A COUP-TF/Svp homolog is highly expressed during vitellogenesis in the mosquito *Aedes aegypti*

K Miura, J Zhu¹, N T Dittmer², L Chen¹ and A S Raikhel¹

Department of Medical Zoology, School of Medicine, Mie University, 2-174 Edobashi, Tsu 514-0001, Japan

¹Department of Entomology, University of California, 3401 Watkins Dr., Riverside, California 92521, USA

²Graduate Program in Genetics, Michigan State University, East Lansing, Michigan 48824, USA

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

(K Miura and J Zhu contributed equally to this work)

(N T Dittmer is now at Department of Biochemistry, Kansas State University, Manhattan, Kansas 66506, USA)

Abstract

In the mosquito *Aedes aegypti*, vitellogenesis is activated via an ecdysteroid hormonal cascade initiated by a blood meal. The functional ecdysone receptor is a heterodimer composed of the ecdysone receptor (EcR) and ultraspiracle, the homolog of the retinoid X receptor. The precise tuning of this hormonal response requires participation of both positive and negative transcriptional regulators. In *Drosophila*, Svp, a homolog of chicken ovalbumin upstream promoter transcription factor (COUP-TF), inhibits ecdysone receptor complex-mediated transactivation *in vitro* and *in vivo*. Here we report the cloning and characterization of the Svp homolog in mosquito *Aedes aegypti*, AaSvp. It possesses a high degree of amino acid sequence similarity to the members of the COUP-TF/Svp subfamily. AaSvp transcripts and protein are present in the fat body at high levels from the state of arrest to about 60 h post blood meal. AaSvp binds strongly to a variety of direct repeats of the sequence AGGTCA, but weakly to inverted repeats such as *hsp27*EcRE. Transient transfection assays in *Drosophila* S2 cells showed that AaSvp was able to repress 20-hydroxyecdysone (20E)-dependent transactivation mediated by the mosquito ecdysteroid receptor complex. These data suggest that AaSvp negatively regulates the 20E signaling in the fat body during mosquito vitellogenesis.

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Introduction

The ecdysteroid 20-hydroxyecdysone (20E) is a key regulator of insect embryogenesis, metamorphosis and reproduction (Dhadialla & Raikhel 1994, Segraves 1994, 1998, Thummel 1995). The anautogenous mosquito, *Aedes aegypti*, represents an outstanding model system to study the 20E-mediated regulation of insect reproduction because its vitellogenesis in the fat body, which is thought to be functionally similar to vertebrate liver (Hoshizaki *et al.* 1994), and oogenesis in the ovary can only be physiologically triggered by blood ingestion (Raikhel 1992). The process of vitellogenesis in the female mosquito fat body is divided into four phases: preparation, arrest, active yolk protein

synthesis and termination (Raikhel 1992). The preparatory, previtellogenic development, which takes 3 days after adult emergence, is followed by a developmental arrest which lasts until blood feeding. The massive yolk protein synthesis peaks around 24 h post blood meal (PBM 24 h), then drops and terminates by PBM 30–32 h. The changing profile of the 20E titer in female mosquitoes is well correlated with the rate of yolk protein production, suggesting 20E regulation on the mosquito vitellogenic process (Hagedorn 1983, 1985, Raikhel 1992). Furthermore, 20E is sufficient to induce the production of yolk protein precursors in the fat body cultured *in vitro* (Deutsch *et al.* 1995).

Ecdysteroid gene regulatory hierarchy plays a crucial role in governing mosquito vitellogenesis

(Pierceall *et al.* 1999, Raikhel *et al.* 1999). At the top of the hierarchy is the ecdysteroid receptor, which is a heterodimer consisting of the 20E-activated nuclear receptor, ecdysone receptor (EcR) and its obligatory partner, the retinoid X receptor (RXR) homolog, *ultraspiracle* (USP) (Thummel 1995). Two isoforms of ecdysone receptor (AaEcR) and two isoforms of USP (AaUSPa and AaUSPb) have been cloned and characterized from *A. aegypti* (Cho *et al.* 1995, Kapitskaya *et al.* 1996, Wang *et al.* 2000a, S F Wang, C Li & A Raikhel, unpublished observations). Wang *et al.* (1998) have reported that *Aedes* EcR-USP exhibits a broad specificity, binding effectively to natural *Drosophila* (for example, *hsp27EcRE*) and synthetic ecdysone response elements (EcRE). In the fat body nuclei, both AaEcR and AaUSP proteins are present from the previtellogenic state-of-arrest to the end of postvitellogenic period of the vitellogenic cycle, whilst the AaEcR-AaUSP heterodimer is detectable only after the onset of vitellogenesis until PBM 30 h, when vitellogenesis declines (Miura *et al.* 1999, Wang *et al.* 2000a, Kokoza *et al.* 2001). These findings suggest that negative regulatory factors may directly target the functional ecdysteroid receptor complex in the fat body during vitellogenesis.

Nuclear receptors (NR) constitute a large family of transcription factors that are characterized by their modular structure consisting of conserved DNA and ligand-binding domains (LBD) (Evans 1988, Green & Chambon 1988, Beato 1989, Truss & Beato 1993, Mangelsdorf *et al.* 1995). This family includes receptors for steroids, retinoids, thyroids, vitamin D, as well as for a large number of structurally related proteins without known ligands (orphan receptors). The NRs exert their effect on expression of target genes through specific binding of DNA sequences, known as hormone response elements (HRE). For non-steroid members of the NR superfamily, HREs consist of a consensus hexade AGGTCA or its degenerate variants (Kastner *et al.* 1995). This hexade is arranged either singly, with some additional residues, or as inverted (palindromes, IR), direct (DR) or everted repeats with various spacing.

