The EMBO Journal Vol.19 No.2 pp.253–262, 2000

AHR38, a homolog of NGFI-B, inhibits formation of the functional ecdysteroid receptor in the mosquito *Aedes aegypti*

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In anautogenous mosquitoes, vitellogenesis, the key event in egg maturation, requires a blood meal. Consequently, mosquitoes are vectors of numerous devastating human diseases. After ingestion of blood, 20-hydroxyecdysone activates yolk protein precursor (*YPP*) genes in the metabolic tissue, the fat body. An important adaptation for anautogenicity is the previtellogenic developmental arrest (the state-of-arrest) preventing the activation of *YPP* genes in previtellogenic females prior to blood feeding. Here, we show that a retinoid X receptor homolog, Ultraspiracle (AaUSP), which is an obligatory partner in the functional ecdysteroid receptor, exists at the state-of-arrest as a heterodimer with the orphan nuclear receptor AHR38, a homolog of *Drosophila* DHR38 and nerve growth factor-induced protein B. Yeast two-hybrid and glutathione *S*-transferase pull-down assays demonstrate that AHR38 can interact strongly with AaUSP. AHR38 also disrupts binding of ecdysteroid receptor to ecdysone response elements. Cell co-transfection of AHR38 with AaEcR and AaUSP inhibits ecdysone-dependent activation of a reporter gene by the ecdysteroid receptor. Co-immunoprecipitation experiments indicate that AaUSP protein associates with AHR38 instead of AaEcR in fat body nuclei at the state-of-arrest.

Keywords: AHR38/20-hydroxyecdysone/mosquito vitellogenesis/nuclear receptor/protein–protein interaction

Introduction

Mosquito-borne diseases are among the most threatening in modern times. Malaria is particularly devastating, taking a heavy toll on the human population in many parts of the world (Collins and Paskewitz, 1995; Bruno *et al.*, 1997; Butler, 1997; Beier, 1998); therefore, there is an urgent need to explore every possible avenue for developing novel control strategies against the disease pathogens and their vectors.

Mosquitoes serve as vectors for many harmful human diseases because they require a blood feeding for their egg development. In these so-called anautogenous mosquitoes, vitellogenesis, the key event in egg maturation, is initiated only after a female mosquito ingests vertebrate blood. A blood meal triggers a hormonal cascade with 20-hydroxyecdysone (20E) as the terminal signal, which in turn activates yolk protein precursor (*YPP*) genes in the metabolic tissue, the fat body (Hagedorn, 1983, 1985; Raikhel, 1992; Dhadialla and Raikhel, 1994; Deitsch *et al.*, 1995).

In the anautogenous mosquito *Aedes aegypti*, which is being used as a model vector, vitellogenesis proceeds through two periods (Figure 1). A preparatory, previtellogenic period, including an increase in ploidy (Dittmann *et al.*, 1989) and ribosome proliferation (Raikhel and Lea, 1990), is required for the fat body to attain both the responsiveness to 20E and competence for massive yolk protein synthesis and secretion. The 3-day-long previtellogenic development of both the ovary and fat body is under the control of juvenile hormone III. Its levels are high during the previtellogenic period and fall immediately following the blood meal (Figure 1). The fat body then produces the yolk protein precursors (YPPs), which are internalized by developing oocytes (Raikhel, 1992). In addition to vitellogenin (Vg), the major YPP, the mosquito fat body synthesizes two other YPPs: vitellogenic carboxypeptidase (VCP) and vitellogenic cathepsin B (VCB) (Cho *et al.*, 1991, 1999). YPP synthesis reaches its maximum levels at 24 h post-blood meal (PBM). Vitellogenesis proceeds until 30–32 h PBM, when it is rapidly terminated (Raikhel, 1992). The hemolymph levels of ecdysteroids in mosquito females correlate with the rate of YPP synthesis in the fat body (Figures 1 and 3A). The 20E levels are only slightly elevated at 4 h PBM; however, they rise sharply at 6–8 h PBM, reaching their maximum level at 18–24 h PBM (Hagedorn *et al.*, 1975).

Consistent with the proposed role of 20E in activating mosquito vitellogenesis (Hagedorn and Fallon, 1973; Hagedorn *et al.*, 1973; Fallon *et al.*, 1974), experiments using an *in vitro* fat body culture have shown that physiological doses of 20E (10^{-7}–10^{-6} M) activate two *YPP* genes: *vg* and *vcp* (Deitsch *et al.*, 1995). Utilization of the protein synthesis inhibitor cycloheximide has demonstrated that activation of *YPP* genes in the mosquito fat body requires protein synthesis (Deitsch *et al.*, 1995). Thus, it is likely that a regulatory cascade similar to those in *Drosophila* development mediates the action of 20E in the mosquito fat body.

The functional ecdysteroid receptor is a heterodimer of the ecdysone receptor (EcR) and a retinoid X receptor (RXR) homolog, Ultraspiracle (USP) (Yao *et al.*, 1992, 1993). In *A.aegypti*, one isoform of the ecdysone receptor (AaEcR) and two USP isoforms (AaUSP-A and AaUSP-B) have been cloned (Cho *et al.*, 1995; Kapitskaya *et al.*, 1996). The mosquito EcR–USP heterodimer has been shown to bind to various ecdysone response elements (EcREs) to modulate ecdysone-responsive target genes

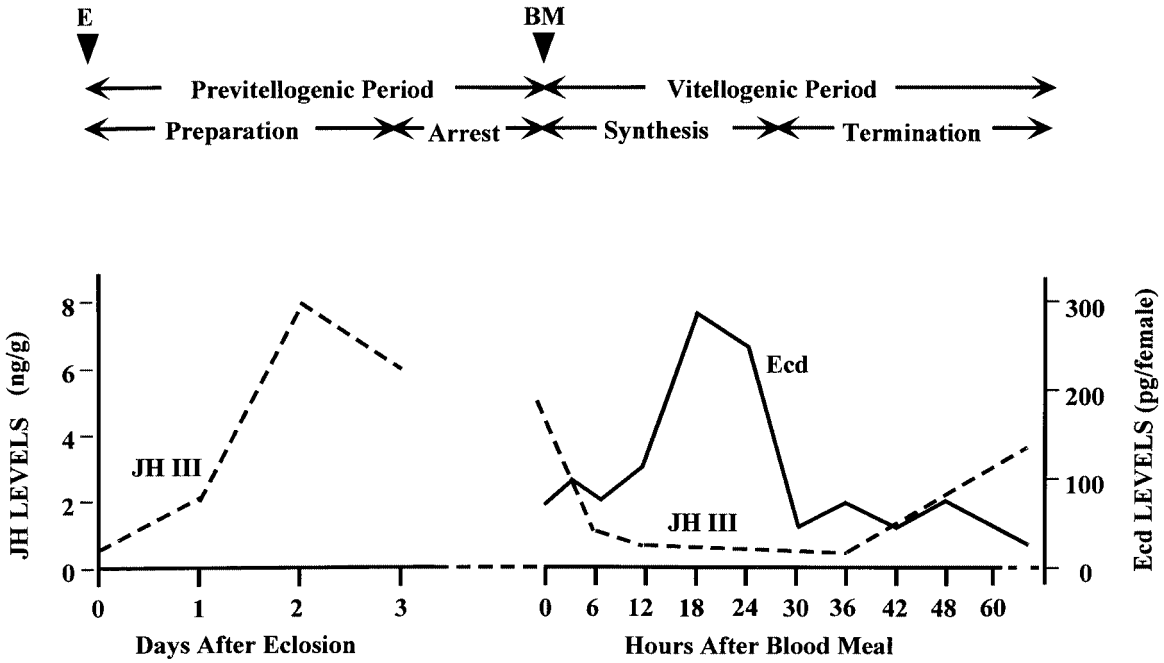

Fig. 1. Hormonal titers during the first vitellogenic cycle of the anautogenous mosquito *A.aegypti.* BM, blood meal; E, eclosion; JH III, juvenile hormone III titers (modified from Shapiro *et al.*, 1986); Ecd, ecdysteroid titers (modified from Hagedorn *et al.*, 1975).

