpression were demonstrated to suppress *yp* gene expression in egg chambers, while Z2 and Z4 had no effect (Terashima and Bownes, 2004). Our study revealed that the mosquito BR proteins modulate stage-specific expression of *Vg*, a 20E target gene, during vitellogenesis in the mosquito female fat body, providing additional evidence that *br* has evolutionarily conserved roles in the insect ecdysone response.

Our functional analyses of *br* by means of isoform-specific RNAi in adult female mosquitoes demonstrated that BR is required for the robust and precisely timed expression of the *Vg* gene after a blood meal. Moreover, individual BR isoforms were shown to play distinct roles in such regulation. We observed Z2 to serve as a transcription activator, essential for the optimal *Vg* expression at 8 and 24 h PBM. Z3 appeared to be functionally similar to Z2, but its effect was limited to 24 h PBM. On the contrary, Z1 and Z4 acted as negative regulators to attenuate the *Vg* expression at 24 and 36 h, and even at 48 h PBM once the 20E titers in the female mosquitoes had reached the maximum at about 20 h PBM. The results of *in vivo* studies are in line with effects of BR on the *Vg* promoter in the cell transfection assays. Importantly, these functions of BR isoforms also appear to be consistent with their expression patterns in the mosquito fat body. Transcription of *Z2* exhibited a sharp rise after the onset of vitellogenesis and reached two peaks, one at 8 h and one at 24 h PBM. Conversely, the *Z3* transcripts remained at a relatively low level throughout the entire vitellogenic period, increasingly slightly at 24 h PBM. mRNA levels of both *Z1* and *Z4* were relatively low during the early vitellogenic stage, but started to rise after 16 h PBM and peaked at 24 h (Chen et al., 2004). In summary, individual

BR isoforms participated in the regulation of *Vg* in response to the 20E pulse after blood ingestion through a coordinated and well-orchestrated effort.

It has been shown in *D. melanogaster* that different BR isoforms can have opposite regulatory functions on the same gene. For example, the larval fat-body-specific expression of the *Fbp-1* gene is activated by the Z3 isoform, but repressed by the Z2 protein (Mugat et al., 2000). Interestingly, functions of an individual isoform are promoter dependent. While Z3 is required for activation of the *Fbp-1* gene, it acts as a repressor for the transcription of a salivary-gland-specific gene Sgs4 (Bayer et al., 1996). A similar phenomenon is also observed in the mosquito 20E response. Our preliminary study suggested that both Z1 and Z4 isoforms are required to upregulate expression of the fat-body-specific lipophorin receptor gene, which is activated at 30-36 h PBM when the 20E titers in the female adults drop to the basal level (Jun and Raikhel, unpublished result). Therefore, it appears that mere existence of the BR proteins cannot explain their diverse properties in terms of transcription regulation. The activities of the BR isoforms probably rely on their binding sequences in the context of native target promoters and/or proteins associated with BR at different scenarios.

The presence of zinc-finger DNA binding domains in BR proteins suggested that they work as transcription regulators and interact directly with cis-acting regulatory elements in target genes. Indeed, there are numerous binding sites for BR isoforms in the mosquito *Vg* promoter. EMSA assays confirmed that BR can bind these sites *in vitro*. When binding sites for Z1 and Z2 were mutagenized in the pVg-Luc reporter construct, the BR proteins lost their influences on the *Vg* promoter in cell transfection assays. Similarly, mutations in the Z4 zinc-finger domain also relieved repression of the *Vg* promoter by this isoform. Thus, these data collectively imply that the BR proteins act through their binding sites in the *Vg* promoter, and that direct DNA-protein interactions are indispensable for appropriate activation of this ecdysone effector gene. It is worth noting that the upregulation of *usp* gene after blood feeding is also modulated by the BR isoforms. This observation not only indicates that BR has profound effects on the vitellogenic 20E response, but also raises the possibility that *Vg* is under direct and indirect control of BR.