HR38 and Svp, two *Drosophila* orphan nuclear receptors (homologs of the nerve growth factor-induced protein B and chicken ovalbumin upstream promoter transcription factor (COUP-TF) subfamilies respectively) have been demonstrated to

interact with USP and inhibit its signaling pathways (Sutherland *et al.* 1995, Zelhof *et al.* 1995). We have shown that in the mosquito at the state-of-arrest USP exists as a heterodimer with a homolog of *Drosophila* HR38, AHR38 which inhibits the response to ecdysone (Zhu *et al.* 2000).

In search of additional negative regulatory factors involved in regulating 20E-mediated timing of the vitellogenic cycle, we isolated the cDNA clone of AaSvp, a mosquito homolog of COUP-TF/Svp. The AaSvp gene is expressed at relatively high levels in the fat body throughout most of the first vitellogenic cycle. Electrophoretic mobility shift assay reveals that AaSvp can bind strongly to a variety of direct repeats of the sequence AGGTCA, but weakly to *hsp27EcRE*. Finally, we show that the AaSvp represses AaEcR-AaUSP-mediated transactivation in *Drosophila* S2 cells, suggesting that AaSvp may also act as a negative regulator of 20E signaling in the mosquito fat body during vitellogenesis.

Materials and methods

Animals

Vitellogenesis in adult females, 3–5 days after eclosion, was initiated by feeding them on rats. Fat bodies were dissected from females at previtellogenic and vitellogenic stages, quickly frozen in liquid nitrogen and stored at -80°C until RNA or nuclear protein isolation occurred.

Isolation of RNA

Total RNA was prepared from the fat body using the guanidine–isothiocyanate method as described previously (Miura *et al.* 1998) with the modification that all isopropanol precipitation steps were done without low-temperature incubation to avoid co-precipitation of glycogen and salts. Poly(A⁺) RNA was isolated utilizing the QuickPrep Micro mRNA Purification Kit (Pharmacia Biotech Inc.). RNA yields were determined spectrophotometrically. A260/A280 and A260/A230 of RNA preparations were always above 1.7 and 2.0 respectively, and quite consistent irrespective of the developmental stages of the mosquitoes.

cDNA cloning of AaSvp

A pair of degenerate primers was designed based on the amino acid sequence alignment of DNA-binding domains (DBD) of COUP-TFs. The degenerate primers used were: Pd-F, SIGGIAAR-CAYTAYGGICARTTYACITG and Pd-R, CK-ICAITAITGICAYTGRTTICKRTGRTG. The positions of these primers appear in Fig. 1. The cDNA fragment corresponding to the DBD of the mosquito Svp homolog (145 bp) was obtained by RT-PCR with 200 pM each of the primers and 2.5 U Taq polymerase (Gibco-BRL), in a volume of 50 µl, using a random hexamer-primed first strand fat body cDNA mixture which corresponds to 0.025 µg total RNA, either from previtellogenic days 3–5 (PV 3–5d) or PBM 24 h, as a template. Thermal cycling conditions were a hot start at 80 °C, then 94 °C for 30 s, followed by 40 cycles of 94 °C for 30 s, 45 °C for 1 min and 55 °C for 1 min. This unusually low extension was determined empirically and proved to be sufficient to amplify the 145 bp fragment, while reducing non-specific, longer products.

Sequencing of this 145 bp fragment confirmed that it encoded the DBD sequence of the mosquito Svp homolog. The 145 bp mosquito Svp cDNA fragment was used to screen a λZAPII cDNA library constructed from the PBM 6–48 h fat body mRNA of the females (Cho & Raikhel 1992), yielding several positive phage clones. The clone bearing the longest cDNA insert was fully sequenced on both strands.

The 5' sequence of the mosquito Svp homolog was obtained by using a 5'-rapid amplification of cDNA ends (5'-RACE) system version 2.0 kit substantially based on the protocol of the supplier (Gibco-BRL) with the following primers: gene-specific primers 1(GSP1), 5'-CGTAGACGACAG AATTG-3', GSP2, 5'-CGACTGGTCGATCGG GCAATTGC-3' and the abridged anchor primer (AAP), 5'-GGCCACGCGTCTGACTAGTACGG GIIGGGIIGGGIIG-3', which came with the kit. Total RNA (2.1 µg) from PV 0–1d fat body was reverse-transcribed at 42 °C, primed with GSP1. The first strand cDNA was purified by Glass Max spun column (Gibco-BRL) and poly-C tailed by terminal deoxynucleotidyl transferase. The tailed cDNA mixture was concentrated by ethanol precipitation, and the whole amount used as a template for RACE PCR. The PCR was carried

out with the GSP2 and AAP and 2.5 U AmpliTaq Gold polymerase (Perkin-Elmer) in a volume of 50 µl. Thermal cycle conditions were: 94 °C for 2 min, followed by 30 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 1.5 min. One-twentieth of the PCR product was fractionated on agarose gel electrophoresis, transferred to a nylon membrane (Amersham), and probed with the cDNA insert obtained by library screening. The corresponding positive DNA fragments were purified from the remainder of the PCR product, and were subcloned into pBluescript-SK (Stratagene) and sequenced.