(Wang *et al.*, 1998). More recently, we have demonstrated that a mosquito homolog of the *Drosophila* early gene *E75* is involved in mediating the ecdysone response during vitellogenesis (Pierceall *et al.*, 1999).

An important adaptation for anautogenicity is the previtellogenic developmental arrest (the state-of-arrest), which prevents the activation of *YPP* genes in previtellogenic females prior to blood feeding. In contrast to *in vitro* incubation of the previtellogenic fat body, in which the *YPP* genes are activated by physiological doses of 20E, only the injection of 20E at supra-physiological dosages could stimulate some expression of these genes *in vivo* (Raikhel, 1992; Deitsch *et al.*, 1995). Therefore, the state-of-arrest can be interpreted as the hindrance of a 20E signaling pathway that may be maintained *in vivo* by undetermined humoral factors; additionally, unidentified humoral factors secreted in response to a blood meal may play a crucial role in the release of vitellogenic tissues from the state-of-arrest. Indeed, we have demonstrated elsewhere that in *A.aegypti* both AaEcR and AaUSP proteins are abundant in nuclei of the previtellogenic female fat body during the state-of-arrest (Wang *et al.*, 2000). In contrast, the EcR–USP heterodimer capable of binding to the specific EcREs is barely detectable in these nuclei (Miura *et al.*, 1999). In this context, we have considered whether the ecdysteroid receptor could be a primary target of the 20E signaling modification in the mosquito target tissues at the state-of-arrest.

One mechanism through which the formation of ecdysteroid receptor might be regulated is by competitive binding of other factors to either EcR or USP. To investigate this possibility, we cloned a cDNA encoding a repressor of 20E signaling identified as AHR38, the *Drosophila* counterpart of which has been reported to dimerize with USP and repress ecdysone-dependent activation of a reporter gene by the ecdysteroid receptor (Sutherland *et al.*, 1995). Here, we report that AHR38 (NR4A4 according to the nomenclature proposed by Nuclear Receptors Nomenclature Committee, 1999), the mosquito homolog of DHR38 and vertebrate nerve growth factor-induced protein B (NGFI-B)/Nurr1 orphan receptors, interacts strongly with the AaUSP protein. Our data show that during the state-of-arrest factors in the fat body nuclei block the dimerization of AaEcR and AaUSP. The evidence presented here suggests that AHR38 is one of the key factors inhibiting the ecdysone response in the fat body of the anautogenous mosquito *A.aegypti* at the state-of-arrest.

Results

Isolation and characterization of the mosquito AHR38 cDNA

To isolate the cDNA encoding a mosquito homolog of DHR38 and NGFI-B, a pair of degenerate primers was designed based on highly conserved regions in the DNA-binding domain of *Drosophila* DHR38 and other NGFI-B members (Pd-F 5′-GGITGIAARGGITTYTTYAARMGI-ACIGTNC-3′, Pd-R 5′-TCIGTICKIACIACYTCYTTIA-CCATNCC-3′). RT–PCR of the fat body total RNA from the adult female at either 0–3 days post-eclosion (PE) or 24 h PBM yielded a 164 bp fragment whose translated sequence predicted a protein that shared significant homology with the DNA-binding domain of DHR38 (data not shown). A putative clone encoding the mosquito AHR38 was obtained by a combination of cDNA library screening and 5′-rapid amplification of cDNA ends (5′-RACE) PCR. These sequence data have been submitted to the DDBJ/EMBL/GenBank database under accession No. AF165528. The AHR38 mRNA extended over 2400 bp and contained

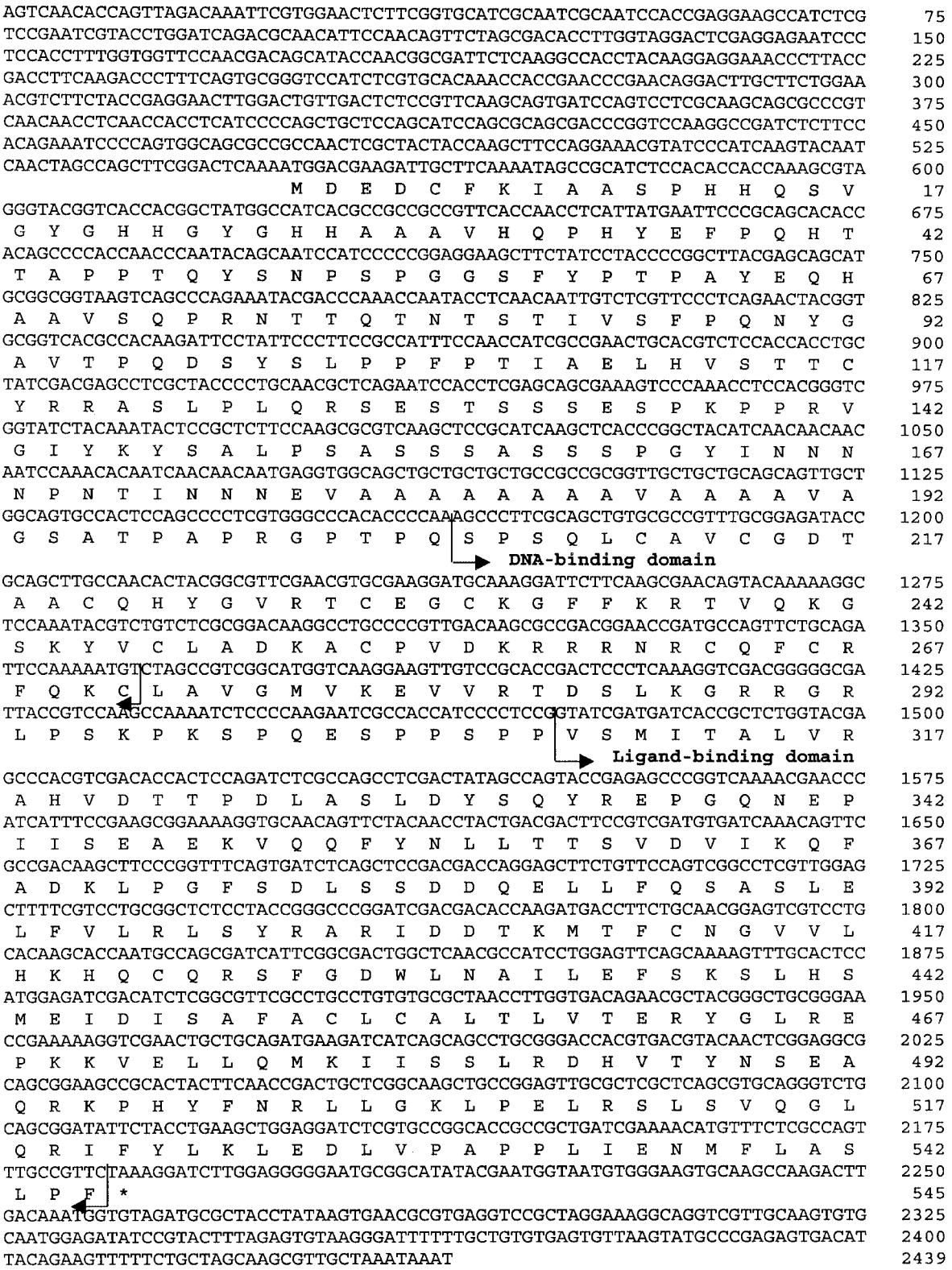

Fig. 2. Nucleotide and deduced amino acid sequences of AHR38 from the mosquito *A.aegypti*. Both nucleotides and deduced amino acids are numbered. Borders of the DNA-binding and ligand-binding domains are marked with bent arrows.

