The early gene *Broad* is involved in the ecdysteroid hierarchy governing vitellogenesis of the mosquito *Aedes aegypti*

L Chen, J Zhu, G Sun and A S Raikhel

Department of Entomology and Institute for Integrative Genome Biology, University of California, Riverside, California 92521, USA

(Requests for offprints should be addressed to A S Raikhel; Email: alexander.raikhel@ucr.edu)

Abstract

The *broad* (*br*) gene, encoding a family of C2H2 type zinc-finger DNA-binding proteins, has been shown to act as a crucial member of the 20-hydroxyecdysone (20E) regulatory hierarchy in the fruitfly, *Drosophila melanogaster* and the moth, *Manduca sexta*. In this study, we have shown that the *br* gene is involved in the 20E-regulatory hierarchy controlling vitellogenesis in the mosquito, *Aedes aegypti*. Unlike *E74* and *E75* early genes, expression of *br* was activated in previtellogenic females, during a juvenile hormone (JH)-dependent period. The levels of Z1, Z2 and Z4 isoform mRNA were elevated in the fat body of 2-day-old females after *in vitro* exposure to JH III. However, JH III repressed 20E activation of *br* in 3- to 5-day-old females, indicating a switch in hormonal commitment. Expression of Z1, Z2 and Z4 was stimulated after blood feeding in both vitellogenic tissues, the fat body and the ovary, corresponding to peaks of ecdysteroid titers. In the fat body, the mRNA profiles of these three isoforms correlated well with those of yolk protein precursor (*YPP*) genes. These BR isoforms were activated by 20E in fat bodies cultured *in vitro* and behaved as early genes, with a self-repressive autoregulatory loop that can be blocked by the protein inhibitor, cyclohexamide. Multiple binding sites for all four BR isoforms were present in the 5' -regulatory region of the major *YPP* gene, *vitellogenin* (*Vg*). Effects of BR isoforms on the expression of *Vg* have been demonstrated by cell transfection analysis. In particular, BR isoforms by themselves had no effects on the *Vg* promoter. However, Z1 and Z4 each repressed *Aedes aegypti* ec dysone receptor (EcR)/*Ultraspiracle* (USP)-mediated 20E activation of the *Vg* promoter, while Z2 enhanced activation of the *Vg* promoter by AaEcR/AaUSP in the presence of 20E. Z3 had no obvious effect in the same experiment. These results suggested that BR isoforms are essential for proper activation and termination of the *Vg* gene in response to 20E. Overall, our study implicated *br* in the regulation of mosquito vitellogenesis.


Introduction

In anautogenous mosquitoes, egg maturation requires a blood meal. As a consequence, mosquitoes are vectors of numerous devastating human diseases. Vitellogenesis is the key process in egg maturation, which involves production of enormous amounts of yolk protein precursors (*YPPs*) by the female fat body, an insect metabolic tissue. This process is mainly governed by two insect hormones: juvenile hormone (JH) III and 20-hydroxyecdysone (20E). Induction of the synthesis of yolk protein precursors in the fat body requires 20E, while JH III prepares newly emerged mosquitoes to accommodate the intense physiological demands of vitellogenesis, and to become competent to respond to signals associated with blood feeding, including 20E (Flanagan & Hagedorn 1977, Shapiro & Hagedorn 1982, Hagedorn 1985). In keeping with the majority practice among researchers in the field, we use ecdysone as the generic term for steroidal insect molting hormones, reserving the term ecdysone for the specific chemical compound $2\beta,3\beta,14\alpha$, etc.
22R,25-pentahydroxy-5β-cholesterol-7-en-6-one, originally known as α-ecdysone. The abbreviation 20E is used to refer to 20-hydroxyecdysone (2β,3β,14α,20R,22R,25-pentahydroxy-5β-cholesterol-7-en-6-one), the highly active ecdysone metabolite believed to serve as the active hormone in most well-characterized responses.

Vitellogenesis is initiated only after a female mosquito ingests vertebrate blood (Hagedorn 1989, Raikhel 1992, Dhadiella & Raikhel 1994). The massive yolk protein synthesis peaks around 24 h post blood meal (24 h PBM), then declines, terminating by 30–36 h PBM. Blood feeding triggers a hormonal cascade, leading to the elevation of 20E, which is secreted by activated ovaries, under the control of egg development neurosecretory hormone (Shapiro & Hagedorn 1982). The changing profile of 20E titers in female mosquitoes is tightly correlated with the expression of YPP genes, rising with yolk protein production and falling when vitellogenesis ceases (Hagedorn 1983, 1985, Raikhel 1992, Dhadiella & Raikhel 1994). The genes encoding three YPPs – vitello-genin (Vg), vitellogenic carboxypeptidase (VCP) and vitellogenic cathepsin B (VCB) – are activated in fat bodies cultured in vitro upon addition of physiological doses of 20E, which suggests that these three genes are regulated by this hormone (Cho & Raikhel 1992, Cho et al. 1999, Deitsch et al. 1995).

The molecular mechanism of 20E action has been dissected in detail during Drosophila and Manduca metamorphosis. In insects, the steroid hormone 20E functions by signaling through a heterodimer of nuclear receptors, consisting of the ecdysone receptor (EcR) and Ultraspiracle (USP). USP is an insect homologue of the mammalian retinoid X receptor (Yao et al. 1992). EcR/USP acts as a ligand-dependent transcription factor which regulates the activity of hormone responsive early genes, including E74, E75 and broad (br). In turn, the expression of these early genes is necessary for the induction of a large number of late genes and repression of the early genes themselves (Ashburner 1972, Burtis et al. 1990, Segraves & Hogness 1990, Cherbas et al. 1991, DiBello et al. 1991, Guay & Guild 1991, Koelle et al. 1991, Yao et al. 1992, 1993, Fletcher & Thummel 1995b).

