

# Synergistic action of E74B and ecdysteroid receptor in activating a 20-hydroxyecdysone effector gene

GuoQiang Sun\*<sup>†</sup>, Jinsong Zhu\*, Li Chen, and Alexander S. Raikhel<sup>‡</sup>

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

Edited by John H. Law, University of Georgia, Athens, GA, and approved September 9, 2005 (received for review April 27, 2005)

A number of insect effector genes activated by the steroid hormone 20-hydroxyecdysone (20E) are dually controlled by the ecdysteroid receptor (EcR/USP) and products of ecdysteroid early responsive genes (*E74*, *E75*, and *Broad*). However, the molecular mechanism of this dual action is poorly understood. Here we examined transcriptional activation of the *vitellogenin* (*Vg*) gene in the yellow fever mosquito, *Aedes aegypti*, by EcR/USP and *E74* in response to an elevation of 20E titers. There are two isoforms of the *Aedes E74* gene, AaE74A and AaE74B, which have a common C-terminal Ets DNA-binding domain and isoform-specific N termini in the female mosquito. Inhibiting expression of AaE74B but not AaE74A by RNA interference led to substantial reduction in the *Vg* gene expression. AaE74B and the ecdysteroid receptor synergistically enhanced 20E-induced transcription of the *Vg* promoter. This action required the *E74*-binding sites and the ecdysone response elements in the *Vg* 5' regulatory region. Two-hybrid assays and coimmunoprecipitation analyses demonstrated direct interaction between AaE74B and AaEcR/AaUSP. Moreover, disruption of this interaction by a dominant negative *E74* mutant abolished the enhanced activation of *Vg*. Therefore, the cooperative interaction between AaE74B and the ecdysteroid receptor is required for high-level expression of the *Vg* gene *in vivo*. The synergistic activation is accomplished through their 20E-dependent protein-protein interaction on the gene promoter. This study reveals how the 20E direct-indirect regulation of an effector gene is achieved at the molecular level.

Ets transcription factor | mosquito egg maturation | nuclear hormone receptor

The steroid hormone ecdysteroid is a key regulator in the coordination of multiple developmental processes in insects, including embryogenesis, metamorphosis, and reproduction (1–6). The molecular mechanism of 20-hydroxyecdysone (20E) action has been studied extensively during metamorphosis in *Drosophila melanogaster* (2, 3, 7). Based on the observations of the effects of 20E on puffing patterns in *Drosophila* polytene chromosomes, Ashburner *et al.* have proposed a hierarchical model of ecdysteroid action (8). Subsequent molecular studies have confirmed and extended this model (2, 3, 7). Ecdysteroid signaling is mediated by the insect ecdysteroid receptor, which comprises the ecdysone receptor (EcR) and Ultraspiracle (USP) (9–11). The ecdysteroid receptor EcR/USP directly induces several early response genes, including *E74*, *E75*, and *Board*. In turn, products of these early genes activate late effector genes that control stage- and tissue-specific developmental responses to 20E (2, 3).

Although this initial hypothesis has postulated indirect action of the hormone on the effector genes via early gene products, further studies have revealed the existence of a more complex direct-indirect mode of effector gene regulation that involves the EcR/USP and one or several products of early genes (3, 5, 6, 12–15). Mechanistic understanding of how the EcR/USP and an early gene product interact during transcription activation of an effector gene with direct-indirect control is still lacking.

The mosquito vitellogenin (*Vg*) gene represents an excellent system to address this question. This gene, which encodes the

major yolk protein precursor for developing eggs, is highly expressed exclusively in the mosquito female fat body, the metabolic tissue analogous to the vertebrate liver. The expression of this gene is initiated only after the mosquito female feeds on vertebrate blood. Blood feeding triggers the production of 20E, which in turn activates the *Vg* gene. 20E is required for both the *Vg* activation and the sustained high level of its expression (5, 6). Because of the intricate link between egg development, blood feeding, and pathogen transmission, detailed understanding of molecular mechanisms governing egg development is essential for future strategies of mosquito control.

Two EcR isoforms (AaEcRa and AaEcRb) and two USP isoforms (AaUSPa and AaUSPb) have been characterized in the mosquito *Aedes aegypti* (16–18). The mosquito AaEcR/AaUSP binds to the ecdysone response elements (EcRE) located in the *Vg* promoter and activates low-level expression of this gene (15). The EcR/USP binding activity occurs in fat body nuclei in a pattern correlating well with *Vg* gene expression (15, 19). Chromatin immunoprecipitation (ChIP) experiments have shown that the AaEcR/AaUSP complex occupies the *Vg* promoter *in vivo* shortly after blood feeding (20).