a 5′ untranslated region (UTR) of at least 550 bp, a continuous open reading frame (ORF) of 1638 bp and a 3′ UTR of at least 252 bp (Figure 2). The complete ORF encoded a protein of 545 amino acids, which exhibited a structural domain organization similar to that of DHR38. The C (DNA-binding), D (hinge) and E/F (dimerization/ligand-binding) domains were highly conserved; AHR38 shared 97, 100 and 79.7% identity with the respective domains of DHR38, and 89.4, 100 and 50.8% identity with rat NGFI-B (data not shown).

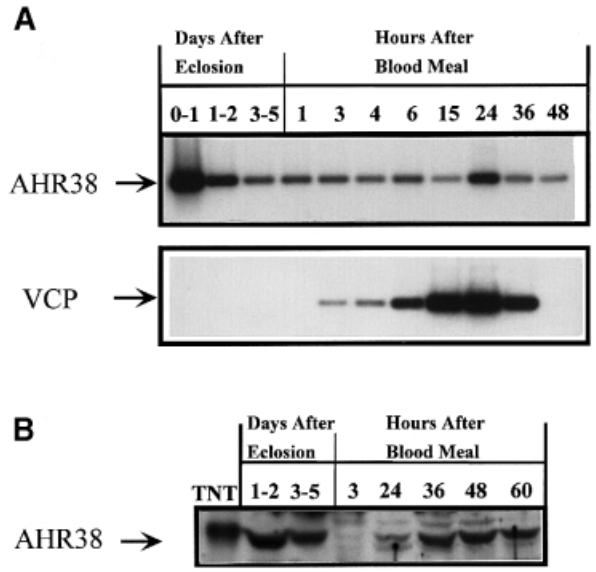

Fig. 3. AHR38 mRNA and protein during the first vitellogenic cycle in the anautogenous mosquito *A.aegypti*. (**A**) AHR38 transcript in the fat body of female adults detected by RT–PCR. RNA was isolated from fat bodies of female *A.aegypti* at different stages. The RT–PCR products were analyzed by agarose electrophoresis and Southern blot hybridization. Primers and RT–PCR conditions are described in Materials and methods. VCP, the transcript of the YPP, vitellogenic carboxypeptidase gene in the fat body. (**B**) The AHR38 protein in the fat body detected by Western blot hybridization. Nuclear extracts were prepared from the fat bodies of 250 adult females for each time point. An aliquot equivalent to 50 mosquitoes was loaded in each lane and resolved by SDS–PAGE. The AHR38 protein was detected using anti-AHR38 polyclonal antibodies. TNT represents the *in vitro* translated AHR38 protein that was used as a control in Western blot analysis.

Developmental kinetics of AHR38 mRNA transcription and protein synthesis during the first vitellogenic cycle

RT–PCR was used to evaluate the levels of AHR38 mRNA in the fat body of female mosquitoes throughout the first vitellogenic cycle. The maximum levels of AHR38 mRNA were present in the fat body of newly emerged females (Figure 3A). The mRNA levels declined during previtellogenic development, declined further following the blood feeding and showed some elevation at 24 h PBM (Figure 3A).

We then investigated the presence of the AHR38 protein in the fat bodies of female mosquitoes at different stages of the vitellogenic cycle. Polyclonal antibodies were prepared against bacterially expressed AHR38 protein. Fat body nuclei were isolated and subjected to Western blot analysis using anti-AHR38 antibodies. These analyses revealed that the levels of AHR38 protein were high in the fat body of previtellogenic females (Figure 3B). However, the levels diminished drastically after blood feeding, and increased slightly at ~24 h PBM. They were restored back to the previtellogenic levels during the post-vitellogenic stage, 36–60 h PBM (Figure 3B).

AHR38 interacts directly with AaUSP

To assess the possibility that AHR38 interacts physically with AaUSP, we used the yeast two-hybrid assay. LexA and B42 fusion constructs were prepared for AaEcR, AaUSPb and AHR38 (Figure 4A); these constructs were tested in the yeast assays. LexA fusions with AaUSPb or

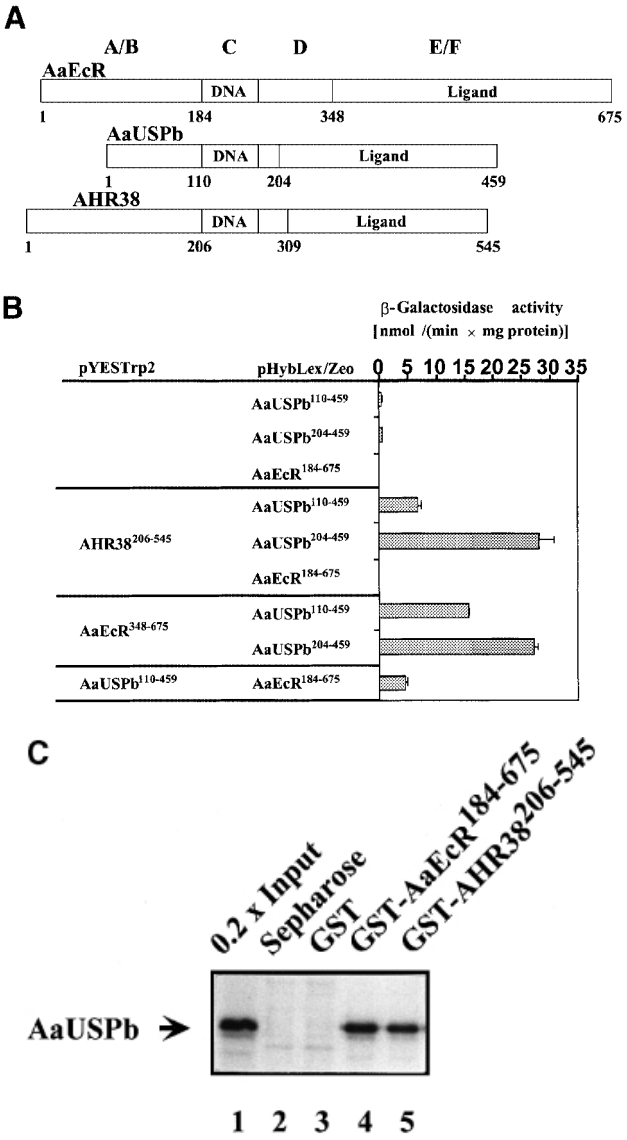

Fig. 4. Interaction of AHR38 with AaUSP. (**A**) Schematic representation of domain structure of AaEcR, AaUSPb and AHR38 used in generating fusion protein constructs for the yeast two-hybrid assays. (**B**) Yeast two-hybrid assays reveal interactions between AHR38 and AaUSP. Yeast cells were co-transformed with the indicated pHybLex/Zeo and pYESTrp2 constructs. β-galactosidase activity was measured and normalized for protein concentration. The data represent means ± SD of three experiments. (**C**) AHR38 binds directly to AaUSP. [^{35}S]methionine-labeled AaUSPb was incubated with glutathione–Sepharose beads (lane 2), beads bound with GST (lane 3), GST–AaEcR (lane 4) or GST–AHR38 (lane 5). The beads were washed in phosphate-buffered saline and collected by centrifugation. Bound proteins were eluted in SDS sample buffer, resolved by SDS–PAGE and visualized by autoradiography.