Finally, an AaSvp cDNA fragment that contained the full-length coding region was obtained by RT-PCR and cloned in pBluescript-SK at PstI site, yielding the plasmid pBS-AaSvp. The primers used in this PCR reaction were: AaSvp F4, 5'-GTCTGCAGTATCAGTAATCCCGGA GA-3' and AaSvp Rev2, 5'-AACTGCAGGT GTCACATCGAAGGTAGATAGG-3'. Thermal cycle conditions were: 94 °C for 2 min, followed by 30 cycles of 94 °C for 30 s, 58 °C for 30 s and 72 °C for 3 min.

Northern blot hybridization

The mRNA was fractionated by electrophoresis in a 1% agarose/formaldehyde gel, and transferred to a nitrocellulose membrane using standard procedures (Ausubel *et al.* 1989, Sambrook *et al.* 1989). Single-strand, antisense DNA probes were generated by PCR using the pBS-AaSvp as the template and were labeled with [α -³²P]dATP (Bej *et al.* 1991). The specific activity of the probe was determined by scintillation counting, and it was added to the hybridization buffer at a concentration of 1×10^6 dpm/ml solution. Hybridization was performed under high stringency conditions using standard procedures (Ausubel *et al.* 1989, Sambrook *et al.* 1989).

Sequence data analyses

Sequence data were edited and analyzed with a commercial program (GENETYX; SDC Software Development Co., Tokyo, Japan). Amino acid sequence alignment was performed with the Clustal W 1.60 program (Thompson *et al.* 1994) and through a WWW server of the DNA Data Bank of Japan (<http://www.ddbj.nig.ac.jp>). Both programs

Figure 1 Nucleotide and predicted amino acid sequences of AaSvp. The combined sequence of the cDNA clone obtained by cDNA library screening and 5'-RACE (nucleotide position 1–1431) is presented. The nucleotide sequence determined by 5'-RACE is indicated by a bent arrow. Consensus polyadenylation signals are double underlined. The locations of primers used in PCR-based homologous cloning (Pd-F and Pd-R) and the 5'-RACE procedure (GSP1 and GSP2) are indicated by arrows.

were run under the default settings for protein analysis.

Antibodies

cDNA fragment encoding the DBD and LBD of AaSvp (amino acids 149–493) was subcloned in pGEX-4T-1 (Pharmacia) to create glutathione S-transferase (GST) fusions. The fusion protein was induced by IPTG in *Escherichia coli* BL21 strain and purified by GST Purification Modules (Pharmacia). Protein was further purified by SDS-PAGE followed by electroelution, and sent to Cocalico Biologicals Inc (PA, USA) where Leghorn chickens were immunized. Antibodies against AaEcR and AaUSP have been described previously (Zhu *et al.* 2000).

Nuclear protein preparation and Western blot assay

Nuclear extracts were prepared from the fat bodies of 250 adult females for each time-point according to the method described by Miura *et al.* (1999). An aliquot equivalent to 50 mosquitoes was loaded in each lane and resolved by 10% SDS-PAGE, followed by immunoblotting. The following dilutions of antibodies were used: anti-AaSvp polyclonal antibodies, 1:50 and anti-chicken IgG antibodies conjugated with horseradish peroxidase, 1:160 000 (Sigma).

Electrophoretic mobility shift assay (EMSA)

Assays were carried out as described by Wang *et al.* (1998). Oligonucleotides used in the EMSA were: DR1 (5'-gatccAGGTCAaAGGTCAg-3'), DR2 (5'-aagcgaaAGGTCAggAGGTCAaggaaat-3'), DR4 (5'-cgatggacaAGGTCAcaggAGGTCActgaagct-3') and *hsp27*EcRE (5'-agcttcaaggGTTCaTGCACtTgtccatcg-3').

Cell culture and transient transfection assay

The reporter plasmids Δ MTV-Eip-Luc (Eip-Luc) and Δ MTV-Hsp-Luc (Hsp-Luc) were kind gifts from M McKeown (Salk Institute, San Diego, CA, USA). Partial sequencing indicated Eip-Luc contained four copies of *eip-28/29* EcRE, whereas Hsp-Luc contained two copies of *hsp-27*EcRE. To

make the reporter construct 2 xDR1-TATA-Luc, two copies of DR1 (5'-AGCTTAGGTCAGAGGTCAAGAGAGGTCAGAGGTCACTCGA-3') were placed in plasmid pLUC-MCS (Stratagene) between XhoI and HindIII. The reporter pAc5-LacZ (Invitrogen) was used to normalize transfection efficiency. The construction of plasmids pAc5-AaEcR and pAc5-AaUSPb has been described elsewhere (Wang *et al.* 2000b). pAc5-AaSvp was constructed by inserting the EcoRV fragments from pBS-AaSvp into the EcoRV site of pAc5/V5/HisA. These expression plasmids utilized the same promoter, actin 5C. All constructs were verified by restriction digestions and partial sequencing.