Intriguingly, BR by itself had no marked effect on the *Vg* promoter in the cell transfection assay. Instead, the BR isoforms either repressed or enhanced transcription of the *Vg* promoter activated by the EcR complex (Chen et al., 2004). The functional interaction between BR and EcR/USP may stem from their physical association that we found *in vitro*. Proteins harboring the BTB domain have been shown to play crucial roles in specifying the overall transcriptional activity of nuclear receptors in response to their cognate ligands. The human promyelocytic leukemia zinc finger (PLZF) protein is a transcriptional repressor belonging to the BTB family. PLZF interacts directly with the Vitamin D3 receptor (VDR) and represses VDR-dependent gene activation (Ward et al., 2001). The ligand-independent physical contact involves the DNA-binding domain of VDR and the BTB domain of PLZF. Likewise, PLZF has been found to bind the retinoic acid receptor (RAR) and to interfere with the transcriptional activity of the RAR-RXR heterodimer (Martin et al., 2003). The zinc finger domain of PLZF and the ligand binding domain of RAR are sufficient for the interaction of these two proteins, while participation of RXR is not required. Unlike BR, DNA binding of PLZF in both cases is not required for its repressive functions. Interestingly, a chromosomal translocation joining the PLZF gene to RARα is associated with a rare syndrome of acute promyelocytic leukemia (Chen et al., 1994). The PLZF-RARα binds to retinoic acid response element as a heterodimeric partner of RXR, interfering with RARα functions by exerting a dominant negative effect (Licht et al., 1996). The functional interactions between members of the nuclear receptor and BTB families thus represent a regulatory mechanism well-conserved in vertebrates and invertebrates.