with AaEcR alone were transcriptionally inert (Figure 4B). However, co-expression of the LexA fusion to AaUSP (LexA–AaUSP$^{110–459}$) with either B42 fusion to AaEcR (B42–AaEcR$^{348–675}$) or to AHR38 (B42–AHR38$^{206–545}$) led to significant activation of the *LacZ* reporter gene, indicating that AaUSP is able to interact directly with both AaEcR and AHR38. Interestingly, utilization of the AaUSP fragment containing only the ligand/dimerization (E/F) domain (LexA–AaUSP$^{204–459}$) resulted in even higher activation of the reporter via either B42 fusion to AaEcR (B42–AaEcR$^{348–675}$) or to AHR38 (B42–

AHR38$^{206–545}$) in similar tests. Testing of the B42 fusion to AaUSPb (B42–AaUSP$^{110–459}$) showed positive interaction with AaEcR (LexA–AaEcR$^{184–675}$). In contrast, no activation of the reporter was observed when LexA–AaEcR$^{184–675}$ was tested with B42–AHR38$^{206–545}$ (Figure 4B).

This AHR38–AaUSP protein interaction was further confirmed by *in vitro* glutathione *S*-transferase (GST) pull-down assays. AaEcR and AHR38 were fused to GST (GST–AaEcR$^{184–675}$ and GST–AHR38$^{206–545}$) and expressed in *Escherichia coli*. These proteins were purified on glutathione–Sepharose beads and used as affinity matrixes to test possible interactions with *in vitro* translated ^{35}S-labeled AaUSP. Whereas no significant binding was observed with GST alone, AaUSPb was efficiently retained by GST–AaEcR$^{184–675}$ or GST–AHR38$^{206–545}$ (Figure 4C). Similar experiments carried out with ^{35}S-labeled AaUSPa showed identical results (data not shown).

Analysis of the AHR38–AaUSP heterodimer interaction with EcRE using electrophoretic mobility shift assays (EMSA)

The interaction between AHR38 and AaUSP in their cooperative DNA binding was examined by EMSA using ^{32}P-labeled oligonucleotides containing consensus EcRE sequences. For these tests, we used *in vitro* translated, as well as bacterially expressed and purified GST fusion proteins of AaEcR, AHR38 and AaUSP. In controls, the AaEcR–AaUSP heterodimer could bind to both the *ng* EcRE (EcRE of *Drosophila ng-1* and *ng-2* intermolt genes, direct repeat of AGGTCA half-sites with a 12 bp spacer; DR-12) and *hsp27* EcRE (EcRE of *Drosophila* heat shock protein-27, inverted repeat of imperfect consensus half-sites with a 1 bp spacer; IR-1) (Figure 5A, lanes 6 and 7 and Figure 5C, lane 2) as observed previously (Wang *et al.*, 1998). AHR38 alone bound to DR-12 (Figure 5A, lane 3), but not to IR-1 (data not shown). Similarly, the AHR38–AaUSP heterodimer was bound to DR-12 (Figure 5A, lanes 8 and 9), but not to IR-1 (Figure 5C, lane 3). The specificity of these binding complexes was confirmed by competition with an excess of unlabeled DR-12 and a putative EcRE of *AaVg* gene (DR-1 with imperfect half-sites), as well as by super-shift analyses with either anti-AHR38 or anti-AaUSP antibodies (Figure 5B). In similar experiments, we also observed the specific binding of AHR38–AaUSP heterodimer to DR-5 (direct repeat of AGGTCA half-sites with a 5 bp spacer) (data not shown).

As shown in Figure 5C, the *in vitro* binding of AaEcR–AaUSPb to IR-1 was inhibited by GST–AHR38$^{206–545}$ in a dose-dependent manner (lanes 4–6), but not by GST alone (lane 7). Addition of 20E to the reaction alleviated this inhibition (Figure 5C, compare lane 6 with lanes 8 and 9). Similar experiments were also carried out with AaUSPa; the results were identical to those seen with AaUSPb (data not shown).

AHR38 attenuates the AaEcR–AaUSP-mediated transactivation

For transient transfection experiments, we employed an *hsp27*-EcRE-*Luc* reporter construct that contains the IR-1 EcRE sequence from the *hsp27* gene. The reporter itself was not activated by the ecdysteroid ponasterone A in