Drosophila cell line S2 (Invitrogen) was maintained at 22–24 °C in Schneider *Drosophila* medium supplemented with 10% heat-inactivated fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 µg/ml) (Gibco-BRL). Transfection was conducted with LipofectACE (Gibco-BRL) with an optimal DNA lipid ratio of 1:20 (wt/wt). Typically, 100 ng luciferase reporter gene, 25 ng reporter pAc5-LacZ, 25 ng pAc5-AaEcR, 25 ng pAc5-AaUSPb, 25–125 ng pAc-AaSvp and 6 µg LipofectACE were mixed in a 24-well plate with a total volume of 40 µl and incubated at room temperature for 30–45 min. The expression vector pAc5/V5/HisA was used as carrier DNA so that each well received 300 ng total DNA. The transfection cocktail was overlaid with 500 µl S2 cells, which were diluted to 10⁶ cells/ml in *Drosophila* serum-free medium (Gibco-BRL). Transfection was terminated 12–15 h later with the addition of 5% fetal bovine serum. After 24 or 36 h of hormone treatment, the medium was aspirated and the cells in suspension and attachment were combined in 200 µl reporter lysis buffer (Promega). Reporter gene assays were conducted as described for the Promega firefly luciferase reporter and β -galactosidase systems. A luminometer (Turner Designs (CA, USA) model TD20e) was used to detect luciferase activity with 10 s delay time and 30 s integration time. The luciferase activity was normalized with β -galactosidase activity. Transfection assays were carried out in triplicate, and each independent experiment was repeated at least three times.

Western blot analyses were carried out with a fraction of the S2 cells transiently transfected as described above. Total soluble proteins (20 µg)

of each sample were separated on an SDS-polyacrylamide gel and tested independently with antibodies against AaEcR, AaUSP and AaSvp.

Results

Cloning the mosquito Svp cDNA

A pair of degenerate primers was designed based on highly conserved regions in the DBD of COUP-TFs/Svps: Pd-F, 5'-SIGGIAARCAYTAYGGICARTTYACITG-3' and Pd-R, 5'-CKICAITAITGICAYTGRTTICKRTGRTG-3'. RT-PCR of the fat body total RNA from the female adult at either 0–3 days post-eclosion or PBM 24 h yielded a single 145 bp product whose translated sequence predicted a protein that shared significant homology with the DBD of *Drosophila* Svp (DmSvp) (data not shown). The 145 bp DNA fragment was used to screen a λ ZAPII cDNA library constructed from the PBM 6–48 h fat body mRNA of the females (Cho & Raikhel 1992). A cDNA clone with an insert of 3.4 kb was isolated, and an additional 1.4 kb 5' sequence of the transcript was obtained by 5'-RACE PCR. Finally, the cDNA fragment containing the entire mosquito Svp (AaSvp) coding region was obtained by RT-PCR. It was then subcloned in pBluescript-SK at the PstI site, yielding the plasmid pBS-AaSvp.

The AaSvp cDNA extended over 4770 bp and contained an open reading frame of 1482 bp, which encoded a protein of 493 amino acids (Fig. 1). The AaSvp cDNA had long 5' and 3' untranslated regions (UTR), 1010 and 2278 nucleotides respectively. Interestingly, the 3'UTR contained two consensus polyadenylation signals, AATAAA, at nucleotide positions 4184–4189 and 4752–4757. The sequence data have been submitted to the DDBJ/EMBL/GenBank database under accession no. AF303224.

Amino acid sequence alignment

AaSvp possessed a high degree of amino acid sequence similarity to the members of the COUP subfamily. The human COUP-TFI (HsCOUP; Wang *et al.* 1989), which is also known as EAR3 (Miyajima *et al.* 1988), zebrafish cognate of HsCOUP (BrSvp44; Fjose *et al.* 1993), chicken COUP-TFII (GgCOUP; Lutz *et al.* 1994), *Xenopus*

COUP-TF (XICOUP; Matharu & Sweeney 1992) and *Drosophila* Svp type1 (DmSvp1; Mlodzik *et al.* 1990) were chosen, and multiple sequence alignments were performed with either overall sequences (Fig. 2A) or domain sequences. Based on these sequence alignments, identity/similarity matrices (Fig. 2B) were constructed.

Figure 2A shows the alignment of full amino acid sequences of the six COUP superfamily members. The most conserved was the C domain, a core of the DBD. In this domain with 66 residues, the AaSvp exhibited 89.4 (to XICOUP) to 95.5% (to DmSvp1) amino acid identity to the other COUP-TF homologs (Fig. 2B). Moreover, this domain of AaSvp showed 100% similarity to human, zebrafish and chicken COUP-TFs while having 98.5% similarity to DmSvp1 due to the Ser residue substitution at the conserved Asn position in DmSvp. The P box, critical for the half-site recognition specificity of NRs (Umesono *et al.* 1991), was identical (EGCKS) in all six of the COUP-TF homologs. The D box sequences, essential for the discrimination of half-site spacing, were somewhat divergent. The COUP-TFs from the human, zebrafish and chicken had identical D box sequences of RANRN, while AaSvp had one conservative substitution.

