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

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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* ecdysone 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.

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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 syn-

thesis 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 ecdysteroid as the generic term for steroidal insect molting hormones, reserving the term ecdysone for the specific chemical compound 2 β ,3 β ,14 α ,

22R,25-pentahydroxy-5 β -cholest-7-en-6-one, originally known as α -ecdysone. The abbreviation 20E is used to refer to 20-hydroxyecdysone (2 β ,3 β ,14 α ,20R,22R,25-pentahydroxy-5 β -cholest-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, Dhadialla & 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, Dhadialla & Raikhel 1994). The genes encoding three YPPs – vitellogenin (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, Deutsch *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 those 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 (Uhlirova *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* (*rbp*) 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*, *rbp* 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 immunoprecipitation 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 Icyler 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: TTCAAACGCTACATATGAGGCC; Z1 reverse primer: CGGCTTCATGTTTCGATGATAG; Z2 forward primer: CGACATATCGCTGATAAACACGC; Z2 reverse primer: CCTGGACGGACTTATGATACG; Z3 forward primer: GCCGCAAGAATGTCCCTACTGTC; Z3 reverse primer: TATACCGGCGGTGGCAGAAT

TC; Z4 forward primer: TTAAACCACTCCA GCGGCGG; Z4 reverse primer: GGTTGCAGG TGCTGTTGCTC.

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'-SIGGIAA RCAYTAYGGICARTTYACITG-3'; Pd reverse, 5'-CKICAITAITGICAYTGRTTICKRTGRTG-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)

EMSAs 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 TAATAAACAAAA (Z1), TTTATCATTT (Z2), TAAACTAAA (Z3) and ATAAACA (Z4) (von Kalm *et al.* 1994). Complementary oligonucleotides were annealed together, and end-labeled by T4 polynucleotide kinase using [γ - 32 P]ATP (Perkin Elmer, Boston, Massachusetts, USA). The AaBR proteins were synthesized *in vitro* with the TNT system (Promega). The gel shift binding buffer (5 $\times$) 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 (2×10^{-6} M in ethanol), JH III (1×10^{-6} M in acetone), and cycloheximide (Calbiochem, San Diego, California, USA; 1×10^{-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 pAc5.1/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

Figure 2 *In vitro* translation analysis. The cDNA sequences encoding AaBR Z1, Z2, Z3 and Z4 were translated by a coupled *in vitro* transcription–translation system and labeled by [³⁵S]methionine. Translation products were separated by SDS-PAGE using a 10% gel followed by exposure to X-ray film. Molecular weight standards are shown on the left-hand side in decreasing order.

(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

Figure 3 Tissue- and stage-specific expression of *A. aegypti* BR isoforms. Total RNAs were isolated from 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.

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 and unfed female mosquitoes, while Z2 mRNA was present to a lesser extent. In blood-fed adult female mosquitoes, expression of Z1, Z2 and Z4 in the fat body was enhanced after blood feeding (24 h PBM), while in ovary or gut, mRNA levels of BR isoforms at 24 h PBM were visibly changed as compared with those without blood feeding (Fig. 3).

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

Figure 4 (A).

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

Figure 4 (B).

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 $\pm$ 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.

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 5 Northern blot analysis of *AaBR* transcripts. Total RNA was extracted from adult mosquitoes. RNA (10 µg) was loaded in each lane. Northern blots were hybridized with *AaBR* isoform-specific probes. Lane M, RNA marker; isoform-specific probes used are shown at the top of each lane.

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

Figure 6 JH III activates *br* gene expression. Fat bodies from 2-day-old female mosquitoes were exposed to 1×10^{-6} M JH III (shaded bars) or acetone (open bars) *in vitro* for 4 or 8 h. Total RNA was then isolated from these samples. Real-time PCR was performed using isoform-specific primers for *AaBR* Z1, Z2, Z3 (not shown) and Z4. The results represent the means of independent triplicate samples $\pm$ S.D., normalized against actin expression.

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 *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

Figure 7 *AaBR* response to 20E in fat body culture. The transcription of *AaBR* in response to 20E was examined in an *in vitro* culture system. Fat bodies from previtellogenic female mosquitoes at 4 days post eclosion were incubated for 4 h at the 20E concentrations indicated. Total RNA was then isolated from these samples. Real-time PCR was performed using isoform-specific primers for *AaBR* Z1, Z2, Z3 and Z4. The results represent the means of independent triplicate samples $\pm$ S.D., normalized against actin expression.

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 (20 μ l out of 100 μ l) 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 titers (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

A

Figure 8 (A).

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

B

Figure 8 (B).

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 *AaBR* are found in the mosquito *Vg* gene, and these binding sites are spread over 2.1 kb

Figure 8 Effects of 20E, CHX and JH III on *AaBR* expression in the fat body cultured *in vitro*. Previtellogenic fat bodies dissected from female mosquitoes (4 days after eclosion) were incubated for 16 h under the different treatments indicated in the diagram. *Vg* gene expression was included as a control. (A) Effects of 20E on *AaBR* expression. CM, culture medium; 20E, medium with 2×10^{-6} M 20E; -20E, 4 h in medium with 2×10^{-6} M 20E and followed by 12 h without 20E. (B) Effects of CHX on 20E-induced *AaBR* expression. CHX, medium with 1×10^{-5} M CHX; 20E/CHX, medium with 2×10^{-6} M 20E and 1×10^{-5} M CHX. (C) Effects of JH III on 20E-induced *AaBR* expression. JH, medium with 1×10^{-6} M JH III; 20E/JH, medium with 2×10^{-6} M 20E and 1×10^{-6} M JH III. RNA was extracted from fat bodies and subjected to real-time PCR. The results show the means of independent triplicate samples $\pm$ S.D., normalized against actin expression. Each panel represents the expression of different genes, which are labeled at the top of each panel.

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

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.

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 10 Schematic illustration of the AaBR-binding sites in the *Vg* promoter. Hormonal- and tissue-responsive unit (EcR-USP, HNF-3/FKH, C/EBP-binding sites), hormonal enhancer unit (E74, E75 -binding sites), and GATA-rich region are presented in the *Vg* promoter, to show the context of the BR isoform-binding sites. HNF-3/FKH, hepatic nuclear factor-3/forkhead; C/EBP, CCAAT enhancer-binding protein, EcRE, ecdysone response element.

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 *AaE75B* 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 (Deutsch *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.*

Figure 11 BR isoforms display different functions on the *Vg* promoter in transfection assays. (A) Effect of BR alone on the *Vg* promoter. In the presence or absence of 20E, the pVg2.1-luc reporter plasmid (100 ng) was co-transfected into L57-3-11 cells with increasing amounts of Z1, Z2, Z3 and Z4. A *renilla* luciferase expression vector pRLCMV (Promega; 100 ng) was co-transfected as an internal control, and *firefly* luciferase activity was normalized to *renilla* luciferase activity (relative luciferase activity). The results represent an average of three independent experiments $\pm$ S.D. (B) BR modulates the transactivation of the *Vg* promoter by AaEcR/AaUSP. The experiment was performed as described above, except that L57-3-11 cells were co-transfected with expression vectors of BR isoforms, AaEcR (100 ng), and AaUSP (100 ng). (C) Western blot analysis with the transfected L57-3-11 cells treated with or without 20E. Antibodies against V5 tag, AaEcR and AaUSP were used in the Western blot and are labeled on the left-hand side. Transfected BR isoforms are indicated at the top of the panel.

sexta. 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 rbp1* 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.

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