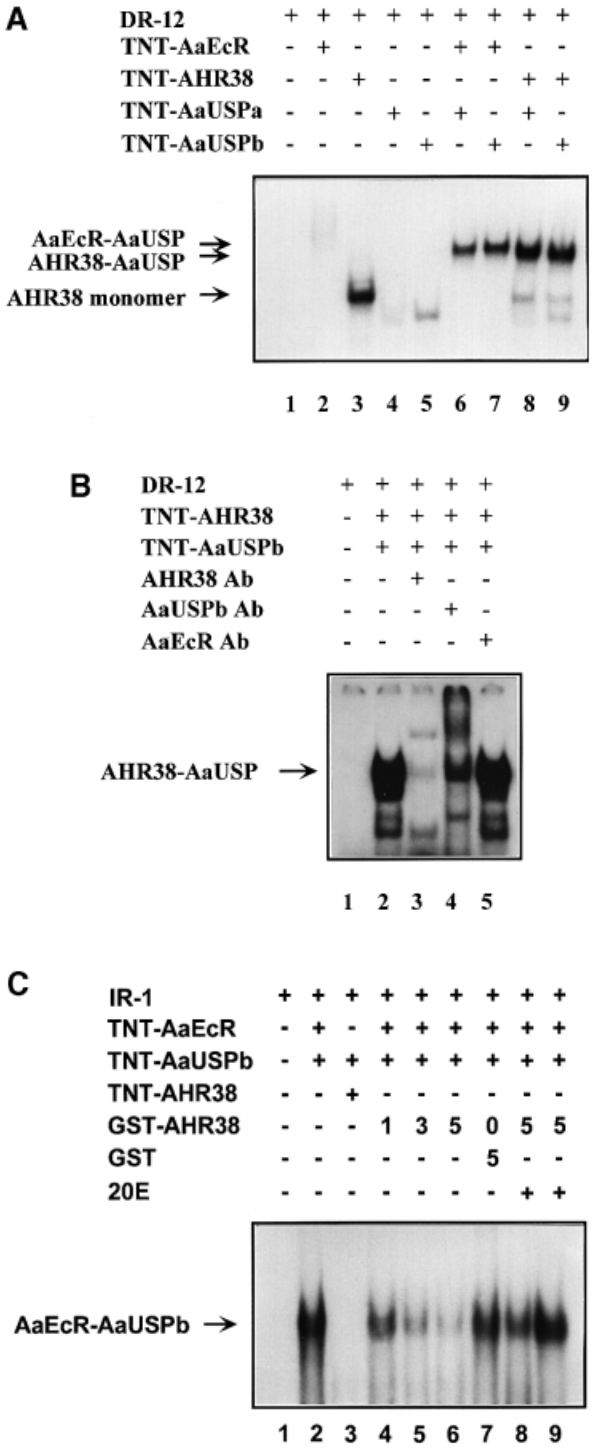

Fig. 5. Effect of AHR38 protein on binding to EcREs. EMSAs were performed using the radiolabeled *ng*-EcRE (DR-12) (A and B) or *hsp27*-EcRE (IR-1) (C). Nuclear receptor proteins were synthesized *in vitro* by the coupled transcription–translation reactions. (**A**) AHR38 was able to bind to DR-12 as either a monomer (lane 3) or an AHR38–AaUSP heterodimer (lanes 8 and 9). (**B**) The binding of AHR38–AaUSP to DR-12 was confirmed by super-shifting with anti-AHR38 (lane 3) and anti-AaUSP (lane 4), but not with anti-AaEcR (lane 5). (**C**) AHR38 repressed the binding of AaEcR–AaUSP to IR-1. Ten nanograms of AaEcR, AaUSP or AHR38 (*in vitro* translated) were used in each reaction. GST and GST–AHR38 fusion proteins were expressed in *E.coli* and partially purified. Increasing amounts of GST–AHR38 protein (1–5 µl, 10 ng/µl, lanes 4–6; 5 µl, lanes 8 and 9), GST protein (5 µl, 10 ng/µl, lane 7) and 20E (2×10^{-7} M, lane 8; 2×10^{-6} M, lane 9) were added to the reactions as indicated.

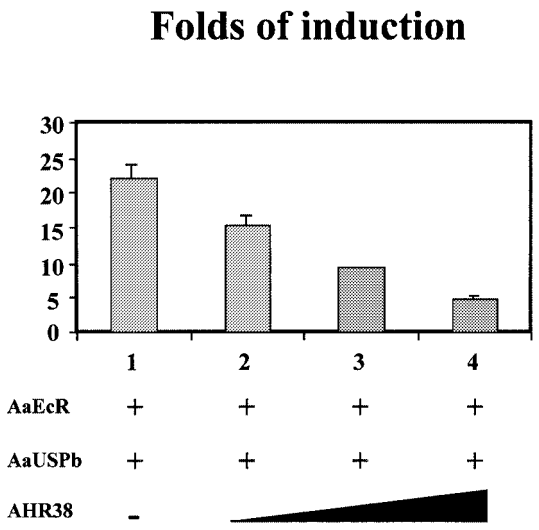

Fig. 6. AHR38 represses AaEcR–AaUSP-mediated transactivation. A combination of 0.3 μg of cytomegalovirus β-galactosidase, 0.6 μg of reporter plasmid ΔMTV-1×*hsp27*-EcRE-*Luc*, and 0.3 μg each of AaEcR and AaUSPb expression vectors was used to transfect CV-1 cells (column 1), with 0.3 μg (column 2), 0.9 μg (column 3) or 1.5 μg (column 4) of AHR38 expression vectors. After transfection, cells were incubated in the presence of the ethanol vehicle or 1×10^{-6} M ponasterone A for 36 h and harvested for luciferase assay. Luciferase activity was normalized by β-galactosidase activity. Data represent means ± SD of triplicate samples.

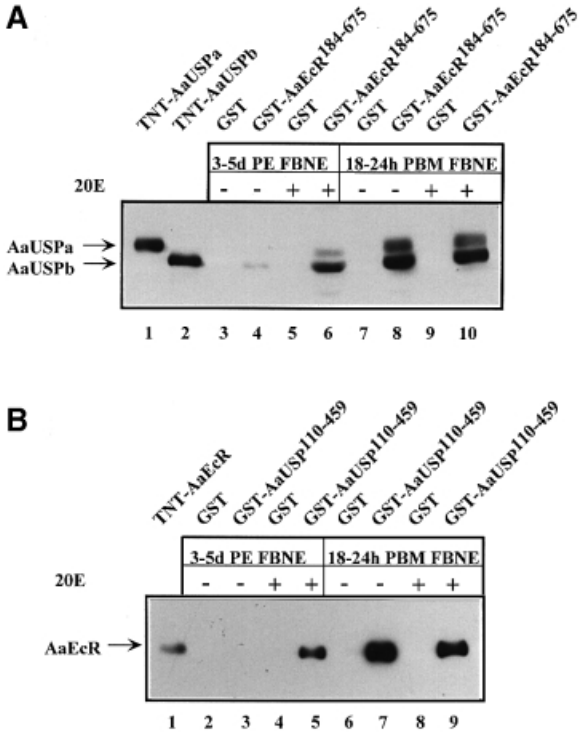

Fig. 7. GST pull-down analysis with nuclear proteins extracted from the fat body of *A.aegypti* at the pre- and vitellogenic periods. Nuclear extracts were prepared from the fat bodies of 250 adult females for each time point. An aliquot equivalent to 50 mosquitoes was incubated with glutathione–Sepharose beads bound with equal amounts of either GST or an indicated GST fusion protein. 20E (5×10^{-6} M) was added to the indicated reactions. Beads were washed, specifically bound material was resolved by SDS–PAGE, and detected on Western blots with anti-AaUSP antibodies (**A**) and anti-AaEcR antibodies (**B**). *In vitro* translated proteins (TNT-AaUSPa, -AaUSPb and -AaEcR) were used as controls in Western blot analysis.

CV-1 cells (data not shown). Co-transfection of AaEcR and AaUSPb led to an ~22-fold activation of the reporter gene expression by 1×10^{-6} M ponasterone A (Figure 6). The addition of increasing amounts of AHR38 resulted in a decrease in the ecdysteroid-dependent transactivation of the reporter gene by AaEcR–AaUSPb in a dose-dependent manner, suggesting that AHR38 was able to inhibit the AaEcR–AaUSP-mediated transactivation in mammalian cells (Figure 6). Co-transfection of AHR38 alone resulted in a weak constitutive stimulation of the luciferase activity (data not shown).