The E region, located at the carboxy-terminal half of proteins and represents a core of the LBD, was the second most conserved portion of COUP-TFs. The multifunctional LBD is responsible for ligand binding and ligand-dependent transcriptional activation in class I and II receptors and, in some cases, transcriptional repression (Mangelsdorf *et al.* 1995), while providing a dimerization interface (Evans 1988, Green & Chambon 1988, Mangelsdorf & Evans 1995). In this region of COUP-TFs, the amino acid sequence identity/similarity scores were more than 86%/92% among the six proteins while AaSvp and DmSvp1 had the highest identity/similarity of 97.2%/98.6%. These two insect proteins shared several specific amino acid or gap substitutions in this region. The 20 amino acid sequence (F, W, Y) (A, S, I) (K, R, E, G) xxxx (F, L) xx (L, V, I) xxx (D, S) (Q, K) xx (L, V) (L, I, F), where x indicates an undefined residue, was a cluster of conserved amino acids over the LBD of NR superfamily members (Wurtz *et al.* 1996). This 'LBD specific signature' was also shared by COUP-TFs, examined here with the one exception of XICOUP

Figure 2 Sequence analyses of AaSvp and COUP-TF family members. (A) Multiple amino acid sequence alignment of six COUP-TF family members. Sequences were aligned by the Clustal W program. The boundaries of respective regions are indicated by bent arrows. The amino acids shared by four or more sequences are represented by black boxes. The positions of P box, D box and the LBD specific signature of NRs are indicated by lines above the sequences. Dots denote inserted gaps. In consensus lines, asterisks, pluses and minuses indicate the following respectively: all identical, above the sequences. Dots denote inserted gaps. Sequences corresponding to α helices 1, 3 to 12 found in hRAR γ LBD are underlined. The putative four or more similar residues and the other cases. Sequences corresponding to α helices 1, 3 to 12 found in hRAR γ LBD are underlined. The putative COUP-TF activation function 2 (AF-2) core motif of COUP-TFs is shown in H12. (B) Identity/similarity matrices of overall or domain sequences of six COUP-TF members. Full or domain sequences were aligned as in (A), and amino acid identity/similarity were calculated on the basis of respective alignment. Percent values to AaSvp are indicated in bold. The length of each sequence is shown on the right while numbers in parentheses indicate total residue numbers in the alignments.

which had Phe substitution at the 11th residue (Fig. 2A).

The D or hinge domain, connecting the cores of DBD and LBD, included a carboxy-terminal extension (CTE) of DBD following the core 66 residues, which corresponded to an amino-terminal portion of the α -helix 1 found in the LBDs of human RXR- α (hRXR α) and human retinoic acid receptor- γ (hRAR γ) (Bourguet *et al.* 1995, Renaud *et al.* 1995) and, in some cases, nuclear localization signals (Picard & Yamamoto 1987). The degree of identity/similarity in this domain of COUP-TFs was not as high as that in C and E domains, mainly because of two amino acid stretch insertions found in the insect proteins (Fig. 2A). Sub-regions that corresponded to the CTE and the amino-terminal portion of the H1 were, however, highly conserved among the COUP-TFs.

The F region in the carboxy-terminal of the NRs, whose function is still uncertain, showed a relatively high degree of sequence homology. BrSvp44 and GgCOUP had identical 14 residue sequences, and HsCOUP two amino acid extensions at the carboxy terminus over the 14 residue sequences. AaSvp and DmSvp1 were the closest receptors having only one difference in 14 residues. XICOUP appeared to be divergent, having 64% identity to the other COUP-TFs.

The amino-terminal A/B domains were quite divergent, both in sequences and lengths. The A/B regions of vertebrate COUP-TFs were relatively well conserved both in sequence and length, whereas the insect COUP-TFs showed less than 10% amino acid identity versus the vertebrate ones. Both insect COUP-TFs had much longer A/B regions with 19.8%/27.8% of identity/similarity between them. Despite the low degree of conservation, however, the A/B domains of COUP-TFs shared characteristic features. The A/B regions of these vertebrate COUP-TFs were proline-rich with 12–15 Pro residues constituting at least 14% of total amino acids. The insect COUP-TFs also contained comparable numbers of Pro residues, although percentage values were lower due to their longer sequences. In addition, the A/B regions of both AaSvp and DmSvp1 were Ser rich, with 24 and 40 residues respectively.

The A/B regions of vertebrate and insect COUP-TFs also shared another common structural feature, which was multiple potential phosphorylation sites (Wang *et al.* 1991). With the exception of

XICOUP, the other five proteins had one to five potential casein kinase II phosphorylation sites (consensus, (S, T) xx (D, E)): HsCOUP, Ser7 and Thr66; BrSvp44, Ser58; GgCOUP, Ser62; AaSvp, Ser63, Thr64, Thr87, Thr115 and Ser142; DmSvp, Ser18, Ser23 and Ser190. Among these potential sites, AaSvp's Thr115 was shared by HsCOUP (Thr66) and BrSvp44 (Thr58), and Ser142 of AaSvp overlapped with Ser190 of DmSvp1 (Fig. 2A). HsCOUP, XICOUP and DmSvp1 also had potential protein kinase C phosphorylation sites (consensus, (S, T) x (R, K)): Ser7, Thr40 and Ser161 respectively.

Expression of AaSvp in the fat body of female adults

To examine the developmental expression of AaSvp during previtellogenic and vitellogenic stages, Northern blot hybridization was carried out using mRNA isolated from the fat body and a 1.2 kb radiolabeled single-stranded cDNA probe (nt. 1455–2641, corresponding to the CDEF domains of AaSvp). A 4.9 kb transcript was predominant and detectable immediately after eclosion. The levels of this transcript peaked at PBM 3 h, then declined slightly and remained relatively steady throughout the rest of the first vitellogenic cycle, while another 4.3 kb transcript was detected only around PBM 30–36 h (Fig. 3A). mRNA from the PBM 36 h fat body was further hybridized with probes corresponding to the 5'UTR region (5' UTR; nt. 24–636), amino terminus of the coding region (NT; nt. 997–1430), carboxy terminus of the coding region (CT; nt. 2228–2495) or 3'UTR region (3' UTR; nt. 4206–4656) of the AaSvp cDNA clone (Fig. 3B). The 4.3 kb transcript could be recognized by NT, CT or 3' UTR probes, but not by the 5' UTR probe, suggesting that these two transcripts differed only at the 5'UTR. Sequence analysis of cDNA clones yielded by 5'-RACE demonstrated that the 4.3 kb transcript had a shorter 5'UTR without the first 819 nucleotides of 4.9 kb transcript. The rest of the 5'UTR and sequences encoding the A/B domain of AaSvp were identical in these two transcripts.