Regulation of *Vg* after blood feeding is complicated, requiring the participation of a lot of transcriptional factors. Another early gene product, E74B, has been shown to work synergistically with the EcR/USP dimer in activating the *Vg* promoter (Sun et al., 2005). This collaboration demands 20E-dependent protein interaction between E74B and EcR/USP, and their direct binding to the *Vg* promoter. In light of the modulatory effects of BR, we believe that BR and E74B bind to the EcR/USP complex and, presumably by altering conformation of the EcR/USP complex, bring to the *Vg* promoter transcriptional cofactors that change local chromosomal structure or formation of the transcription apparatus. The requirement of DNA binding increases local concentrations of these proteins on the *Vg* promoter and also provides an additional layer of specificity for such protein interactions. Future studies should determine which domains of BR and EcR/USP are involved in the protein interaction, and whether EcR/USP sequentially or simultaneously binds BR and E74B on the *Vg* promoter. It should also be of interest to elucidate the molecular details of how BR regulates a target gene when the EcRE is not present in the promoter.

Acknowledgement

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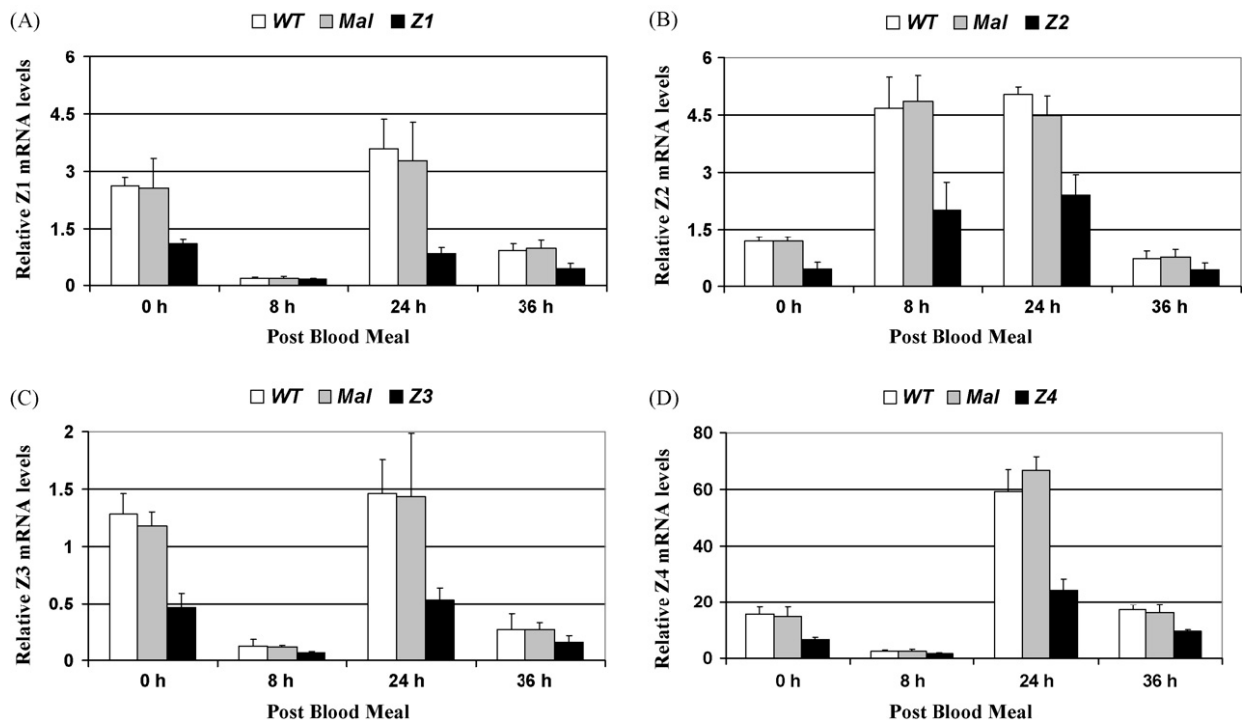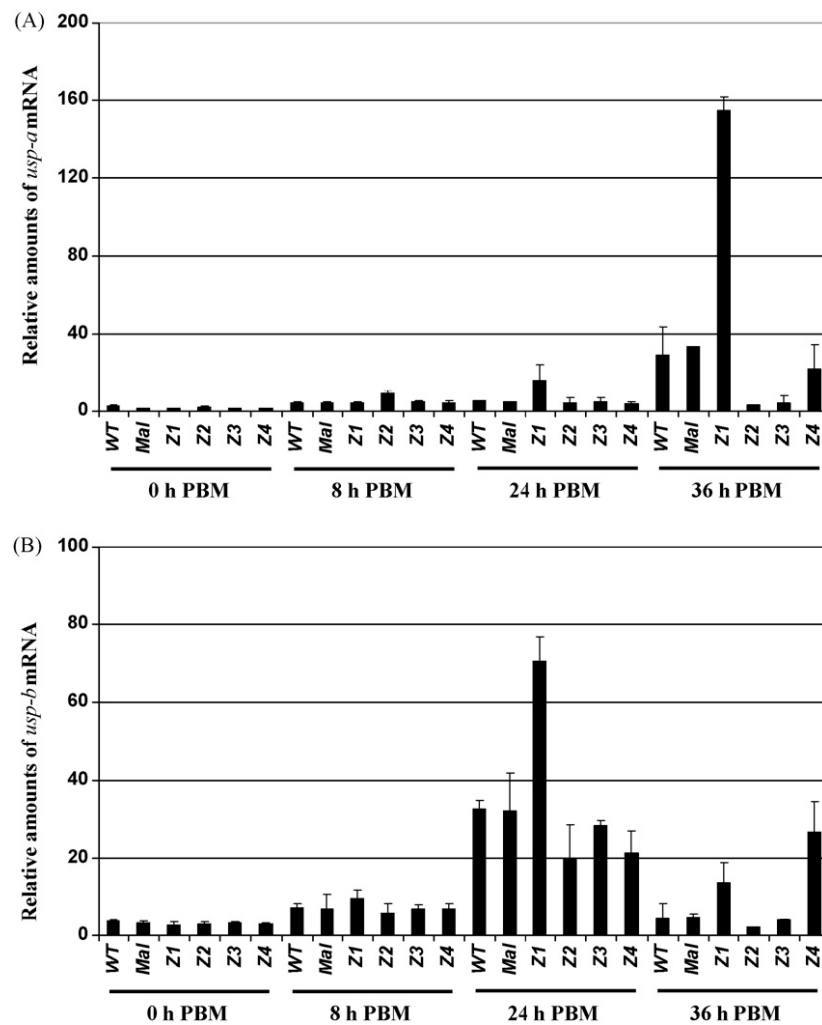
Fig 1.