AaEcR and AaUSP proteins are sequestered by some factors during the previtellogenic state of developmental arrest

RT–PCR and Western blot analyses have shown that AaEcR and AaUSP are present at both the mRNA and protein levels in the fat body of female mosquitoes during previtellogenic developmental arrest, 3–5 days PE (Wang *et al.*, 2000). Therefore, we investigated whether the interaction between AaEcR and AaUSP was inhibited by some factors active in the fat body at this stage. We utilized GST pull-down assays, which were performed by incubating GST fusion proteins of AaEcR or AaUSP with fat body nuclear extracts (FBNE). GST–AaEcR$^{184–675}$ was able to retain AaUSP protein, predominantly AaUSPb, from FBNE of 18–24 h PBM mosquitoes. However, the interaction between GST–AaEcR and AaUSP from FBNE of 3–5 days PE female mosquitoes was not detected unless 5×10^{-6} M 20E was added to the incubation buffer. Under these conditions, interaction with AaUSPb was observed (Figure 7A). Similarly, GST–AaUSP$^{110–459}$ was able to bind to AaEcR from FBNE of female mosquitoes 18–24 h PBM. However, no interactions were detected from FBNE of previtellogenic females 3–5 days PE. Addition of 5×10^{-6} M 20E to the incubation buffer enabled GST–AaUSP$^{110–459}$ to interact with AaEcR from FBNE of 3–5 days PE mosquitoes (Figure 7B). These results suggested that both AaEcR and AaUSP proteins were sequestered by some other factors during the state-of-arrest, and that the presence of 20E helped the release of AaEcR and AaUSP.

Co-immunoprecipitation experiments implicated AHR38 as blocking the ecdysone signaling during the previtellogenic arrest via dimerization with AaUSP

To investigate whether AHR38 interacts directly with AaUSP during the previtellogenic period in the *A.aegypti* female, immunoprecipitation studies were performed using FBNE from mosquitoes 3–5 days PE. FBNE from vitellogenic mosquitoes 24 h PBM were used as a control. First, the putative complex was immunoprecipitated using the anti-AHR38 antibody and analyzed by Western blotting with anti-AaUSP antibodies (Figure 8A); in the second test, we used the anti-AaUSP antibody to precipitate the putative complex and anti-AHR38 antibody for the Western blot (Figure 8B). Both of these tests showed that the AHR38–AaUSP interactions occurred in the fat body nuclei of previtellogenic female mosquitoes during the state of arrest. In contrast, no AHR38–AaUSP complex was found in the FBNE of vitellogenic female mosquitoes

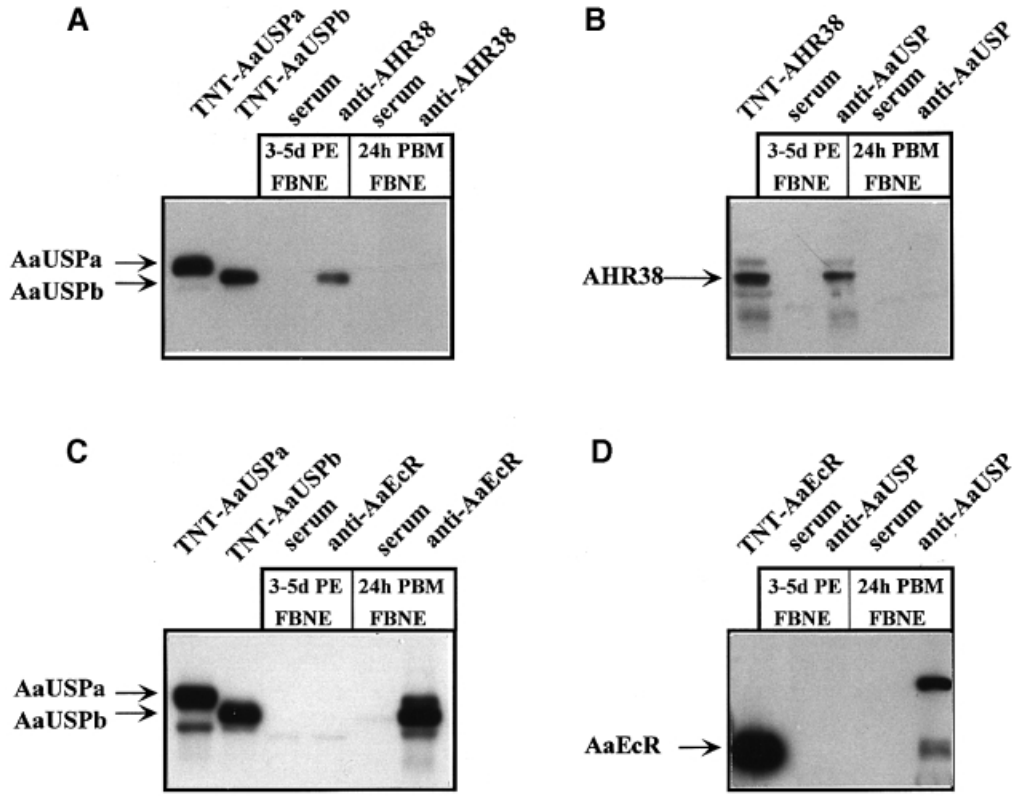

Fig. 8. Co-immunoprecipitation analyses of interactions between AHR38 and AaUSP, AaEcR and AaUSP in the fat body of *A.aegypti* at the pre- and vitellogenic periods. Nuclear extracts were prepared from the fat bodies of 250 adult females for each time point. An aliquot equivalent to 100 mosquitoes was pre-cleared with protein A–agarose and then incubated with rabbit polyclonal anti-AHR38 (**A**), mouse monoclonal anti-DmUSP (**B** and **D**), rabbit polyclonal anti-AaEcR antibodies (**C**) or the respective pre-immune sera (serum). The resulting immune complexes were then precipitated by the addition of protein A–agarose beads. After extensive washing, immune complexes were dissociated by boiling the beads in 1× SDS sample buffer. Protein samples were separated by SDS–PAGE, followed by immunoblot analyses with mouse anti-DmUSP (A and C), rabbit anti-AHR38 (B) or rabbit anti-AaEcR (D) antibodies. *In vitro* translated proteins (TNT-AaUSPa, -AaUSPb, -AHR38 and -AaEcR) were used as controls in Western blot analysis.

18–24 h PBM (Figure 8A and B). To detect the presence of the AaEcR–AaUSP heterodimer in FBNE, we used the anti-AaEcR and anti-AaUSP antibodies (Figure 8C and D). In contrast to the AHR38–AaUSP complex, the AaEcR–AaUSP complex only immunoprecipitated from FBNE of vitellogenic female mosquitoes, but not from FBNE of previtellogenic females (Figure 8C and D). The results were the same irrespective of which antibodies were used for immunoprecipitations. These analyses suggested that in the fat body of a previtellogenic female mosquito during the state-of-arrest, AaUSP predominantly existed in a complex with AHR38 and not with AaEcR. On the contrary, in the fat body of vitellogenic female mosquitoes AaUSP was able to form a complex with AaEcR. Interestingly, Western blot analyses indicated that in both stages AaUSPb was the predominant isoform in these complexes (Figure 8A and C), while for AaEcR the analyses showed the existence of two immunopositive polypeptides in the vitellogenic fat bodies (Figure 8D). The smaller polypeptide corresponds to AaEcR; however, the identity of the larger immunopositive polypeptide remains to be elucidated.

Discussion

In this paper, we report the cloning of AHR38, the mosquito homolog of the *Drosophila* nuclear receptor DHR38. DHR38 and AHR38 are both insect members of the NGFI-B/Nurr1/Nor1 subgroup of nuclear receptors (Sutherland *et al.*, 1995). The mosquito homolog exhibits structural organization typical for a nuclear receptor with the C (DNA-binding), D (hinge) and E/F (dimerization/ligand-binding) domains highly conserved with those of DHR38 and NGFI-B receptors. The evolutionary conservation of this nuclear receptor subgroup has been demonstrated by the finding of NGFI-B homologs not only in insects but also in *Caenorhabditis elegans* (Kostrouch *et al.*, 1995).