Genetic studies using *Drosophila* and *Aedes* transformation have identified a region in the 5' regulatory region of the *Vg* gene that contains putative binding sites for *E74* and is essential for stage-specific hormonal enhancement of *Vg* gene expression (14). The protein product of the *E74* gene belongs to the Ets domain transcription factor family, which binds to the core DNA motif 5'-GGAA/T-3' (21). The *Aedes E74* gene encodes two isoforms, AaE74A and AaE74B, which have a common C-terminal Ets DNA-binding domain and isoform-specific N termini (22). AaE74B activates the *Vg* gene *in vitro*, strongly suggesting that it is a positive regulator of this gene *in vivo* (23). Furthermore, the DNA binding activities of AaE74B and AaEcR/AaUSP tested with their respective binding sites are temporally similar in the nuclear extract of vitellogenic fat bodies (23).

We report here that a high level of *Vg* expression requires synergistic action of the EcR/USP and AaE74B through their direct protein-protein interaction on the *Vg* promoter. This study reveals how the 20E direct-indirect regulation of an effector gene is achieved at the molecular level.

## Materials and Methods

**Animals.** Mosquitoes of the *A. aegypti* Rockefeller/UGAL strain were used in this study. They were reared as described in ref. 24. Vitellogenesis was initiated by feeding 3- to 5-day-old (post-mergence) females on white laboratory rats.

This paper was submitted directly (Track II) to the PNAS office.

Abbreviations: 20E, 20-hydroxyecdysone; dsMal, *malE* dsRNA-injected female adult *A. aegypti*; EcR, ecdysone receptor; EcRE, ecdysone response elements; PBM, postblood meal; RNAi, RNA interference; USP, Ultraspiracle.

\*G.S. and J.Z. contributed equally to this work.

<sup>†</sup>Present address: Neurosciences Division, Beckman Research Institute, City of Hope, Duarte, CA 91010.

<sup>‡</sup>To whom correspondence should be addressed at: University of California, Department of Entomology, 3401 Watkins Drive, Riverside, CA 92521. E-mail: alexander.raikhel@ucr.edu.

© 2005 by The National Academy of Sciences of the USA

**RNA Interference (RNAi) Assay.** Synthesis of dsRNAs and microinjection were performed as described in ref. 25. The dsRNA corresponding to the AaE74B isoform-specific region, where no other significant homology was found by BLAST search (National Center for Biotechnology Information, Bethesda), was prepared by using the HiScribe RNAi Transcription Kit (New England Biolabs) following the manufacturer's instructions. The integrity of the dsRNA was assessed by gel electrophoresis, and concentration was determined by spectrophotometry. Two days after emergence, mosquito females were each microinjected intrathoracically with  $\approx 1 \mu\text{g}$  of dsRNA. The injected mosquitoes were allowed a period of 2 days for recovery and then were fed on blood. The transcription of each gene was analyzed by real-time PCR as described in ref. 25.

**Plasmid Constructs.** Three reporter constructs,  $Vg^{1071}\Delta$ Cluster-luciferase (Luc) with mutation of E74 cluster,  $Vg^{1071}\Delta$ C10-Luc with mutation of C10, and double-mutation construct  $Vg^{1071}\Delta$ DB-Luc, were generated by using the QuikChange Site-Directed Mutagenesis System (Stratagene) to change the E74 core recognition sequence GGAA to TTAA. These mutations in the E74 binding sites abolished their binding to E74B (data not shown). The EcRE-deletion plasmid,  $Vg^{1071}\Delta$  EcRE-Luc, was created by insertion of a fragment from the  $Vg$  promoter ( $-1,071$  to  $-618$  bp) into a vector  $Vg\Delta^{RE1,2}$ -Luc, described in ref. 15. The serial deletions of  $Vg$  5' region luciferase reporter constructs  $Vg^{2100}$ -Luc,  $Vg^{1071}$ -Luc,  $Vg^{618}$ -Luc,  $Vg^{348}$ -Luc, pAcAaE74B, pAcAaEcR, and pAcAaUSP have been described elsewhere (15, 26). The reporter pRL-CMV (Promega) was used to normalize transfection efficiency.

DNA fragments encoding the isoform-specific region or common region of AaE74B were subcloned into the pM vector (Clontech) to create GAL4-AaE74B fusion proteins. All plasmids were confirmed by restriction digestion and partial sequencing.

**Cell Culture and Transient Transfection Assay.** Transfection of the *Drosophila* S2 cell line (Invitrogen) was conducted as described in ref. 15. EcR-deficient *D. melanogaster* L57-3-11 cell line, generously provided by Lucy Cherbas (Indiana University, Bloomington), was transfected according to instructions of Hu *et al.* (27). The green African monkey kidney CV-1 cell line (American Type Culture Collection) was used for the mammalian two-hybrid assay and transfected as described elsewhere (25).