Knockdown of *br* expression in the adult female mosquito. dsRNAs corresponding to individual BR isoforms were injected into thoraces of female mosquitoes as described in Section 2. mRNA expression of *Z1* (A), *Z2* (B), *Z3* (C) and *Z4* (D) was measured by means of real-time PCR at the indicated time points after blood feeding. Each sample was analyzed in triplicate and normalized to the internal control, β-*actin* mRNA. *WT*, uninjected *A. aegypti* Rockefeller/UGAL strain; *Mal RNAi*, injected with double-stranded RNA complementary to bacterial *malE*; *Z1-Z4*, injected with dsRNA corresponding to the isoform-specific regions of the mosquito BR isoforms.
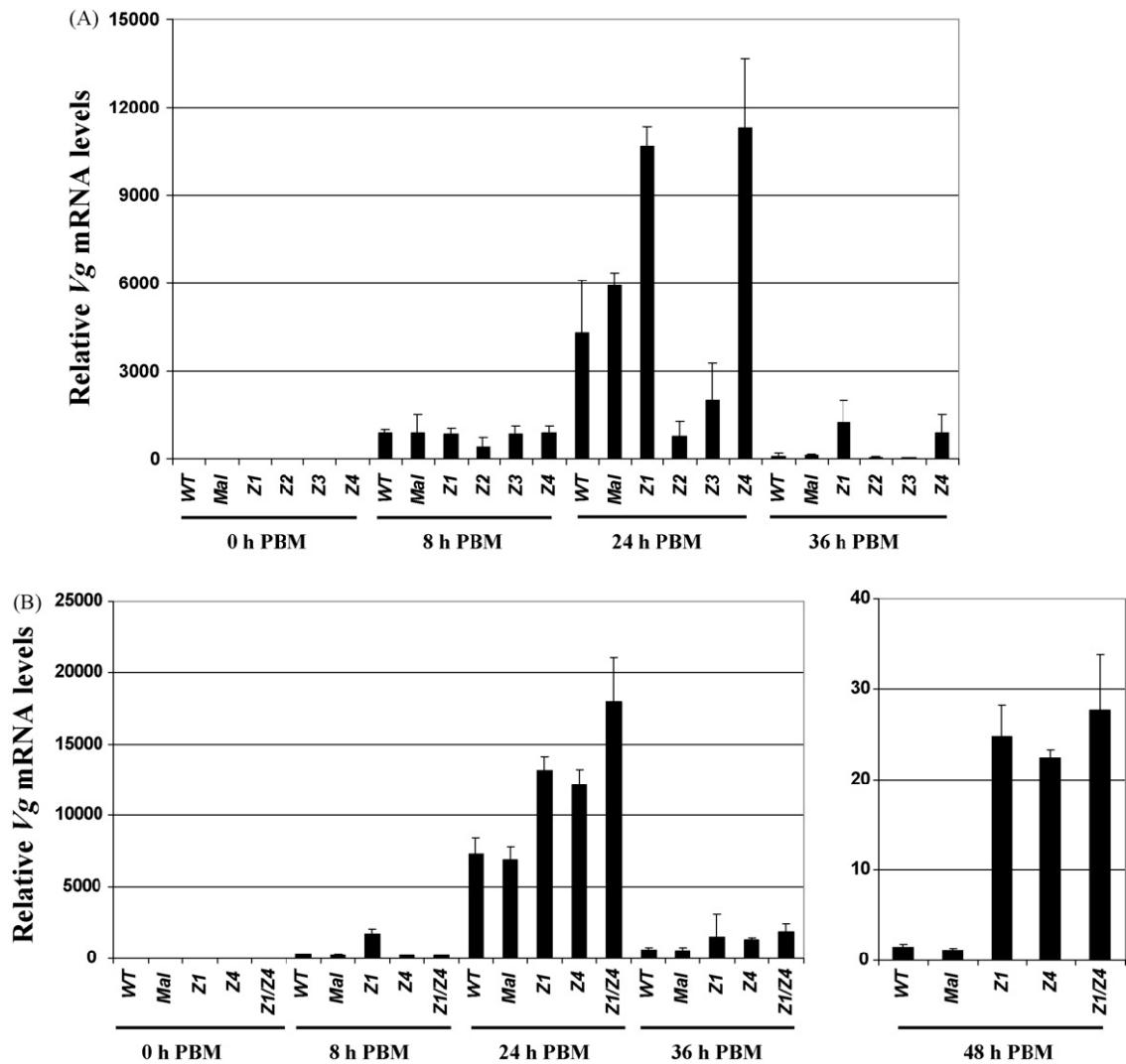
Fig 2.