The br gene has been identified as a key gene required for insect molting, metamorphosis and oogenesis. In both Drosophila and Manduca, br induction only occurs in the final larval instar. BR protein activates the pupal program and suppresses both the larval and adult programs. JH application at the onset of the adult molt causes re-expression of br and the formation of a second pupal cuticle, suggesting that BR is sufficient to mediate the status quo action of JH (Zhou & Riddiford 2002). Similarly, RNAi knock-down of br in the silkworm Bombyx mori results in the failure of animals to complete the larval–pupal transition or in later morphogenetic defects (Uhlbrova et al. 2003). The br gene is expressed in a dynamic pattern during oogenesis. It is activated by ecdysteroid in follicle cells at stage 6 of oogenesis, where it is essential for the control of endoreplication and chorion gene amplification. Later, br is also involved in cell migration, as well as morphogenesis of chorionic appendages (Tzolovsky et al. 1999).

Br encodes a family of four classes of protein isoforms (Z1–Z4), which share a common amino-terminal core domain, alternatively spliced to four distinct carboxy-terminal domains bearing pairs of zinc-finger DNA-binding domains (DiBello et al. 1991, Bayer et al. 1996). The common core region contains a highly conserved 120 amino acids, called the BTB or POZ domain; this appears to be involved in protein–protein interaction (Bardwell & Treisman 1994, Zollman et al. 1994). The br gene is defined by mutations that comprise three classical complementation groups, br, reduced bristles on palpus (rhp) and 2Bc, as well as a non-complementing nonpupariating (npr) class (Kiss et al. 1988). The BR isoforms are critical mediators of the ecdysteroid hierarchy because they are required in the regulation of intermolt, early and late gene activities in Drosophila (Belyaeva et al. 1980, Karim et al. 1993). The npr mutant lacks all of the functional BR isoforms, and larvae are viable and initiate wandering behavior at the end of the third instar, but die without pupariating (Stewart et al. 1972, Kiss et al. 1978). Alleles that belong to the br, rhp and 2Bc complementing functions result in lethality somewhat later in development, with severe morphological defects (Belyaeva et al. 1980, Kiss et al. 1988, Restifo & Merrill 1994). Mutations that reduce or eliminate br function disrupt the expression of all classes of 20E-responsive genes (Belyaeva et al. 1981, Lepesant et al. 1986, Guay & Guild 1991, Nelson et al. 1991, Karim et al. 1993).
The ecdysteroid-triggered regulatory hierarchy during mosquito vitellogenesis is reminiscent of the one utilized in *Drosophila* metamorphosis. However, the individual participants in the hierarchy are uniquely utilized to respond to the biological needs of the reproducing insects (Zhu et al. 2003a,b). DNA-binding sites of EcR/USP, E74 and E75 have all been identified in the 5′-regulatory region of the Vg gene (Kokoza et al. 2001). Both genetic studies of the Vg gene utilizing *Aedes* transformation and cell transfection assays have indicated that EcR/USP is required for initiation of stage-specific, 20E-mediated, Vg gene expression (Martin et al. 2001). Moreover, chromatin immuno-precipitation assays have demonstrated that the Vg promoter was loaded with EcR/USP heterodimer in vivo shortly after blood feeding (Zhu et al. 2003a). E74 and E75 are induced by the elevation of 20E titers after blood feeding in the fat body, and their protein products are essential for the high level expression of the Vg gene in response to 20E during vitellogenesis (Pierceall et al. 1999, Kokoza et al. 2001, Sun et al. 2002, 2004). Therefore, the Vg gene is the target of direct and indirect regulation by 20E (Raikhel et al. 2003).

Sequence analysis of the *A. aegypti* Vg regulatory region also revealed the presence of putative binding sites of BR, prompting us to isolate and characterize the mosquito homologue of BR. Here, we report the cloning of four mosquito BR isoforms (*Aedes aegypti* (Aa) BR Z1–Z4). Transcripts of AaBR increase in the fat body and ovary following the blood meal. *In vitro* culture experiments demonstrated that br expression was induced by 20E in the fat body. In cell transfection assays, AaBR isoforms showed distinct AaEcR/AaUSP-mediated effects on the Vg promoter activity: AaBR Z2 as an activator and AaBR Z1 and AaBR Z4 as repressors. These findings suggest that AaBR is involved in mediating the 20E response in the fat body and ovaries during mosquito vitellogenesis.

**Materials and Methods**

**Animals**

Mosquitoes, *A. aegypti*, were raised as described by Hays & Raikhel (1990). Larvae were fed a standard diet (Lea 1964) and adults were fed on 10% sucrose solution continuously by wick. Adult females, 3–5 days after eclosion, were fed on anesthetized white rats to initiate vitellogenesis. All dissections were performed in *Aedes* physiological saline (Hagedorn et al. 1977) at room temperature.

**RNA extraction, RT and real-time PCR**

Dissected fat bodies were homogenized with a motor-driven pellet pestle mixer (Kontes, Vineland, New Jersey, USA) and lysed by Trizol reagent (Invitrogen). RNA was isolated following the manufacturer’s protocol. Contaminating genomic DNA was removed by treatment with RNase-free DNase I (Invitrogen). RT was carried out using an Omniscript reverse transcriptase kit (Qiagen) in a 20 µl reaction mixture, containing random primers and 1 µg total RNA at 37 °C for 1 h. Two microliters of cDNA from this reaction were subjected to PCR reaction by using *AaBR* isoform-specific primers (30 cycles of 95 °C for 30s, 56 °C for 30s, 68 °C for 60s). PCR products were separated on a 1% agarose gel and confirmed by Southern analysis.