**Antibodies.** Generation of rabbit polyclonal antibodies against AaUSP and AaEcR, as well as against AaE74A and AaE74B, was reported in refs. 20 and 23. The monoclonal antibody against DmUSP (anti-DmUSP) was a gift from Fotis C. Kafatos (European Molecular Biological Laboratories, Heidelberg, Germany).

**EMSA.** EMSAs for E74 were performed as described in ref. 22. For competition reactions, unlabeled oligonucleotides were added in excess of 50-fold.

**Coimmunoprecipitation Assay.** *In vitro* expressed and  $^{35}\text{S}$ -labeled proteins or *Drosophila* S2 cell nuclear extracts were used as protein sources. Expression vectors of AaE74B, AaEcR, and AaUSP were cotransfected into *Drosophila* S2 cells in the presence or absence of 20E. The nuclear extracts were prepared from the transfected S2 cells as described in ref. 19 and incubated with AaEcR, AaUSP, AaE74A, or AaE74B proteins that were synthesized *in vitro* and labeled with  $^{35}\text{S}$  methionine. Antibodies against AaEcR, AaE74B, and *Drosophila* USP were used for this analysis. Assays were performed by using the Immunoprecipitation Kit (Roche Applied Science, Indianapolis) following the manufacturer's instructions. The resulting immune complexes

were precipitated by addition of protein A-agarose beads. After extensive washing, the complexes were fractionated by SDS/PAGE and subjected to autoradiography. When the experiment was conducted with 20E, 20E was added into the binding and washing buffers.

## Results

**Functional Analysis of AaE74 during Vitellogenesis.** The two AaE74 isoforms show distinct expression profiles after blood ingestion in mosquitoes, implying that AaE74B is a potential activator of  $Vg$  gene expression whereas AaE74A is a negative regulator (22, 23). To elucidate the physiological functions of the two AaE74 isoforms *in vivo*, we used RNAi to attenuate expression of the AaE74 isoforms. dsRNA, corresponding to the isoform-specific region of either AaE74A or AaE74B, was injected into the thorax of female mosquitoes 2 days postemergence. In parallel, dsRNA complementary to the bacterial *malE* gene was used as a negative control. After a 2-day recovery, these mosquitoes were given a blood meal, and the fat bodies were subsequently collected at various time points and subjected to the analysis of gene expression.

In the *AaE74B* RNAi mosquitoes, the mRNA levels of AaE74B declined dramatically at 12 h and 24 h postblood meal (PBM) compared with the uninjected (WT) and *malE* (dsMal) dsRNA-injected female adults (Fig. 1A). Significantly, a substantial reduction in the expression of the  $Vg$  gene was observed in *AaE74B* RNAi mosquitoes at 12 h and 24 h PBM (Fig. 1B). In control mosquitoes injected with *malE* dsRNA, expression profiles of AaE74B and  $Vg$  were similar to those in the untreated mosquitoes. These experiments indicated that AaE74B expression was effectively suppressed by RNAi, which resulted in virtual shutdown of  $Vg$  gene expression. We concluded that AaE74B is required for activation and sustained expression of this gene *in vivo*.