The BR isoforms are required for proper *Vg* expression in the fat body after blood ingestion. dsRNAs corresponding to individual (A) or a combination of two (B) BR isoforms were injected into the thoraces of newly emerged female mosquitoes. The transcripts of *Vg* in female mosquitoes were then measured using real-time RT-PCR at the indicated time after blood feeding and were normalized to β-*actin* expression. Please note the different *Y*-axis scale used in Panel B for animals at 48 h PBM. *WT*, uninjected *A. aegypti* Rockefeller/UGAL strain; *Mal*, injected with double-stranded RNA complementary to bacterial *malE*; *Z1*, *Z2*, *Z3* and *Z4*, injected with BR isoform-specific dsRNA; *Z1/Z4*, injected with a mixture of dsRNA for *Z1* and *Z4*. Representative data (mean ± S.E.M.) from at least three independent experiments are shown.

Fig 3.
RNAi-mediated reduction in mosquito BR leads to abnormal expression of the USP gene. dsRNAs corresponding to individual BR isoforms were injected into thoraces of newly emerged female mosquitoes. The *usp-a* (A) and *usp-b* (B) transcripts in the fat body were then measured using real-time RT-PCR at the indicated time after blood feeding as described in Section 2. *WT*, uninjected *A. aegypti* Rockefeller/UGAL strain; *Mal*, injected with double-stranded RNA complementary to bacterial *malE*; *Z1*, *Z2*, *Z3* and *Z4*, injected with BR isoform-specific dsRNA.
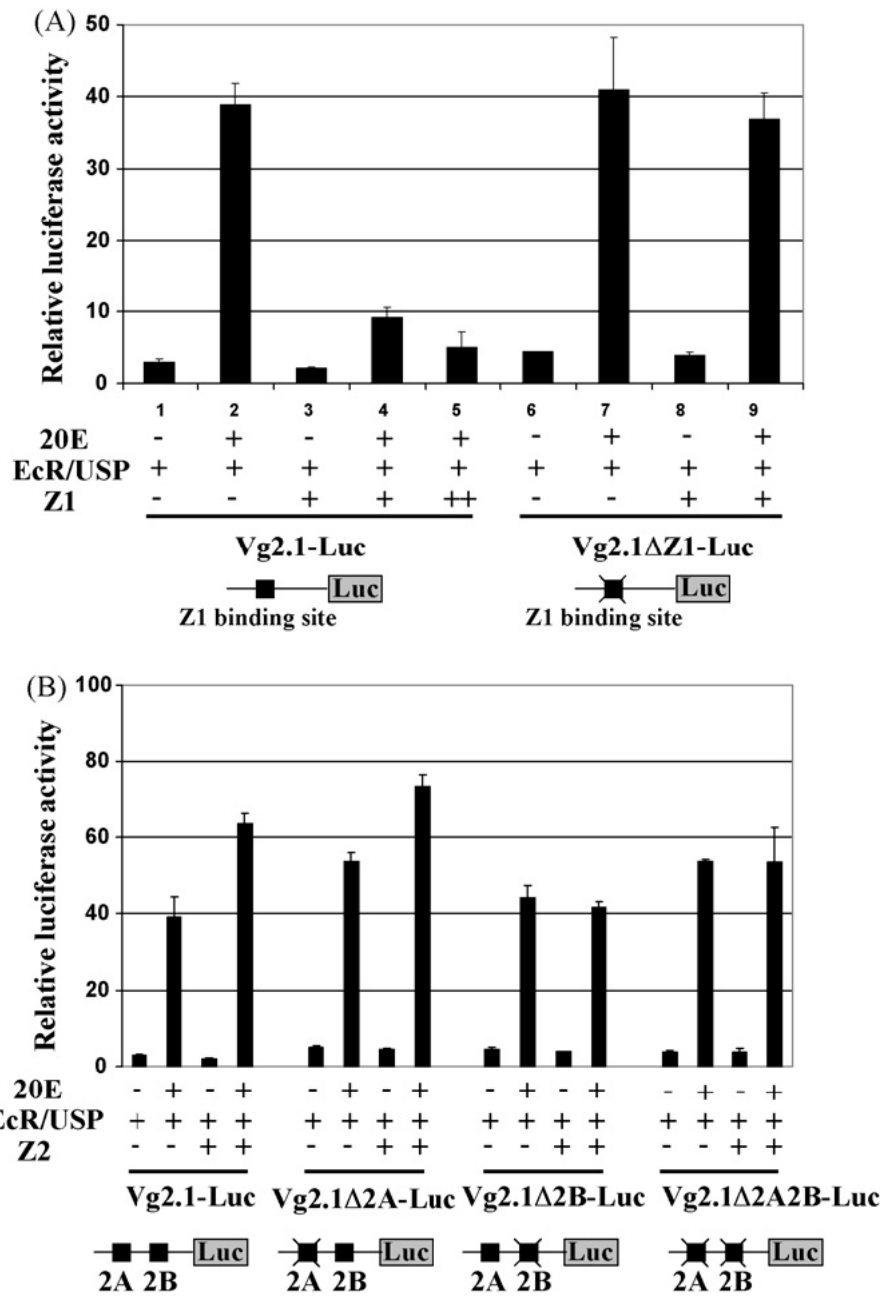
Fig 4.
The BR binding sites on the *Vg* promoter are essential for transcriptional regulation by the BR isoforms. *Drosophila* L57 cells were transfected with 100 ng phRL-CMV (*Renilla* luciferase control vector), 100 ng of different reporter plasmids and 100 ng expression vectors for AaEcR-B, AaUSP-B, AaBR-Z1 (A) and AaBR-Z2 (B). A schematic diagram of the reporter constructs is depicted at the bottom of each panel. Transfections with increasing amounts of AaBR-Z1 are indicated as "+" (100 ng) and "++" (300 ng). After transfection, cells were cultured in medium with or without 1×10^{-6} M of 20E. Data represent the ratios of *firefly* luciferase to *Renilla* luciferase activity (relative luciferase activity). Values are means of triplicate independent transfection experiments, with error bars representing the S.D. of the mean.
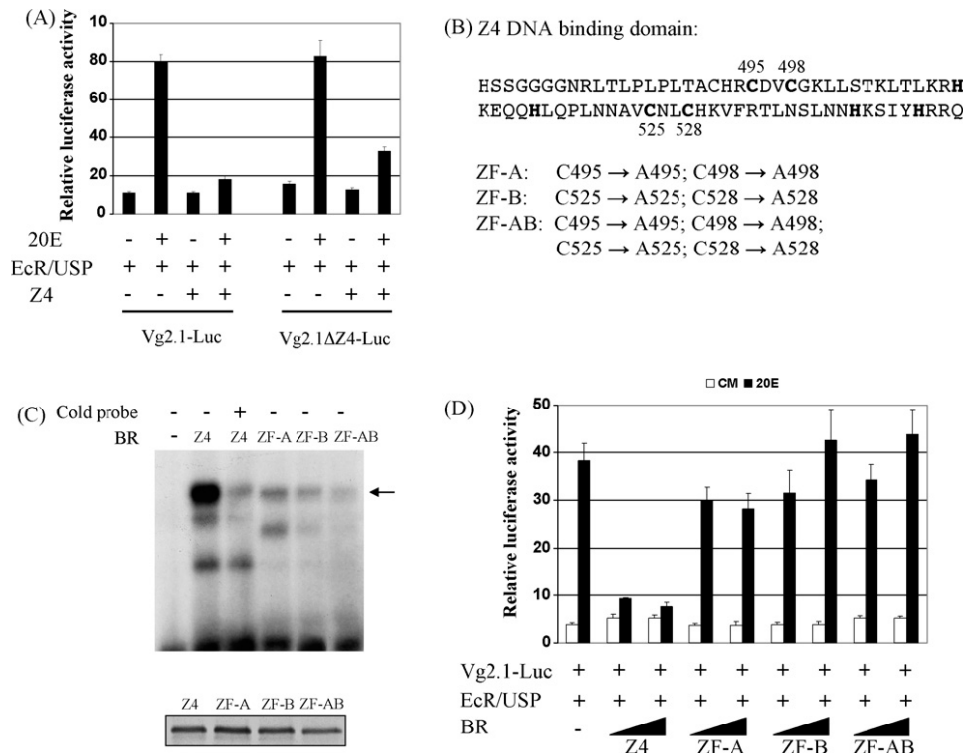
Fig 5.