Real-time PCR was performed using the iCycler iQ system (Bio-Rad, Hercules, California, USA), as previously reported (Zhu et al. 2003b). Reactions were performed in 96-well plates with a Quantitect SYBR PCR kit (Qiagen). In order to quantify relative gene expression, standard curves were generated, using 10-fold serial dilutions of cDNA pools containing high concentrations of the gene of interest. The protocol used for amplifying the cDNA product was 40 cycles of 95 °C for 30s, 59 °C for 45 s, followed by melting curve analysis to detect specific product amplification. Samples from three individual organ culture experiments were used for real-time PCR. Each sample was analyzed in triplicate and normalized to the internal control, β-actin mRNA. Real-time data were collected by the Icycler IQ Real Time Detection System Software V.3.0 for Windows. Raw data were exported to Excel (Microsoft, Seattle, WN, USA) for analysis. Real-time PCR primers: Z1 forward primer: TTCAAACGTACATAGGCCC; Z1 reverse primer: CGGCTTCATGTGTCGATGAT AG; Z2 forward primer: CGACATATCGCCTGA TAAACACGC; Z2 reverse primer: CCGGGACG GGACCTATAGTACG; Z3 forward primer: GCCGCAAGAATGTCCCTACTGTC; Z3 reverse primer: TATACCGGGGTGGCAGAATG.
TC; Z4 forward primer: TTAACCACTCCA GCGGCAGG; Z4 reverse primer: GGTTGCAGG TGCTGTGCTGC.

Cloning and sequencing of cDNAs

Drosophila BR shares significant sequence similarity with its counterparts in Manduca at BTB domains and C-terminal zinc-finger regions (Zhou et al. 1998). This allowed us to isolate mosquito homologues of BR by utilizing a PCR-based strategy. First, a pair of degenerate primers (Pd) was designed based on highly conserved regions in the BTB domain of BR: Pd forward, 5'-SIGGGIAA RCAYTAAGGCARTTYACITG-3'; Pd reverse, 5'-CKICAITAIGCATYTGRTTICKRTGRTG-3' (Zollman et al. 1994). RT-PCR with the fat body total RNA from the female adult at 3–24 h PBM yielded a single 260 bp product, the putative amino acid sequence of which shared 97% identity with BTB domains of Drosophila BR and 100% identity with BTB domain of Anopheles gambiae (Fig. 1A). The sequence of BR BTB domain of Anopheles gambiae was obtained from the National Center for Biotechnology Information (NCBI). Next, degenerate primers corresponding to the isoform-specific zinc-finger regions were designed based on alignment of the C-terminal regions of BR from Drosophila and Manduca (Bayer et al. 1996, Zhou et al. 1998). These were then used in RT-PCR, in combination with a primer derived from the BTB-domain coding sequence. Four 1·6 kb different fragments were obtained, and DNA sequence analyses predicted that they encoded four different C2H2 zinc-finger domains homologous to those of Z1 (96%), Z2 (96%), Z3 (100%) and Z4 (98%) of Drosophila (Fig. 1B). Those sequences also showed high percentage identity to the zinc-finger domains of BR from Anopheles gambiae and Manduca (Fig. 1B). After that, the 5' and 3' ends of individual cDNA clones were determined, using 5'- and 3'-rapid amplification of cDNA ends PCR. The 5'- and 3'-untranslated regions are isoform specific. Finally, cDNA fragments containing the entire coding regions of mosquito BR isoforms were obtained by RT-PCR. The sequence data of four mosquito BR isoforms have been submitted to the DDBJ/EMBL/GenBank database under accession numbers AY499537–AY499540.

DNA sequences were analyzed by the SeqWeb (San Diego, California, USA) and BLAST NCBI, NIH, Bethesda, MD, USA). AaBR sequence alignments were performed using the ‘Multiple Sequence Alignments’ program from the website, http://www.justbio.com.

Electrophoretic mobility shift assays (EMSA)

EMSA were carried out as described previously (Wang et al. 1998). Drosophila BR consensus binding sequences were used to determine the DNA-binding activities of the AaBR isoforms. The consensus sequences that we used were TAATAACAAAA (Z1), TTTATCATTT (Z2), TAAAATCAA (Z3) and ATAAACA (Z4) (von Kalm et al. 1994). Complementary oligonucleotides were annealed together, and end-labeled by T4 polynucleotide kinase using [γ-32P]ATP (Perkin Elmer, Boston, Massachusetts, USA). The AaBR proteins were synthesized in vitro with the TNT system (Promega). The gel shift binding buffer (5 x) was purchased from Promega.

In vitro fat body culture

20-E and JH III (Sigma) were dissolved in ethanol and acetone respectively. A medium containing JH III was prepared as described by Riddiford et al. (1979), and containers and culture plates were coated with Sigmacote (Sigma). Abdominal walls with adhering fat bodies (hereafter referred to as fat bodies), which were from previtellogenic female mosquitoes (2 days and 4 days after eclosion), were incubated in an organ culture system as previously described (Deitsch et al. 1995). The dissected fat bodies were treated with 20E (2 x 10^-6 M in ethanol), JH III (1 x 10^-6 M in acetone), and cycloheximide (Calbiochem, San Diego, California, USA; 1 x 10^-5 M in water) (a protein synthesis inhibitor) for 4–16 h, and three groups of nine fat bodies were collected at 4-h intervals. As a control, fat bodies were incubated with 0·1% ethanol or acetone in the culture medium.