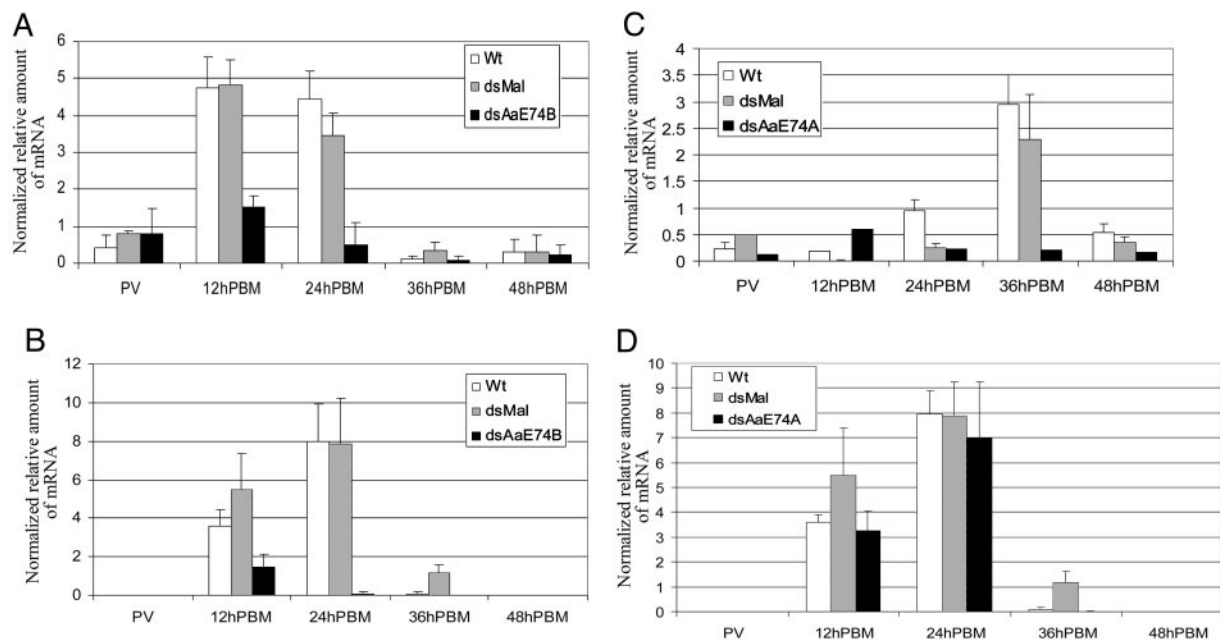
In mosquitoes injected with the AaE74A-specific dsRNA, the levels of AaE74A mRNA showed dramatic reduction at 36 h PBM when the AaE74A mRNA level was normally high in the untreated mosquitoes. Such a reduction in the AaE74A mRNA level was not observed in *malE* dsRNA-injected females (Fig. 1C). However, RNAi-dependent silencing of AaE74A expression exhibited no effect on  $Vg$  gene expression at 12 h and 24 h PBM (Fig. 1D). From these reverse-genetic analyses, it appeared that AaE74A was not required for the expression of the  $Vg$  gene. Therefore, we focused on investigating the role of AaE74B in  $Vg$  gene expression.

## Characterization of E74-Binding Sites in the $Vg$ 5' Regulatory Region.

To determine whether the  $Vg$  gene is a direct target of E74, we studied transcriptional activation of the  $Vg$  promoter by E74B by using a transient transfection assay. Four luciferase reporter constructs,  $Vg^{2100}$ -Luc,  $Vg^{1071}$ -Luc,  $Vg^{618}$ -Luc, and  $Vg^{348}$ -Luc, were analyzed in *Drosophila* Schneider 2 cells (S2). Each construct contains progressive deletions of the 5' regulatory region of  $Vg$  gene (Fig. 6A and B, which is published as supporting information on the PNAS web site). The results indicated that the region from  $-618$  bp to  $-1,071$  bp is critical for the regulation of  $Vg$  by AaE74B.

Sequence analysis of the 2.1-kb  $Vg$  5' regulatory region revealed 10 potential E74 binding sites, named C1–C10. The EMSA competition assay showed that the order of binding ability to AaE74B is C10, C5 > C2, C3, C4, C9 > C1, C6, C7, and C8 (Fig. 6C). The binding sites C3, C4, and C5 form a cluster  $\approx 50$  bp long located at  $-963$  bp to  $-912$  bp and were designated the E74 cluster. It is worth noting that both the E74 cluster and C10 are located in the median region of the  $Vg$  promoter ( $-1,071$  bp to  $-618$  bp), which is essential for E74B-dependent transcriptional activation in cell transfection experiments.

To further investigate the function of the putative E74-binding



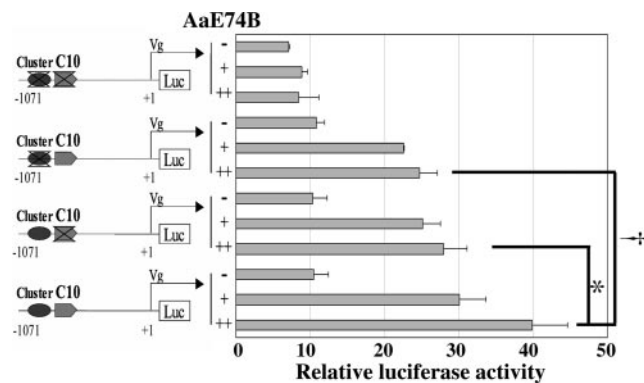
**Fig. 1.** Aae74 RNAi assay. dsRNAs of Aae74B or Aae74A were injected into thoraces of female mosquitoes as described in *Materials and Methods*. mRNA expression was measured by real-time PCR at the indicated time points after blood feeding. Each sample was analyzed in triplicate and normalized to the internal control,  $\beta$ -actin mRNA (25). (A) Aae74B mRNA levels in mosquitoes injected with Aae74B dsRNA. (B) Vg mRNA levels in Aae74B RNAi mosquitoes. (C) Aae74A mRNA levels in Aae74A RNAi mosquitoes. (D) Vg mRNA levels in mosquitoes injected with Aae74A dsRNA. Wt, uninjected *A. aegypti* Rockefeller/UGAL strain; dsMal, injected with dsRNA complementary to bacterial *malE*; dsAae74B, injected with Aae74B dsRNA; dsAae74A, injected with Aae74A dsRNA; PV, previtellogenic.