To investigate the levels of AaSvp protein in the fat body, polyclonal antibodies were raised against the bacterially expressed DBD and LBD of AaSvp in a Leghorn chicken. Fat body nuclei were isolated

Figure 3 Northern blot analysis of AaSvp mRNA in the fat body of the female mosquito during the first vitellogenic cycle. (A) Northern hybridization of mRNA prepared from dissected fat bodies at the indicated times. In I and III, 35 mosquito-equivalents were loaded per lane and in II, 25 mosquito-equivalents were loaded per lane. After hybridization with AaSvp cDNA (nt. 1455–2641; Fig. 1), the membranes were stripped and successively probed with actin cDNA and the yolk protein precursor vitellogenic carboxypeptidase (VCP) cDNA. An RNA ladder (in kb) is noted on the left; d, days post-eclosion; h, hours PBM. The arrows point to the two AaSvp transcripts. (B) mRNA from PBM 36 h fat body was hybridized with probes corresponding to 5'UTR region (5'UTR; nt. 24–636), amino terminus of the coding region (NT; nt. 997–1430), carboxy terminus of the coding region (CT; nt. 2228–2495) or 3'UTR region (3'UTR; nt. 4206–4656). Fifty mosquito-equivalents were loaded in each lane.

Figure 4 The AaSvp protein in the fat body detected by Western blot assay. 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 AaSvp protein was detected using anti-AaSvp polyclonal antibodies. TNT represents the *in vitro* translated proteins that were used as controls in Western blot assay.

and subjected to Western blot analysis using anti-AaSvp antibodies. The result indicated that AaSvp was barely detectable shortly after eclosion, increased dramatically during the previtellogenic state of arrest and remained at relatively high constant levels until PBM 44–48 h and then decreased at about PBM 60–64 h (Fig. 4).

DNA binding properties of AaSvp

The DNA-binding specificity of AaSvp was determined by EMSAs with AaSvp protein produced by a coupled *in vitro* transcription and translation. First, we used a binding site composed of two tandem copies of the half-site sequence AGGTCA separated by one nucleotide spacer, namely DR1 probe, which has been shown to be optimal for COUP-TF/Svp family members (Cooney *et al.* 1992, Kliewer *et al.* 1992, Tran *et al.* 1992, Jonk *et al.* 1994, Lutz *et al.* 1994, Zelhof *et al.* 1995). AaSvp was able to bind DR1 (Fig. 5, lane 2), while the reticulocyte lysate without the plasmid did not interact with the DR1 probe (Fig. 5, lane 1).

To learn whether or not AaSvp binds to other DNA motifs, such as inverted (IR) and direct (DR) repeats of the half-site consensus sequence AGGTCA separated by spacers of variable length, we conducted competition experiments. Binding of AaSvp to DR1 was performed as above, but in the presence of 20-fold molar excess of competitor oligonucleotide during incubation. The formation

Figure 5 The DNA binding ability of AaSvp. EMSAs were done by incubating *in vitro* synthesized AaSvp with ³²P-labeled DR1 in the absence (lane 2) or presence of a 20-fold molar excess of unlabeled *hsp27EcRE* (lane 3), DR1 (lane 4), DR2 (lane 5), DR4 (lane 6) or non-specific DNA (lane 7). Polyclonal antibodies (AB) against AaSvp (lane 8) or pre-immune serum (lane 9) were added to EMSA incubations to check the identity of the proteins bound to DR1 probe.

of AaSvp-DR1 complex was completely inhibited by DR1, DR2 and DR4 (Fig. 5, lanes 4–6). However, *hsp27EcRE* (a degenerate IR1) competed to a lesser degree (Fig. 5, lane 3), indicating that the affinity of AaSvp to direct repeats was higher than

that to inverted repeats. Moreover, an oligonucleotide of similar length, but containing only a single AGGTCA half site, did not compete against the DR1 for binding to AaSvp, suggesting that AaSvp was binding as a homodimer and not as a monomer (data not shown). The specificity of AaSvp-DR1 binding was additionally confirmed by adding polyclonal antibodies against AaSvp to the EMSA incubation. AaSvp antibodies effectively abolished this DNA-protein complex (Fig. 5, lane 8), whereas pre-immune serum had no significant effect (Fig. 5, lane 9).