Cell culture and transient transfection assay in the Drosophila L57–3–11 cell line

The coding region sequences of the four BR isoforms were inserted into the pAcE3·1/V5/HisA (Invitrogen) vector and fused to a V5 and a His tag. Vg 2·1 kb promoter was inserted into pGL3/firefly luciferase vector (Promega) to form the reporter
Figure 1  Alignment of predicted amino acid sequences of *Aedes aegypti* (Aa), *Anopheles gambiae* (Ag), *Drosophila melanogaster* (Dm) and *Manduca sexta* (Ms) BR. (A) Alignment of the BTB domains. (B) Alignment of the zinc-finger domains. The conserved Cys and His residues in the zinc-finger domains are highlighted in bold. (C) Schematic representation of sequence identity of structural domains between AaBR and DmBR. The predicted amino acid (aa) sequences of *Aedes aegypti* BR were compared with the GenBank database using a BLAST search. GenBank accession numbers for the cDNA sequences of Z1, Z2, Z3 and Z4 are AY499537, AY499538, AY499539 and AY499540 respectively. *, identical residues; :, conserved substitutions; ., semi-conserved substitutions.
construct pVg2·1–luc. The construction of plasmids pAc5-AaEcR and pAc5-AaUSP has been described elsewhere (Wang et al. 1998).

The Drosophila L57–3–11 cell line (a kind gift from Dr Lucy Cherbas, Indiana University, USA) was maintained at 26–28 °C in Schneider Drosophila medium and supplemented with 5% fetal bovine serum. L57–3–11 is an EcR-deficient clone, derived from the Kc167 cell line by parahomologous targeting (Hu et al. 2003). Transfection was conducted using CellFECTIN (Invitrogen) with an optimal DNA–lipid ratio of 1:4 (w/v), following the manufacturer’s instructions. Typically, increasing amounts of BR (100, 300 and 900 ng), 100 ng pVg2·1–luc, 100 ng pRLCMV/renilla luciferase (Promega) and CellFECTIN were mixed in a 24-well plate with a total volume of 250 µl serum-free medium, and incubated at room temperature for 20 min. The expression vector pAc5·1/V5/HisA was used as carrier DNA so that each well received an equal amount of total DNA. Renilla luciferase served as an internal control for transfection efficiency. The cells transfected with empty expression vectors pAc5·1/V5/HisA were used as a negative control. The transfection cocktail was added to L57–3–11 cells for 6 h at 27 °C. Transfection mixtures were then removed, replaced with fresh growth media, and allowed to incubate with or without ecdysteroid application (2×10^{-6}M). After 36–48 h of incubation, the medium was aspirated and cells were lysed in 100 µl passive lysis buffer (Promega). Dual luciferase activities were measured by Lumimark (Bio-Rad). The relative luciferase activity was obtained by normalization of the firefly luciferase activity against renilla luciferase activity.

Western blot analyses were carried out with a fraction of the L57–3–11 cells transiently transfected as described above. Twenty microliters of each sample were separated by SDS-PAGE and tested independently with antibodies against AaEcR, AaUSP and V5 (Invitrogen).

Results
Cloning Aedes aegypti BR isoforms

The four isoforms Z1, Z2, Z3 and Z4 were cloned from Aedes aegypti, utilizing a PCR-based strategy. These isoforms share an N-terminal core region of 459 amino acids, except that isoforms Z2 and Z3 contain seven more amino acids at position 272 (Fig. 1C). The regions between the N-terminal BTB domain and the C-terminal zinc-finger domain were divergent among the four isoforms and showed no similarity to D. melanogaster, A. gambiae and M. sexta BR. Among the isoforms, the Z1 and Z4 zinc-finger domains showed a high degree of homology. This suggests that they diverged most recently and may have similar functions. The Z1, Z2, Z3 and Z4 proteins have a predicted molecular mass of 67 kDa (561 amino acids (aa)), 64 kDa (530 aa), 73 kDa (607 aa) and 73 kDa (607 aa) respectively. The conceptual translation of AaBR isoforms was generally supported by in vitro-coupled transcription and translation assay (Fig. 2).

Expression of br in the fat body and ovary of Aedes aegypti females

In order to examine temporal and spatial expression patterns of the BR isoforms, RT-PCR
experiments were first performed with total RNAs isolated from mosquitoes at different developmental stages. Relatively abundant transcripts of Z1, Z3 and Z4 isoforms were found in early 4th instar larvae (12–24 h post 3rd molt), early pupae (12–24 h post pupation), adult male (4 days post eclosion), unfed (PV) female mosquitoes (4 days post eclosion) and blood-fed (V) adult female mosquitoes (24 h PBM), and RT-PCR was performed. Isoform-specific probes were used to detect AaBR expression by Southern blot. Actin RNA level is shown as a control.

To determine the expression profiles of BR isoforms in detail during vitellogenesis, real-time PCR was applied to measure the mRNA levels of the BR isoforms. In the fat body, Z1 transcripts showed a peak at the previtellogenic 84–96 h period (Fig. 4A). After a blood meal, Z1 transcripts gradually increased again and reached a second peak at 24 h PBM. Z4 mRNA displayed a similar profile to the Z1 transcript; it was, however, considerably more abundant than Z1 mRNA and had a larger peak at 24 h PBM. The Z2 transcript level also increased during the previtellogenic period; however, it exhibited a sharp rise after the onset of vitellogenesis and reached an early peak at 8 h PBM and the level of Z2 mRNA declined after that and rose again at 24 h. Z3 transcript in the fat body remained at a relatively low level throughout the vitellogenic period, having two small peaks of expression during the previtellogenic period (84–96 h) and at 24 h PBM. The expression of BR isoforms in the fat body returned to basal levels by 36–40 h PBM (Fig. 4A).