sites in the median region of the *Vg* promoter, we made three derivative reporter constructs:  $Vg^{1071}\Delta$ cluster-Luc with mutation of the E74 cluster,  $Vg^{1071}\Delta$ C10-Luc with mutation of the C10 site, and  $Vg^{1071}\Delta$ DB-Luc with mutations of both the E74 cluster and the C10 site. In S2 cells cotransfected with the Aae74B expression vector, both  $Vg^{1071}\Delta$ cluster-Luc and  $Vg^{1071}\Delta$ C10-Luc displayed significantly reduced transcription in comparison with the intact construct  $Vg^{1071}$ -Luc (Fig. 2). Transactivation by E74 was abolished in the assay with the double-mutation construct  $Vg^{1071}\Delta$ DB-Luc. Collectively, these experiments indicated that transactivation of the *Vg* gene by E74B required both the E74 cluster and the C10 binding site in the *Vg* 5' regulatory region.

**Synergistic Activation of *Vg* Expression by Aae74B and AaeEcR/AaUSP.** A functional ecdysteroid response element (*Vg*EcRE) in the *Vg* 5' regulatory region was identified as necessary to elicit transcriptional induction by 20E (15). Moreover, the *Vg*EcRE was shown to be occupied by the AaeEcR/AaUSP complex in fat body nuclei in the early vitellogenic stage (3–12 h PBM) (20). Because the functional Aae74B protein was also observed at this stage (23), Aae74B might act in concert with AaeEcR/AaUSP on the *Vg* promoter. This possibility was explored with a transient transfection assay. EcR-deficient *D. melanogaster* L57-3-11 cells transfected with the  $Vg^{1071}$ -Luc reporter (Fig. 3A) construct alone did not respond to  $10^{-6}$  M 20E (Fig. 3B, lanes 1 and 2). AaeEcR/AaUSP up-regulated the reporter activity by  $\approx$ 9-fold in response to 20E treatment (Fig. 3B, lanes 3 and 4). Similarly, transcriptional activity of the *Vg* promoter was induced 4-fold by cotransfection with Aae74B (Fig. 3B, lanes 5 and 6). Importantly, cotransfection of Aae74B together with AaeEcR/AaUSP resulted in 42-fold activation of the *Vg* promoter after addition of 20E (Fig. 3B, lanes 7 and 8).

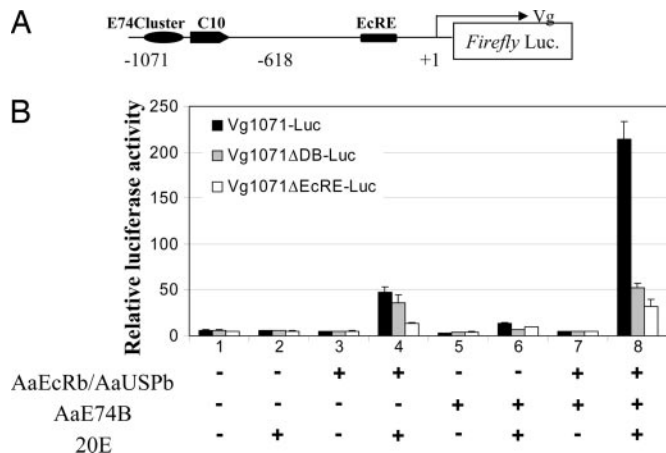
To evaluate whether the E74 binding sites (E74 cluster and C10) and *Vg*EcRE in the *Vg* promoter are indispensable for this synergistic activation, we used two reporter constructs:

$Vg^{1071}\Delta$ DB-Luc and  $Vg^{1071}\Delta$ EcRE-Luc with mutations of the E74 binding sites (precise base pair changes in E74 cluster and C10) or *Vg*EcRE, respectively. The cotransfection of Aae74B and AaeEcR/AaUSP led to no synergistic induction with either of these reporter constructs (Fig. 3B, lane 8). Thus, both the E74 binding sites and *Vg*EcRE were required for robust activation of the *Vg* gene by Aae74B and AaeEcR/AaUSP.