The NGFI-B, Nurr1 and Nor1 orphan nuclear receptors have been implicated as transcriptional regulators, which exert their effects via binding as monomers to a DNA recognition sequence called the NGFI-B-response element, AAAGGTCA (Wilson *et al.*, 1993; Glass, 1994; Zetterstrom *et al.*, 1996). A recent report, which has determined an important role of DHR38 in the formation of the adult insect cuticle, has also suggested that it may act as a monomer (Kozlova *et al.*, 1998).

The unique feature of the NGFI-B/Nurr1/Nor1 orphan nuclear receptors is that in addition to acting as monomers they can heterodimerize with RXR and bind to recognition response elements arranged as direct repeats of AGGTCA spaced by five nucleotides (Forman *et al.*, 1995; Perlmann and Jansson, 1995; Zetterstrom *et al.*, 1996). RXR is a promiscuous nuclear receptor, which serves as a hetero-

dimeric partner for the retinoic acid receptor (RAR), the thyroid hormone receptor (TR), the vitamin D receptor (VDR) and the fatty acid/peroxisome proliferator-activated receptor (PPAR) (Ribeiro *et al.*, 1993; Glass, 1994; Tsai and O'Malley, 1994; Kastner *et al.*, 1995; Mangelsdorf and Evans, 1995; Mangelsdorf *et al.*, 1995). Likewise, DHR38 is an alternative partner of USP, the insect homolog of RXR, which is an obligatory partner of EcR (Sutherland *et al.*, 1995). DHR38 can compete *in vitro* with EcR for dimerization with USP and consequently disrupts EcR–USP binding to an EcRE. Transfection experiments in Schneider cells show that DHR38 can repress ecdysteroid-dependent transcriptional activation (Sutherland *et al.*, 1995). Interestingly, both DHR38–USP and EcR–USP heterodimers can bind to EcRE of *Drosophila ng-1* and *ng-2* intermolt genes, containing directly repeated half-sites spaced by 12 bp (Crispi *et al.*, 1998). This suggests that DHR38 may interfere with the ecdysone-signaling pathway not only by disrupting the formation of the EcR–USP heterodimer, but also by competing for binding to selected EcREs. Similarly, we demonstrated here that AHR38 interacted strongly with AaUSP, and that AHR38–AaUSP was able to recognize EcREs such as the DR-12 of the *ng* genes and DR-5. DNA-binding and transient transfection assays showed that AHR38 disrupted the binding of the AaEcR–AaUSP heterodimer to a specific EcRE and inhibited the transactivation of a reporter gene by the AaEcR–AaUSP heterodimer.

Sutherland *et al.* (1995) have demonstrated that DHR38 inhibits ecdysone action through its heterodimerization with USP; however, its biological role in *Drosophila* remains obscure. We propose here that in the anautogenous mosquito AHR38 plays an important function by blocking ecdysone responsiveness in the target tissues at the state-of-arrest through heterodimerization with AaUSP as well as by possibly competing for binding EcREs.

Data on the developmental kinetics of AHR38 at both the RNA and protein levels support the presumed role of this mosquito nuclear receptor during the state-of-arrest. Maximal levels of AHR38 mRNA were present in the fat body of newly emerged females, the mRNA levels declined during previtellogenic development, and levels of its protein were high during the previtellogenic developmental stage, including the state-of-arrest. The AHR38 protein levels, which decreased after blood feeding, were restored to the previtellogenic levels during post-vitellogenic stage 36–60 h PBM. This suggests that AHR38 may play a similar role in preventing the ecdysone response via heterodimerization with AaUSP in post-vitellogenic mosquitoes until another blood meal activates a second vitellogenic cycle.

GST pull-down assays utilizing GST fusion proteins of AaEcR or AaUSP and FBNE showed that GST–AaEcR was able to bind AaUSP from FBNE of vitellogenic mosquitoes. However, the interaction between GST–AaEcR and AaUSP, from FBNE of previtellogenic female mosquitoes during the state-of-arrest, was not detectable. The same was also true for the interaction of GST–AaUSP and AaEcR from FBNE. These results suggest that the AaEcR and AaUSP proteins may be sequestered by other partners in the fat body of previtellogenic female mosquitoes during the state-of-arrest and therefore are not capable of forming the AaEcR–AaUSP heterodimers. Co-immunoprecipitation experiments clearly showed that AaUSP protein interacted with AHR38 instead of AaEcR in nuclei of the fat body at that stage.

Interestingly, the interaction between GST–AaEcR and AaUSP from FBNE of 3–5 days PE female mosquitoes was detectable in the presence of 5×10^{-6} M 20E. Likewise, GST–AaUSP was able to bind to AaEcR from this FBNE after the addition of 20E. This observation suggests that 20E is capable of shifting the preference of AaUSP to heterodimerization with AaEcR. However, *in vivo* in the fat body of previtellogenic female mosquitoes at the state-of-arrest, an undetermined factor probably maintains AaUSP binding to AHR38. It has been shown that RXR, which is a silent heterodimeric partner with RAR and TR with respect to its own ligand-binding activity, is active in a complex with NGFI-B or Nurr1 (Kurokawa *et al.*, 1994; Forman *et al.*, 1995; Perlmann and Jansson, 1995). At present, it is not known whether heterodimerization of AaUSP with AHR38 could impart similar allosteric changes on the USP LBD, modifying the USP into an active ligand-binding receptor. Elucidation of the nature of this possible USP-specific ligand represents a challenging question for future studies.

Materials and methods

Animals

Aedes aegypti mosquitoes were reared as described in Hays and Raikhel (1990). Vitellogenesis was initiated by allowing females 3–5 days PE to feed on an anesthetized white rat. Fat bodies were dissected from females at previtellogenic and vitellogenic stages, quickly frozen in liquid nitrogen and stored at –80°C.

RNA isolation and RT–PCR/Southern blot analysis

Fat bodies were dissected from female mosquitoes at different time points ranging from 0 to 5 days PE at the previtellogenic stage or from the vitellogenic stage ranging from 1 to 60 h PBM. Total RNA was prepared from the fat body throughout the first vitellogenic cycle, using the guanidine isothiocyanate method as described previously (Miura *et al.*, 1998) with the modification that all isopropanol precipitation steps were performed without low-temperature incubation to avoid co-precipitation of glycogen and salts. Total RNA was also extracted with Trizol reagent (Gibco-BRL). RNA yields were determined spectrophotometrically. A_{260}/A_{280} and A_{260}/A_{230} of RNA preparations were always above 1.7 and 2.0, respectively, and quite consistent irrespective of developmental stages of the mosquitoes. The temporal profile of AHR38 transcripts in the mosquito fat body was examined using RT–PCR followed by Southern blotting with an AHR38-specific radioactive probe. Five microgram aliquots of each RNA preparation were reverse-transcribed by the Superscript II reverse transcriptase (Gibco-BRL) and random hexamer (Promega) in a reaction volume of 20 µl. The reverse transcription product was diluted to 40 µl with TE buffer (10 mM Tris–HCl, 1 mM EDTA pH 8.0) and stored at –20°C as a cDNA pool until use. For the developmental profile analysis, 0.025 fat body equivalents of cDNA pools were used as PCR templates. A 285 bp AHR38-specific fragment was amplified with the following primer pair: forward, 5′-AGCTCACCCGGCTACATCAAC-3′; and reverse, 5′-GCAGGCCTT-GTCCGCGAGAC-3′. A 463 bp VCP-specific fragment (Cho *et al.*, 1991) was amplified with the following primer pair: forward, 5′-AGCGCCCATTCTTGTTTGG-3′; and reverse, 5′-CAGCTCATACAG-GTATTCTCC-3′.