In the ovary, expression of Z1 and Z4 isoforms was augmented after blood feeding (Fig. 4B). mRNAs of both isoforms were abundant between 6 and 24 h PBM, reaching a broad peak between 16 and 24 h PBM, then gradually declining and completely disappearing by 36 h PBM. Z2 expression reached a peak by 6 h PBM and then declined to the background level by 36 h PBM. Interestingly, Z3 transcript was most abundant in ovaries of newly emerged mosquitoes, vanishing by 24 h post eclosion (Fig. 4B).

Transcripts of BR isoforms were examined by Northern blot analysis. Total RNA derived from mosquitoes at 24 h PBM was subjected to Northern blot analysis by using probes corresponding to the isoform-specific region of BR. Results showed that Z1 and Z4 isoforms were each represented by only one transcript, while Z2 and Z3 each had two transcripts, of unequal size. All transcripts were of different size, and each was larger than 9·5 kb (Fig. 5).

**Hormonal responses of mosquito br gene in mosquito fat body culture**

In the mosquito fat body, early peaks of BR transcript levels occurred during the previtellogenic period, which is under the control of JH III (Zhu et al. 2003b). We tested whether the br gene is directly responsive to this hormone, using fat body culture. We cultured fat bodies of female mosquitoes 2 days after eclosion, which corresponded to the beginning of the previtellogenic rise in BR transcript levels, in the presence of JH III (1×10^{-6} M) or with solvent (acetone) alone. We examined the responsiveness of br to JH III in fat bodies cultured for 4 and 8 h. Total RNA was isolated from these samples and transcripts of BR isoforms were measured using real-time PCR. Figure 6 shows that after 8 h of incubation in the presence of JH III, transcript levels of Z1, Z2
and Z4 were considerably elevated. Z3 was not responsive to JH III in this experiment (not shown). Thus, the br gene is responsive to JH III during the JH-dependent previtellogenic stage of mosquito fat body maturation.

The increase in BR isoform transcripts in both the fat body and ovaries after a blood meal suggested that br was induced by elevated ecdysteroid titers. We therefore examined the effect of 20E on the expression of BR isoforms utilizing an in vitro fat body culture system. Fat bodies were dissected from 3- to 4-day-old previtellogenic female mosquitoes, which are fully competent for 20E response, and cultured in medium with different concentrations of 20E for 4 h. Total RNA was isolated from these samples and transcripts of BR isoforms were measured by real-time PCR. The analyses revealed that the mRNA levels of all four isoforms were stimulated by 20E in a dose-dependent manner (Fig. 7). The level of BR Z4 was dramatically increased, while lower levels were observed for transcripts for Z1, Z2 and Z3 isoforms. The BR transcripts reached their maximal expression with 10⁻⁷ M 20E.

Next, we examined the effect of 20E (1×10⁻⁶ M) on the expression of BR isoforms during 16 h of culturing. Vg gene expression was monitored as a control for these in vitro fat body culture experiments. The Vg mRNA level increased in the presence of 20E, but declined if 20E
was withdrawn at 4 h of incubation (Fig. 8A). Thus, the Vg gene required the continuous presence of 20E for its expression. In contrast, in fat bodies cultured in the constant presence of 20E levels, all four BR isoform transcripts increased during the first 4 h but then declined to a basal level (Fig. 8A). However, if 20E was withdrawn from the medium after the first 4 h of incubation, transcripts of all BR isoforms increased again. We found that mRNA levels of all four isoforms were greatly augmented by CHX alone at 4 h after the start of incubation (Fig. 8B). A similar phenomenon has been described in the Drosophila imaginal discs in vitro culture experiment, in which the br gene is repressed in the absence of ecdysteroid, and that repression requires protein synthesis (Bayer et al. 1996). When mosquito fat bodies were treated with both 20E and CHX, Z1 and Z4 mRNA increased over time to levels higher than in those treated with either 20E or CHX alone. The level of Z2 transcript was elevated after 8 h of incubation with 20E and CHX, but decreased with longer incubations. On the other hand, Z3 mRNA levels decreased to a basal level after 8 h of incubation with either CHX alone or both 20E and CHX. These results indicated that these isoforms were under distinct regulatory mechanisms. However, despite these differences the expression of br isoform transcripts in the mosquito fat body is under the control of a 20E negative feedback control, characteristic of 20E-regulated early response genes.

We also tested whether or not responsiveness to JH III was altered in the fat bodies which have already achieved competence for 20E responsiveness. We cultured fat bodies dissected from 3- to 4-day-old previtellogenic female mosquitoes. When JH III alone (1×10^{-6} M) was added to the culture medium, transcription of BR isoforms was not elevated above the background levels after 16 h of incubation (Fig. 8C). However, if JH III and 20E were added together, JH prevented the rise in

**Figure 4** Developmental profiles of AaBR in the fat body and ovary during vitellogenesis. Real-time PCR was performed to quantify mRNA levels of four BR isoforms in the fat body and ovary during vitellogenesis. Total RNA (0.05 µg) of each sample was used for real-time PCR. The results are means of independent triplicate samples±S.D., normalized against actin expression. (A) Developmental profiles of AaBR and Vg mRNA in the fat body during vitellogenesis. (B) Developmental profiles of AaBR mRNA in the ovary during vitellogenesis.
20E-induced expression of each of the BR isoforms that occurred at 4 h after the start of incubation. At later time-points of incubation, there was no apparent inhibitory effect of JH on \( br \) expression. This was in contrast to the effect of JH and 20E on \( Vg \) expression, where JH prevents the increase for at least 16 h (Fig. 8C, top panel).