**Fig. 2.** The E74 cluster and C10 are required for transcriptional activation of the *Vg* promoter by Aae74B. A schematic diagram of the deletion reporter constructs is depicted in *Left*. *Drosophila* S2 cells were transfected with 100 ng of pRL-CMV (*Renilla* luciferase control vector), 100 ng of different reporter plasmids, and expression vector pAcAae74B. Transfections without the pAcAae74B are indicated as “–,” and those with increasing amounts of Aae74B are indicated as “+” (50 ng) and “++” (100 ng). Data in *Right* represent the ratios of firefly luciferase to *Renilla* luciferase activity (relative luciferase activity). Significant differences (*t* test) between single deletion and intact reporter constructs are indicated (\* and †,  $P < 0.05$ ). Values are means of triplicate independent transfection experiments, with error bars representing the SD of the mean.





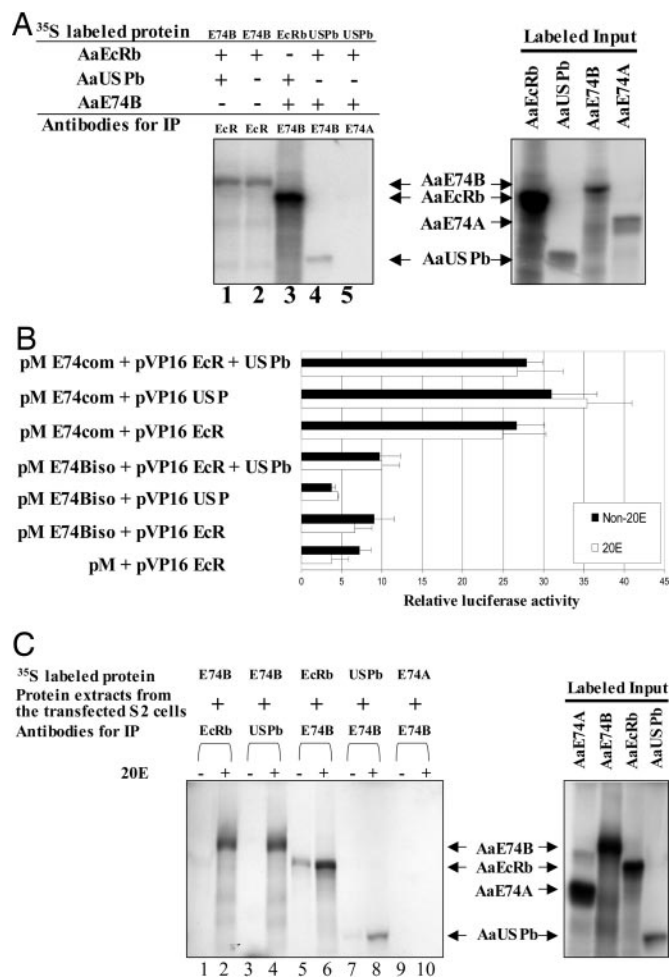
**Fig. 3.** AaE74B acts synergistically with AaEcR/AaUSP in activating the *Vg* promoter. (A) Schematic illustration of the luciferase reporter construct *Vg*<sup>1071</sup>-Luc. (B) Activation of the *Vg* promoter by AaEcR/AaUSP and AaE74B. *Drosophila* L57-3-11 cells were transfected by expression vectors as indicated, together with different reporter constructs: *Vg*<sup>1071</sup>-Luc, *Vg*<sup>1071</sup>ΔDB-Luc, and *Vg*<sup>1071</sup>ΔEcRE-Luc. *Vg*<sup>1071</sup>ΔDB-Luc denotes a *Vg*<sup>1071</sup>-Luc derivative with mutations in the E74 cluster and C10, and *Vg*<sup>1071</sup>ΔEcRE-Luc represents a *Vg*<sup>1071</sup>-Luc derivative that lacks the functional EcRE as identified in ref. 15. After transfection, cells were cultured in medium with or without  $1 \times 10^{-6}$  M 20E. Data represent ratios of firefly luciferase to *Renilla* luciferase activity (relative luciferase activity). Shown are mean values  $\pm$  SD from three independent transfection experiments.

**Protein-Protein Interaction of AaE74B with AaEcR/AaUSP.** To examine whether AaE74B directly interacts with AaEcR/AaUSP during transactivation of the *Vg* gene, we used coimmunoprecipitation and two-hybrid analyses.

In the first series of coimmunoprecipitation experiments, we used *in vitro* synthesized and labeled AaE74B, AaE74A, AaEcR, and AaUSP proteins. The proteins were mixed in pair-wise combinations, incubated in the presence or absence of 20E, and precipitated with the antibodies specific to AaE74B, AaEcR, or AaUSP. The results indicated that AaE74B bound either AaEcR or AaUSP in the absence of 20E (Fig. 4A).