Thermal cycling conditions were as follows: the reaction was incubated at 94°C for 3 min followed by 17 cycles of 94°C for 30 s, 60°C for 30 s and 72°C for 40 s. As a reference of the developmental changes of the fat body, the same cDNA was subjected to the 10–15 cycles of PCR with a primer pair specific to the mosquito VCP. After PCR amplification, 10 µl out of the total 25 µl reaction were fractionated on a 2% agarose gel, and transferred onto Hybond N^+ membrane (Amersham) under alkaline conditions. The RT–PCR products were visualized by Southern hybridization with AHR38- or VCP-specific probes.

5′-RACE

A Gibco-BRL 5′-RACE kit was used according to the manufacturer's protocol. The primers used were: gene-specific primer1 (GSP1) (5′-TGGAATCTGCAGAACTG-3′); gene-specific primer2 (GSP2) (5′-CGT-TCGAACGCCGTAGTGTTG-3′); and abridged anchor primer (AP) (5′-GGCCACGCGTCGACTAGTACGGIIGGGIIGGGIIG-3′).

Antibodies

cDNA fragments encoding AaEcR, AaUSP (Cho *et al*., 1995; Kapitskaya *et al.*, 1996) and AHR38 were individually subcloned in pGEX-4T-1 (Pharmacia) to create GST fusions. The fusion proteins were induced by isopropyl-β-D-thiogalactopyranoside (IPTG) in *E.coli* strain BL21 and purified by GST Purification Modules from Pharmacia. Proteins were further purified by SDS–PAGE followed by electroelution, and sent to Cocalico Biologicals Inc., where New Zealand white rabbits and Leghorn chickens were immunized. The monoclonal antibody against DmUSP (anti-DmUSP), described in Christianson *et al.* (1992), was a gift from Dr F.C.Kafatos (European Molecular Biological Laboratories, Heidelberg, Germany). For Western blot assays, the following dilutions of antibodies were used: anti-AaEcR polyclonal antibodies, 1:100; anti-AaUSP polyclonal antibodies, 1:100; anti-DmUSP monoclonal antibodies, 1:200; anti-AHR38 polyclonal antibodies, 1:100; anti-rabbit IgG antibodies conjugated with horseradish peroxidase (HRP), 1:5000 (Cappel); anti-mouse IgG antibodies conjugated with HRP, 1:5000 (Cappel); anti-chicken IgG antibodies conjugated with HRP, 1:160 000 (Sigma).

GST pull-down assay

GST fusion proteins immobilized on glutathione–Sepharose 4B beads (Pharmacia) were resuspended in the binding buffer [20 mM HEPES pH 7.9, 100 mM NaCl, 1 mM EDTA, 4 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 0.02% NP-40, 10% glycerol supplemented before use with 1 mg/ml bovine serum albumin, 0.5 mM phenylmethylsulfonyl fluoride, and 5 mg/ml each of antipain, leupeptin and pepstatin, 9 mg/ml aprotinin and 2 mM benzamidine], and incubated for 2–12 h at 4°C with FBNE (see below) or proteins labeled with [^{35}S]methionine by a coupled *in vitro* transcription–translation protocol (TNT; Promega). After extensive washing, the bound proteins were recovered by boiling the beads in 1× SDS sample buffer and separated by SDS–PAGE. The proteins were examined by autoradiography or immunoblot analysis. 20E (5×10^{-6} M) was included in the incubation mixture, while only the ethanol vehicle was added to the control incubation.

Electrophoretic mobility shift assay

Assays were carried out as described by Wang *et al.* (1998). Oligonucleotides used in EMSA were: DR-5, 5′-AGCGGATCCAGGTCACCGAAA-GGTCAGGATCCGCG-3′; *hsp27*-EcRE, 5′-AGCTTCAAGGGTTCA-TGCACTTGTCCATCG-3′; *ng*-EcRE, 5′-GCGAAAGGTCAAGAG-GCCAAATGAAGGTCAGGAA-3′; and *vg*-EcRE, 5′-AGCGGGAGG-CCAATGGTCGAGTGAATCT-3′.

Transient transfections

The *Kpn*I–*Not*I fragment of the AHR38 cDNA was cloned into the corresponding sites of the expression vector pcDNA 3.1/Zeo(+) (Invitrogen). The green African monkey kidney CV-1 cell line (American Tissue Culture Collection, Bethesda, MD) was transfected as described elsewhere (Wang *et al.*, 1998).

Yeast two-hybrid assay

cDNA fragments of AHR38, AaEcR and AaUSPb were inserted into either pHybLex/Zeo or pYESTrp vector (Invitrogen) to form fusion proteins with the DNA-binding domain of LexA or the activation domain of B42, respectively. The fusion constructs were used to co-transform the L40 strain of *Saccharomyces cerevisiae* [*MAT***a** *his3D200 trp1-901 leu2-3112 ade2 LYS2::(4lexAop-HIS3) URA3::(8lexAop-lacZ) GAL4*], which has integrated LexA operator-driven *HIS3* and *lacZ* reporter genes. The strength of protein–protein interactions in the yeast nuclei was estimated by either the filter assay with X-gal (Breeden and Nasmyth, 1985) or the liquid β-galactosidase assay with the substrate *o*-nitrophenyl-β-D-galactoside according to Mori *et al*. (1992).

Co-immunoprecipitation experiments

FBNE were prepared as described (Miura *et al.*, 1999). The soluble nuclear proteins were diluted to reduce the NaCl concentration to 150 mM and pre-cleared with Sepharose beads (Pharmacia) for 1–2 h at 4°C. The supernatant was then incubated with antiserum or pre-immune serum for 12 h at 4°C followed by incubation with protein A–agarose beads (Boehringer Mannheim) for 3–4 h at 4°C. The binding buffer was composed of 25 mM HEPES pH 7.9, 150 mM NaCl, 10% glycerol, 0.1 mM DTT, 0.2 mM EDTA, 0.1% NP-40 and 1 mM (2-aminoethyl)benzenesulfonyl fluoride (AEBSF). The precipitate was washed three times with binding buffer and once with a solution of 0.1 M Tris (pH 6.7) containing protease inhibitors. The bound proteins were recovered by boiling the beads in 1× SDS sample buffer and separated by SDS–PAGE followed by immunoblotting. Ponceau-S staining of Western blots confirmed the presence of equal amounts of precipitating antibody in each sample. The antigen–antibody complex was detected by chemiluminescence using the SuperSignal Substrate system (Pierce).

Acknowledgements

The authors thank Dr M.Fluck for permission to use her laboratory for cell transfection assays and Dr F.C.Kafatos for a generous gift of anti-USP monoclonal antibodies. We are also grateful to Drs S.-F.Wang and J.Sun, and Mr N.Dittmer and Mr C.Ontiveros for critical reading of the manuscript. This work was supported by a grant from the National Institutes of Health (RO1 AI-36959).

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Received September 3, 1999; revised November 9, 1999; accepted November 11, 1999