**DNA-binding sites of BR isoforms in the vitellogenin gene 5′-regulatory region**

The potential binding sites of BR isoforms in the mosquito vitellogenin gene (\( Vg \)) 5′-regulatory region, simply called here the \( Vg \) promoter, were screened with consensus DNA-binding sequences identified in \( Drosophila \) (von Kalm et al. 1994), and examined using EMSA. Double-stranded oligonucleotides containing the consensus BR isoform-binding sites were labeled with \(^{32}P\) and used as probes. They formed specific DNA–protein complexes with \textit{in vitro} translated BR isoforms, but not with the control reticulocyte lysate. Oligonucleotides containing distinct putative BR-binding sites in the \( Vg \) promoter were added to reactions as competitors. For example, two putative binding sites for isoform Z1, 1A and 1B were found in the \( Vg \) promoter. Interaction between a consensus sequence and Z1 was competitively abolished by the 100-fold excess of 1A site, but not by 1B site (Fig. 9A). In addition, 1A and 1B sites were tested by direct binding of Z1, and the results were consistent with those of the competition binding experiments (data not shown). Similar competitive
and direct binding experiments were performed with Z2, Z3 and Z4. We found two binding sites for Z2, three binding sites for Z3 and seven binding sites for Z4 in the Vg promoter (Fig. 9B, C and D). In vitro-expressed Z4 protein showed a high affinity to 4A and 4F, but a low affinity to other potential binding sites (Fig. 9D). Direct binding experiments confirmed the results of competitive binding assays for Z2, Z3 and Z4 (data not shown). Potential binding sites for BR isoforms on the 5′-regulatory region Vg gene are shown in Fig. 10. The presence of these numerous potential binding sites suggests that BR isoforms are directly involved in 20E-mediated regulation of the Vg gene during mosquito egg maturation.

Different functions of BR isoforms on Vg promoter in Drosophila L57–3–11 cells

To investigate the possible effect of BR isoforms on the Vg promoter, we used a cell transfection assay. The reporter plasmid pVg2·1–luc was transfected into L57–3–11 cells, alone, with the expression plasmids of one of the BR isoforms or with AaEcR and AaUSP. When the reporter and individual BR isoform expression vectors were transfected into cells, no significant change of luciferase activity was detected (Fig. 11A). Co-transfection of pVg2·1–luc and AaEcR/AaUSP resulted in a sevenfold increase of luciferase activity, when treated with 20E, compared with no 20E treatment (Fig. 11B lanes 3 and 4). When Z1 was co-transfected along with AaEcR/AaUSP, no significant change of luciferase was detected without 20E treatment (Fig. 11B, lane 5). However, in the presence of 20E, Z1 repressed AaEcR/AaUSP-mediated activation of the Vg promoter. Increasing the amount of BR resulted in a further decrease in EcR/USP-dependent transactivation of the reporter gene (Fig. 11B, lanes 6–8). Similar results were observed when Z4 was co-transfected with pVg2·1–luc and AaEcR/AaUSP (Fig. 11B, lanes 17–20). In contrast, when Z2 was co-transfected with pVg2·1–luc and AaEcR/AaUSP, Z2 enhanced EcR/USP-mediated activation of the reporter gene in the presence of 20E and showed no effect on the reporter gene in the absence of 20E (Fig. 11B, lanes 9–12). Z3 only marginally increased the effect of EcR/USP in the presence of 20E (Fig. 11B, lanes 13–16).

In order to demonstrate that the observed BR effects on the reporter gene were not due to alteration of AaEcR/AaUSP translation, Western blot analysis was performed. An aliquot of each sample (20µl out of 100µl) was examined with antibodies against AaEcR, AaUSP and V5 tag (Fig. 11C). All exogenous proteins were detected after transfection, and the endogenous Drosophila USP was seen as well. Importantly, levels of AaEcR and AaUSP proteins did not change significantly after co-transfection with BR plasmids. Thus, Western blot analysis indicated that BR acts as a true modulator of the ecdysteroid receptor action.

Discussion

In this study, we have shown that the br gene is involved in the 20E regulatory hierarchy controlling vitellogenesis in the mosquito A. aegypti. Our conclusion is substantiated by several lines of evidence. First, expression of Z1, Z2 and Z4 isoforms was stimulated after blood feeding in both vitellogenic tissues, the fat body and the ovary, corresponding to the peaks of ecdysteroid tilters (Hagedorn et al. 1975). In the fat body, the mRNA profiles of these three isoforms correlated well with those of YPP genes, Vg, VCP and VCB (Cho et al. 1991, 1999, Cho & Raikhel 1992). Secondly, these
BR isoforms were activated by 20E in an in vitro fat body system and behaved as early genes, having a negative autoregulatory loop, which could be blocked by the protein inhibitor CHX. Thirdly, multiple binding sites for all four BR isoforms were present in the 5' regulatory region of the Vg gene. Finally, effects of BR isoforms on Vg gene expression have been demonstrated by cell transfection analysis.