Regulation of the *Vg* promoter requires intact DNA binding domains of BR. (A) *Drosophila* L57 cells were co-transfected with expression vectors for AaEcR-B, AaUSP-B, AaBR-Z4, and indicated reporter plasmids. In pVg2.1ΔZ4-Luc, all seven identified potential Z4 binding sites were removed by point mutagenesis. After transfection, cells were cultured in medium with or without 1×10^{-6} M of 20E. Data represent the ratios of *firefly* luciferase to *Renilla* luciferase activity (relative luciferase activity). (B) Point mutations were introduced into the Z4 DNA binding domain. The cystidines targeted in the zinc fingers are highlighted in the amino acid sequence. (C) EMSA was performed using ^{32}P-labeled oligonucleotide containing consensus *Drosophila* BR-Z4 binding site and *in vitro*-synthesized mosquito Z4 and its mutants. A 100-fold molar excess of unlabeled probe was added as specific competitor. When the Z4 proteins were synthesized, an *in vitro* coupled transcription and translation (TNT) reaction was performed in parallel in the presence of ^{35}S-methionine. The autoradiograph of the TNT products is shown at the bottom, indicating that comparable amounts of individual Z4 proteins were added in the EMSA experiment. (D) *Drosophila* L57 cells were co-transfected with pVg2.1-Luc reporter, expression vectors for AaEcR-B and AaUSP-B, and indicated AaBR-Z4 constructs. The solid triangle represents increasing amounts of the Z4 expression plasmids.
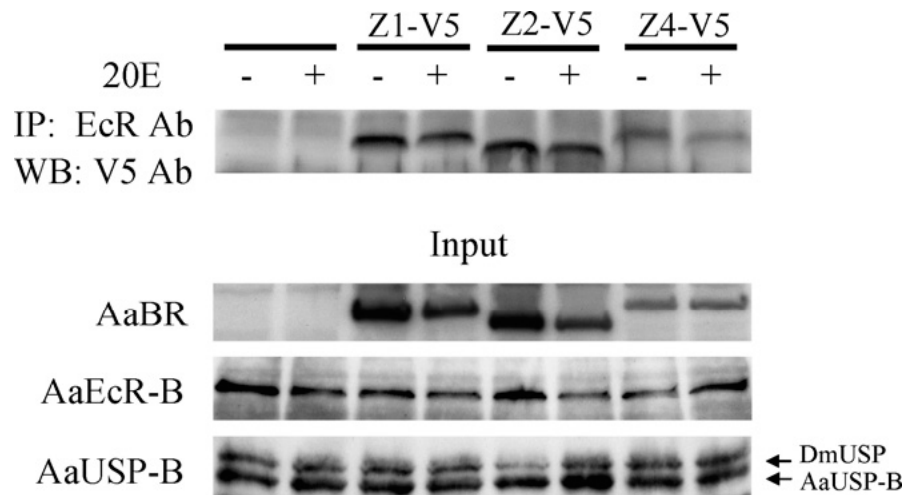
Fig 6.
BR is physically associated with EcR/USP *in vitro*. L57 cells were co-transfected with expression vectors for AaEcR-B, AaUSP-B and individual BR-V5 fusion proteins. Cell extracts were incubated with antibodies against EcR, and the precipitated proteins were subjected to Western-blot analysis with V5 antibody. At the bottom, AaEcR-B, AaUSP-B and AaBR proteins in the inputs were examined by mean of immunoblotting using antibodies against AaEcR, DmUSP and V5 tag, respectively. When the experiment was conducted using 20E, 1×10^{-6} M 20E was added into the binding and washing buffers.