To further investigate the interaction of AaE74B with AaEcR and AaUSP, we performed two-hybrid analysis in CV1 cells, which have low levels of RXR (the USP homolog) but no endogenous E74, EcR, or USP protein. The N-terminal AaE74B isoform-specific region (E74Biso) or the C-terminal region common for both E74 isoforms (E74com) was fused to the GAL4 DNA binding domain (DBD). Conversely, AaEcR and AaUSP truncated forms (pV16EcR and pV16USP) containing the DNA-binding and ligand-binding domains (domains C-F) were expressed as fusions to the acidic activation domain of the herpes simplex virus transcriptional activator VP16. No interaction was observed between the AaE74B isoform-specific region and EcR or USP (Fig. 4B). However, the reporter activity increased significantly when pM-E74com was expressed together with either pV16EcR or pV16USP, suggesting that the C-terminal region of E74B was required for protein-protein contact with EcR and USP. Most importantly, the interaction between E74B and AaEcR was not affected by either the addition of full-length AaUSP or the presence of 20E (Fig. 4B).

We also studied the interactions of AaE74B, AaEcRb, and AaUSPb with proteins expressed in *Drosophila* S2 cells. AaE74B, AaE74A, AaEcRb, and AaUSPb proteins were *in vitro*-synthesized and labeled with [<sup>35</sup>S]methionine. The labeled proteins were incubated individually with nuclear extract from S2 cells cotransfected concurrently with AaE74B, AaEcRb, and



**Fig. 4.** Association of AaE74B with AaEcR and AaUSP. (A) Proteins were synthesized and were labeled by using the TnT Coupled Transcription/Translation System. These proteins were then incubated for 4 h in the presence or absence of  $1 \times 10^{-6}$  M 20E, followed by immunoprecipitation with the indicated antibodies. The resulting immune complexes were precipitated by addition of protein A-agarose beads. After extensive washing, the complexes were analyzed by SDS/PAGE and autoradiography. (B) Interaction of AaE74B with AaEcR and AaUSP in mammalian two-hybrid assay. CV-1 cells were cotransfected with control reporter pCMV-Luc and reporter construct pFR-Luc together with indicated plasmids. Luciferase activity was normalized with *Renilla* luciferase activity. The results represent the mean values obtained from three independent experiments. (C) *Drosophila* S2 cells were cotransfected collectively by vectors for AaE74B, AaEcRb, and AaUSPb and cultured in the presence or absence of 20E. Nuclear extracts prepared from the transfected cells were incubated with [<sup>35</sup>S]methionine-labeled AaEcRb, AaUSPb, or AaE74B, followed by immunoprecipitation.

AaUSPb expression plasmids, followed by immunoprecipitation with the antibodies specific to AaEcR, AaUSP, or AaE74B (Fig. 4C). The AaEcR and AaUSP antibodies precipitated radioactively labeled AaE74B in the presence of 20E but not without 20E (Fig. 4C, lanes 1-4). Similarly, AaE74B was associated with AaUSPb only in the presence of 20E (Fig. 4C, lanes 7 and 8). A low level of AaEcRb was precipitated by AaE74B antibodies in the absence of 20E (Fig. 4C, lane 5). However, the intensity of precipitated AaEcRb was enhanced dramatically after the addition of 20E (Fig. 4C, lane 6). AaE74A could not be recognized by the AaE74B antibody, indicating that the interactions were specific (Fig. 4C, lanes 9 and 10). We concluded that in S2 cells AaE74B specifically interacted with AaEcR/AaUSP in a 20E-dependent manner. This difference in hormonal dependence



contrast, AaEcRb and AaUSPb expressed in *Drosophila* S2 cells bind AaE74B weakly in the absence of 20E, whereas this association is enhanced by the presence of 20E. This discrepancy in hormonal dependence suggests that additional signaling transduction and unknown auxiliary factors may be implicated in mediating the interaction between E74B and EcR/

USP *in vivo*. It is of great importance to elucidate how 20E modulates this specific protein–protein interaction in the future.

This work was supported by National Institutes of Health Grant AI-36959 (to A.S.R.).