AaSvp represses the EcR-USP-mediated transactivation in *Drosophila* S2 cells

To investigate the possible effect of AaSvp on ecdysteroid-mediated gene transactivation, we used a cell transfection assay. The reporter plasmid Hsp-Luc was transfected into *Drosophila* S2 cells alone or along with expression plasmids carrying AaEcR and AaUSP. After transfection, cells were incubated either in the absence of hormone or in the presence of 20E at 5×10^{-6} M. When challenged with 20E, cells receiving Hsp-Luc alone exhibited a low level of activation, which was presumably mediated by the endogenous *Drosophila* EcR-USP heterodimer (Fig. 6A, column 1). Co-transfection of AaSvp resulted in no significant change of the luciferase activity (Fig. 6A, columns 2–4). After co-transfection along with AaEcR and AaUSP, the induction was more robust in response to 20E (Fig. 6A, column 5). The further addition of increasing amounts of AaSvp resulted in a noteworthy decrease in the ecdysone-dependent transactivation of the reporter gene by AaEcR-AaUSPb in a dose-dependent manner (Fig. 6A, columns 6–8). A similar experiment was carried out with Eip-Luc instead of Hsp-Luc (Eip is a complex EcRE containing IR1 and DR6). The activation of the luciferase gene in Eip-Luc by the mosquito EcR-USP heterodimer was not as strong as that in Hsp-Luc. However, AaSvp also repressed this activation (data not shown).

Next, we tested the effect of AaSvp on the activation of the 2 xDR1-TATA-Luc reporter construct, containing two copies of DR1, the response element which had maximal binding to AaSvp in the EMSA experiments (Fig. 6B). In the transfection assay, AaSvp inhibited the EcR-USP-mediated activation of this reporter in a similar

manner as it did that of the Hsp-Luc (Fig. 6B, columns 2–4 and 6–8), suggesting that the direct binding of AaSvp to EcREs did not play the major role in repressing the function of AaEcR-AaUSP heterodimer function.

In order to demonstrate that the observed AaSvp inhibitory effect was not due to inhibition of EcR or USP translation, Western blot analyses were carried out. These analyses were performed for experiments utilizing the Hsp-Luc and 2 xDR1-TATA-Luc reporter genes. An aliquot of each sample was examined independently with anti-serum against AaEcR, AaUSP and AaSvp. As shown in the bottom panels of Fig. 6A and B, all three proteins were expressed after transfection. In addition, the endogenous *Drosophila* USP (DmUSP) was seen as well. Importantly, levels of AaEcR and AaUSP proteins did not change significantly after co-transfection with various doses of the AaSvp plasmid. The level of AaSvp protein corresponded to expected dose-dependent increase of its transfected plasmid. Thus, Western blot analyses indicated that the AaSvp inhibitory effect observed in these transfection experiments was not the artifact of EcR or USP translation inhibition in the cell transfection but rather a true repression of the ecdysteroid receptor action.

Discussion

COUP-TFs have been first identified and purified from chicken oviduct cells (Bagchi *et al.* 1987). They represent the most highly conserved subclass of the orphan receptors, from sea urchins to *Drosophila* to humans (Miyajima *et al.* 1988, Wang *et al.* 1989, Mlodzik *et al.* 1990, Chan *et al.* 1992, Fjose *et al.* 1993, Lutz *et al.* 1994, Jonk *et al.* 1994). The COUP-TFs are expressed primarily in the developing nervous system of *Drosophila*, zebrafish and mice, as well as in other tissues or organs (Mlodzik *et al.* 1990, Fjose *et al.* 1993, Jonk *et al.* 1994). The *Drosophila* COUP-TF homolog (seven-up, *svp*) was first isolated as a gene required for correct cell type differentiation during eye development (Mlodzik *et al.* 1990). In addition to its critical roles in the development and differentiation in nervous systems, Kerber *et al.* (1998) have recently reported that DmSvp regulates cell proliferation in Malpighian tubules, an insect excretory system.

A homodimer of COUP-TFs is capable of binding of a wide variety of HREs, to DRs and palindromes of AGGTCA with various spacing (Cooney *et al.* 1992, Tran *et al.* 1992). These include the HREs for the vitamin D receptor (VDR), thyroid hormone receptor (TR) and the RAR that require the RXR as a heterodimer partner. Based on this promiscuous binding, COUP-TFs are thought to act as negative regulators in the RXR-mediated transactivation by VDR, TR and RAR both *in vivo* and *in vitro* (Kliewer *et al.* 1992, Tran *et al.* 1992, Cooney *et al.* 1993, Jonk *et al.* 1994, Qiu *et al.* 1996). In mice, COUP-TFI null animals die prenatally because of improper neuronal development (Qiu *et al.* 1997). The mechanism underneath the repression of RXR-based transcription by COUP-TFs is considered to include either competition for binding to common HREs or heterodimer formation with RXR (Cooney *et al.* 1993, Qiu *et al.* 1996). Similarly, the *Drosophila* Svp can inhibit 20E-dependent transactivation mediated by the ecdysteroid receptor complex, a heterodimer composed of EcR and a homolog of RXR, USP, possibly through both DNA-binding competition and heterodimerization with EcR (Zelhof *et al.* 1995).

The considerable sequence homology among the LBD of COUP-TFs, from insects to sea urchin and to human, suggests that COUP-TFs may have unknown, cognate ligands. However, identifying them has been unsuccessful so far (Power *et al.* 1991). Wurtz *et al.* (1996) aligned the LBDs of 86 members of the NR superfamily by utilizing published three-dimensional structure information of the unliganded hRXR α and liganded hRAR γ (Bourguet *et al.* 1995, Renaud *et al.* 1995). Based on their framework, positions of α -helices H1 to H12 of hRAR γ were superimposed on the COUP multiple sequence alignment, while omitting the H2 that is found only in hRXR α (Fig. 2A). The α -helix H1 encompassing the junction between

regions D and E was first reported to be essential for interaction with the nuclear receptor corepressor (N-CoR) in TR and RAR (Horlein *et al.* 1995, Kurokawa *et al.* 1995). The corresponding regions of the COUP-TFs are highly conserved among them, especially in the carboxy-terminal half. Shibata *et al.* (1997) have established that this region in the LBD of COUP-TFI mediates the interaction with N-CoR and silencing mediator for retinoic acid receptor and thyroid hormone receptor. Thus, it is likely that the repressor domains of insect Svps are also involved in the gene silencing through co-repressor proteins (Tsai *et al.* 1999).