The BR sequence analyses have shown that the BTB domain and isoform-specific DNA-binding domains are highly conserved in BR from A. aegypti, A. gambiae, D. melanogaster, and M. sexta. In Drosophila, there are six different isoforms, four of which vary in the zinc-finger domains. Three isoforms have been found in Manduca (Z2, Z3 and Z4) and in Anopheles gambiae (Z1, Z2 and Z3). The BR isoforms have various C2H2 zinc-finger regions that can bind to different A/T-rich consensus sequences. The high level of variability of these A/T-rich sequences suggests that additional determinants are important for specific DNA recognition. The BTB/POZ domain that is present in all BR isoforms appears to be such a...
determinant. The crystal structure of the BTB domain of promyelocytic leukemia zinc finger was determined and revealed to form a homodimer (Ahmad et al. 1998). The domain mediates a functionally relevant dimerization in vivo, promoting strong co-operative DNA binding to multiple sites (Bardwell & Treisman 1994). The BTB/POZ domains are thus likely to play a critical role in targeting proteins to specific chromosomal loci.

The presence of the zinc-finger motifs suggests that BR action is mediated by direct sequence-specific interactions with DNA. Based on the use of recombinant Drosophila BR isoforms produced in E. coli for in vitro footprint experiments, several studies have reported a direct in vitro binding of these proteins to promoter regions of the BR-regulated Sgs4 (Von Kalm et al. 1994), L71 (Crossgrove et al. 1996), Fbp1 (Mugat et al. 2000) and the first introns of the Dopa decarboxylase (Ddc) gene (Hodgetts et al. 1995). In the salivary gland of Drosophila, both the BR-Z1 and E74A proteins contribute to L71 late gene activation and directly regulate L71–6 (Fletcher & Thummel 1995a, Urness & Thummel 1995). Because one of the E74A-binding sites at L71–6 overlaps with BR Z1-binding sites 1 and 2, it is possible that direct interactions between these proteins are necessary for L71–6 activation. Likewise, multiple putative binding sites of AeBR are found in the mosquito Vg gene, and these binding sites are spread over 2·1 kb.

**Figure 8 (B).**
of its 5’-regulatory region, with some binding sites close to those of E75 and E74 (Fig. 10). Gel shift assays confirmed that AaBR isoforms bind to these sites in vitro, suggesting that BR isoforms may function on the Vg promoter by direct binding.

In both Manduca and Drosophila, ecdysteroid induces and co-ordinates molting, and JH controls some characteristics of the molt. In both species, it is the appearance of br transcripts in the final instar that correlates with commitment from larval to pupal differentiation. These transcripts are
normally induced by 20E, and JH blocks their appearance in larval Manduca (Zhou & Riddiford 2002). In contrast, juvenoid treatment of a young pupa of Manduca or the white puparium of Drosophila results in the reappearance of BR leading to the formation of a Manduca second pupa or a Drosophila second pupal abdomen (Zhou & Riddiford 2002). In our experiments with in vitro culture of fat bodies from previtellogenic female mosquitoes, we detected different effects of JH III on the expression of br, depending on the age of females. The fat body reaches its complete 20E responsiveness by 72 h after eclosion, when 20E is capable of eliciting the highest level of activation of both early and late genes (Zhu et al. 2003b). Significantly, the br gene was responsive to JH III, which activated its expression in 2-day-old (48 h post eclosion) females. In contrast, br was not responsive to JH III alone in the fat bodies of fully competent mosquitoes (3–4 days post eclosion). In these fat bodies, however, 20E induction of br expression was blocked by JH III. Thus, br is responsive to JH activation at the previtellogenic stage when the reproductive tissues are preparing for 20E-regulated vitellogenesis. After reaching the stage of complete competence to 20E, br responsiveness to JH is switched off. Further studies should elucidate the molecular mechanism underlying this switch in br responsiveness to JH.

In the mosquito fat body, the early genes E74 and E75 are expressed only after the initiation of vitellogenesis and an increase in ecdysteroid titers (Pierceall et al. 1999, Sun et al. 2002). In contrast, levels of all BR isoform transcripts increased by the completion of the previtellogenic stage, indicating that the presence of BR is important for activation of 20E-regulated target effector genes. Our preliminary gel-shift analysis of fat body nuclear extracts indicated the presence of active BR proteins in previtellogenic females (L Chen, J Zhu & A S Raikhel, unpublished results). This situation is similar to that with EcR and USP proteins, which are also present in the previtellogenic fat body nuclei (Zhu et al. 2003b).

The PBM profile of AaBR Z2 mRNA in the fat body is reminiscent of those of E75 isoform transcripts, which exhibit early and late peaks (Pierceall et al. 1999). The early peak of expression is also characteristic for AaEcRB (Wang et al. 2002). The timing of these early peaks closely correlates with the first small peak of the ecdysteroid titer.

**Figure 9** Competitive EMSA analyses of AaBR-binding properties to various binding sites in the Vg promoter. Drosophila BR consensus DNA-binding sequences were labeled as probes, and EMSA was performed with in vitro-translated (A) AaBR Z1, (B) Z2, (C) Z3 and (D) Z4. A 100-fold molar excess of unlabeled potential AaBR DNA-binding sequences was added as specific competitors. Arrows indicate the specific retardation complexes. TNT, in vitro coupled transcription and translation.
in vitellogenic female mosquitoes, 4–6 h PBM (Hagedorn et al. 1975). In contrast, during the vitellogenic period, AaBR Z1, Z3 and Z4 transcripts have only one late peak at 24–32 h PBM that correlates with the expression profiles of AaE74B, AaUSPB and the YPP genes, Vg, VCP and VCB (Cho & Raikhel 1992, Cho et al. 1999, Wang et al. 2000, Sun et al. 2002).