1. Riddiford, L. M. (1993) in *The Development of Drosophila melanogaster*, eds. Bate, M. & Martinez-Arias, A. (Cold Spring Harbor Lab. Press, Cold Spring Harbor, NY), pp. 899–939.
2. Thummel, C. S. (1996) *Trends Genet.* **12**, 306–310.
3. Thummel, C. S. (2002) *Insect Biochem. Mol. Biol.* **32**, 113–120.
4. Kozlova T. & Thummel, C. S. (2003) *Science* **301**, 1911–1914.
5. Raikhel, A. S., Kokoza, V. A., Zhu, J. S., Martin, D., Wang, S. F., Li, C., Sun, G. Q., Ahmed, A., Dittmer, N. & Attardo, G. (2002) *Insect Biochem. Mol. Biol.* **32**, 1275–1286.
6. Raikhel, A. S., McGurk, L. & Bownes, M. (2003) in *Encyclopedia of Hormones*, eds. Henry, H. L. & Norman, A. W. (Academic, San Diego), Vol. 1, pp. 451–459.
7. Bender, M. (2003) in *Encyclopedia of Hormones*, eds. Henry, H. L. & Norman, A. W. (Academic, San Diego), Vol. 1, pp. 438–446.
8. Ashburner, M., Chihara, C., Meltzer, P. & Richards, G. (1974) *Cold Spring Harbor Symp. Quant. Biol.* **38**, 655–662.
9. Yao, T. P., Segraves, W. A., Oro, A. E., McKeown, M. & Evans, R. M. (1992) *Cell* **71**, 63–72.
10. Yao, T. P., Forman, B. M., Jiang, Z., Cherbas, L., Chen, J. D., McKeown, M., Cherbas, P. & Evans, R. M. (1993) *Nature* **366**, 476–479.
11. Thomas, H. E., Stunnenberg, H. G. & Stewart, A. F. (1993) *Nature* **362**, 471–475.
12. Antoniewski, C., Laval, M., Dahan, A. & Lepesant, J. A. (1994) *Mol. Cell. Biol.* **14**, 4465–4474.
13. Lehmann, M. & Korge, G. (1995) *EMBO J.* **14**, 716–726.
14. Kokoza, V. A., Martin, D., Mienaltowski, M. J., Ahmed, A., Morton, C. M. & Raikhel, A. S. (2001) *Gene* **274**, 47–65.
15. Martin, D., Wang, S. F. & Raikhel, A. S. (2001) *Mol. Cell. Endocrinol.* **173**, 75–86.
16. Kapitskaya, M., Wang, S., Cress, D. E., Dhadialla, T. S. & Raikhel, A. S. (1996) *Mol. Cell. Endocrinol.* **121**, 119–132.
17. Wang, S. F., Li, C., Zhu, J., Miura, K., Miksicek, R. J. & Raikhel, A. S. (2000) *Dev. Biol.* **218**, 99–113.
18. Wang, S. F., Li, C., Sun, G., Zhu, J. & Raikhel, A. S. (2002) *Mol. Cell. Endocrinol.* **196**, 29–42.
19. Miura, K., Wang, S. F. & Raikhel, A. S. (1999) *Mol. Cell. Endocrinol.* **156**, 111–120.
20. Zhu, J., Miura, K., Chen, L. & Raikhel, A. S. (2003) *Proc. Natl. Acad. Sci. USA* **100**, 544–549.
21. Urness, L. D. & Thummel, C. S. (1995) *EMBO J.* **14**, 6239–6246.
22. Sun, G. Q., Zhu, J. S., Li, C., Tu, Z. J. & Raikhel, A. S. (2002) *Mol. Cell. Endocrinol.* **190**, 147–157.
23. Sun, G. Q., Zhu, J. S., Li, C. & Raikhel, A. S. (2004) *Mol. Cell. Endocrinol.* **218**, 95–105.
24. Hays, A. R. & Raikhel, A. S. (1990) *Roux's Arch. Dev. Biol.* **199**, 114–121.
25. Zhu, J., Chen, L. & Raikhel, A. S. (2003) *Proc. Natl. Acad. Sci. USA* **100**, 13338–13343.
26. Wang, S. F., Miura, K., Miksicek, R. J., Segraves, W.A. & Raikhel, A. S. (1998) *J. Biol. Chem.* **273**, 27531–27540.
27. Hu, X., Cherbas, L. & Cherbas, P. (2003) *Mol. Endocrinol.* **17**, 716–731.
28. Miley, G. R., Fantz, D., Glossip, D., Lu, X., Saito, R. M., Palmer, R. E., Inoue, T., Van Den Heuvel, S., Sternberg, P. W. & Kornfeld, K. (2004) *Genetics* **167**, 1697–1709.
29. Blandin, S., Moita, L. F., Kocher, T., Wilm, M., Kafatos, F. C. & Levashina, E. A. (2002) *EMBO Rep.* **3**, 852–856.
30. Hansen, I. A., Attardo, G. M., Park, J.-H., Peng, Q. & Raikhel, A. S. (2004) *Proc. Natl. Acad. Sci. USA* **101**, 10626–10631.
31. Li, R., Pei, H. & Watson, D. K. (2000) *Oncogene* **19**, 6514–6523.
32. Darby, T. G., Meissner, J. D., Ruhlmann, A., Mueller, W. H. & Scheibe, R. J. (1997) *Oncogene* **15**, 3067–3082.
33. Tolon, R. M., Castillo, A. I., Jimenez-Lara, A. M. & Aranda, A. (2000) *Mol. Cell. Biol.* **20**, 8793–8802.