As an initial step to explore the function of mosquito COUP-TF/Svp homolog, we cloned and characterized AaSvp. The AaSvp cDNA sequence of 4770 bp was obtained by a combination of conventional cDNA library screening and 5'-RACE procedure. Two AaSvp transcripts are detected in fat body RNA, with a predominant one having an apparent size of 4.9 kb and a minor one of 4.3 kb. Thus, the AaSvp cDNA represents an almost complete sequence of the predominant transcript of 4.9 kb in length. More detailed Northern blot analysis and sequencing of 5'-RACE products indicated that these transcripts differed at the 5' ends and appeared to be formed by differential usage of two transcription initiation sites. The physiological significance of this phenomenon is not clear since the protein levels of Svp in the fat body nuclei do not change considerably after the appearance of the second transcript. In *Drosophila*, the *svp* gene encodes two distinct proteins, DmSvp1 and DmSvp2. DmSvp1 shares the common feature of COUP-TF subfamily members whereas, in DmSvp2, a carboxy-terminal half of the LBD is truncated and replaced by unrelated amino acid sequences (Mlodzik *et al.* 1990). However, such isoforms were not found during cDNA cloning of mosquito Svp.

Figure 6 AaSvp represses AaEcR-AaUSP-mediated transactivation. S2 cells were transfected with 25 ng reporter pAc5-LacZ, 25 ng pAc5-AaEcR, 25 ng pAc5-AaUSPb and 100 ng reporter plasmid Hsp-Luc (A) or 2 xDR1-TATA-Luc (B). AaSvp (pAc-AaSvp) (25–125 ng) was added as indicated. After transfection, cells were incubated either in the absence of hormone or in the presence of 5×10^{-6} M 20E for 24 h and harvested for β -galactosidase and luciferase activities. Luciferase activity was normalized with β -galactosidase activity. The results are expressed as fold induction of the luciferase activity from cells treated with hormone over that from cells treated with the control vehicle ethanol. The bottom panels of (A) and (B) are results of Western blot analyses with the transfected S2 cells treated with 20E. Total soluble proteins (50 μ g) were loaded in each lane. Transfected S2 cells treated with ethanol exhibited similar protein levels and results are not shown here.

Analyses on the deduced amino acid sequence of AaSvp clearly indicate that it displays the characteristic structure of the NR superfamily members and falls in the COUP-TF subfamily. The P box sequences in DBDs are identical in the COUP-TFs examined. COUP-TFs from the human, mouse and *Drosophila* bind as homodimers to DRs and IRs of AGGTCA with various spacing, with the highest affinity to DR1 (Cooney *et al.* 1992, Kliewer *et al.* 1992, Tran *et al.* 1992, Jonk *et al.* 1994, Lutz *et al.* 1994, Zelhof *et al.* 1995). The CTE in region D that follows the core of DBD is also highly conserved among these vertebrate and insect COUP-TFs. Therefore, it is likely that the AaSvp possesses similar target DNA specificity. Here our EMSA experiments showed that AaSvp homodimer binds strongly to DR1, DR2 and DR4, but weakly to *hsp27EcRE*.

The USP-mediated 20E signaling plays a crucial role in the regulation of mosquito vitellogenesis. Massive synthesis of yolk protein precursors is inhibited during the state of arrest and shuts down swiftly during the termination stage (Hagedorn 1985, Raikhel 1992). It is quite possible that a mosquito counterpart of COUP-TF/Svp is among negative regulatory factors that directly target the functional ecdysteroid receptor complex in this process. Both mRNA transcripts and protein of AaSvp are detected in the fat body during the first vitellogenic cycle, and the expression levels are much higher than those of AaEcR and AaUSP. The capacity of AaSvp to repress 20E signaling in transfection assays suggests its possible involvement in regulating the vitellogenic response to 20E in the female fat body. However, since AaSvp protein is abundant in the fat body nuclei throughout the first vitellogenic cycle, some additional factors may be required to regulate the timing and extent of such repression.

Despite the striking difference in AaSvp binding to DR1 versus to *hsp27EcRE* (IR1), it displayed a similar inhibitory effect on either Hsp-Luc or 2 xDR1-TATA-Luc reporters. This suggests that the inhibitory function of AaSvp in the 20E signaling is unlikely to be due to competition for binding to EcREs. The mechanism governing repression of 20E signaling by AaSvp is being investigated. In mammals, COUP-TF is reported to form a heterodimer with RXR (the vertebrate counterpart of insect USP) on DR1 (Kliewer *et al.* 1992), but not with VDR, TR or RAR (Cooney *et al.* 1993). On

the other hand, the *Drosophila* Svp is found to interact with EcR and not with USP in the yeast two-hybrid assay (Zelhof *et al.* 1995). Our next goals are to determine whether AaSvp directly interacts with either AaEcR or AaUSP *in vivo* and to study the mechanism by which AaSvp is involved in maintaining the cyclicity of ecdysteroid regulation of mosquito vitellogenesis.

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