Despite differences in expression profiles in vivo, mRNA transcripts of all four isoforms accumulate rapidly in the fat body in response to 20E, but decline after 4 h when tested for induction in tissue culture. The sensitivity of BR transcripts to 20E are similar to those of other early genes AaE74B and AaUSPB as well as to AaUSPB, which all reach their maximal expression at 10^{-7} M 20E (Pierceall et al. 1999, Wang et al. 2000, Sun et al. 2004). In contrast, the 20E concentration required for maximal induction of the target genes, VCP and Vg, is 10-fold higher (Deitsch et al. 1995). Interestingly, AaE74 isoform mRNAs, which also have very different expression profiles in the fat body, respond simultaneously to 20E activation in the fat body culture in vitro (Sun et al. 2002, 2004). The differences between in vivo mRNA profiles of BR and E74 isoforms and their in vitro 20E induction in organ culture are probably due to the lack of a complete set of humoral factors in the culture medium, which are present in the hemolymph of vitellogenic females. Although the identity of such additional factors remains to be determined, involvement of the CREB transcription factor in the termination of Vg gene expression strongly supports the action of a peptide hormone on the fat body during late vitellogenesis (Dittmer et al. 2003). In vivo action of such additional hormones can affect the expression patterns of individual early genes in a unique way. It is also possible that the remaining JH present after blood feeding causes the br expression delay in vivo.

In Drosophila, the br gene is crucial at several stages of oogenesis (Deng & Bownes 1997). Its expression regulates endoreplication of the polyploid follicle cells, specific amplification of the chorion genes and later positioning of the chorionic appendages. All BR isoforms are expressed in oogenesis, while Z1 is the sole isoform expressed at high levels in the appendage-producing cells (Tzolovsky et al. 1999). The early induction of br is controlled by 20E, similar to its expression during metamorphosis as an early ecdysteroid-response gene. mRNA expression of AaBR isoforms Z1, Z2 and Z4 in the ovary is induced after a blood meal and has different profiles during vitellogenesis. This suggests that AaBR may display similar functions during oogenesis in A. aegypti.

Individual BR isoforms play distinct roles during molts and metamorphosis in D. melanogaster and M.
In the imaginal discs and the abdominal epidermis of *Drosophila* during the formation of the pupal cuticle, Z1 is the predominant isoform and is needed for pupal cuticle formation (Bayer *et al.* 1996, 1997). In contrast, Z4 is predominant in the epidermis during pupal cuticle formation in *M. sexta* (Zhou *et al.* 1998, Zhou & Riddiford 2001). The *Manduca* Z4 transgene can partially substitute for *Drosophila* Z1 in the *Drosophila rbp* mutant (Bayer *et al.* 2003). Likewise, Z4 expression is predominant in the fat body and ovary of the adult *A. aegypti* females. During the molt to the final larval instar in *Manduca*, misexpression of Z1 causes the reappearance of a pupal cuticle gene *Edg78E* and suppresses a larval cuticle gene *Lcp65A-b*. Z3 and Z4 both suppress the larval cuticle gene, while Z2 activates the pupal cuticle gene. During the adult molt, misexpression of Z1 activates the pupal cuticle gene and inhibits an adult cuticle gene *Acp65A*. These data indicate that different isoforms have varying effects on these cuticle genes. It also has been shown in *Drosophila melanogaster* that different BR isoforms can have opposite regulatory functions on the same gene. For example, in regulating the *Ddc* gene at the end of the molt, Z2 functions as an activator, while either Z1 or Z4 acts as a repressor (Andres *et al.* 1993, Hodgetts *et al.* 1995).

Effects of mosquito BR isoforms on *Vg* gene expression have been tested by the cell transfection analysis. This analysis confirmed the ability of BR isoforms to alter transactivation of the *Vg* gene. Significantly, all BR isoforms have no functions by themselves on the *Vg* promoter. However, they can enhance or attenuate AaEcR/AaUSP-mediated 20E activation of the *Vg* promoter. Z1 and Z4 have very similar protein structure and expression profiles during vitellogenesis and both of them repressed AaEcR/AaUSP-mediated activation of the *Vg* promoter in the cell transfection assay. In contrast, Z2 enhanced AaEcR/AaUSP-mediated activation of the *Vg* promoter. Z3 had no obvious effect on the *Vg* promoter in cell transfection assay. Together, these results suggest that the BR isoforms are essential for proper activation and termination of the *Vg* gene in response to 20E.

**Acknowledgements**

This work was supported by a grant AI-36959 from the National Institutes of Health to A S R. We
thank Mr Andrew Hufford for editing the manuscript.

References

Ahmad KF, Engel CK & Prive GG 1998 Crystal structure of the BTB domain from PLZF. *PNAS* 95 12123–12128.


Fletcher JC & Thummel CS 1995a The *Drosophila* E74 gene is required for the proper stage-specific and tissue-specific transcription of ecdysone-regulated genes at the onset of metamorphosis. *Development* 121 1411–1421.


Zhou BH & Riddiford LM 2001 Hormonal regulation and patterning of the Broad-Complex in the epidermis and wing discs of the tobacco hornworm, Manduca sexta. Developmental Biology 231 125–137.


Zhu J, Miura K, Chen L & Raikhel AS 2003a Cyclicity of mosquito vitellogenic ecdysteroid-mediated signaling is modulated by alternative dimerization of the RXR homologue Ultraspireacle. PNAS 100 544–549.

Zhu J, Chen L & Raikhel AS 2003b Posttranscriptional control of the competence factor betaTIZ-F1 by juvenile hormone in the mosquito Aedes aegypti. PNAS 100 13338–13343.

Zollman S, Godt D, Prive GG, Couldec, JL & Laski FA 1994 The BTB domain, found primarily in zinc finger proteins, defines an evolutionarily conserved family that includes several developmentally regulated genes in Drosophila. PNAS 91 10717–10721.

Received 31 August 2004
Accepted 14 September 2004
Made available online as an Accepted Preprint 27 September 2004