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Regulation of gene expression by 20-hydroxyecdysone in the fat body of *Aedes aegypti* (Diptera: Culicidae)

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Abstract. In response to a blood meal, the fat body of the female mosquito produces several yolk proteins (YP) which are accumulated by the developing oocytes. 20-hydroxyecdysone (20E) stimulates high levels of YP gene expression in fat bodies cultured in vitro, but initiation of this expression was eliminated with cycloheximide. In the 1.5 kb upstream region of the gene encoding vitellogenic carboxypeptidase, a mosquito YP, there are several putative regulatory sequences that resemble steroid response elements (SRE), but they do not match the ecdysteroid response element (EcRE) consensus. A cDNA encoding a mosquito ecdysteroid receptor (AaEcR) has recently been cloned and found to possess P-Box and D-Box domains nearly identical to those found in the *Drosophila* and *Chironomus* EcRs, indicating that its DNA binding sequence will likely match the EcRE consensus. Taken together, these results indicate that the control of YP genes in the vitellogenic mosquito fat body by 20E is indirect. It may involve several factors in a hierarchy, with a factor other than the AaEcR directly controlling the YP genes.

In the mosquito fat body, lysosomal enzymes are involved in termination of YP secretion. The mRNA of a mosquito lysosomal aspartic protease (LAP) reaches its peak 12 hr before the peak of LAP protein and enzymatic activity. The level of LAP protein rises as the titer of 20E declines. This suggests a translational inhibition of LAP mRNA by a high titer of 20E. We obtained direct experimental confirmation of this novel function of 20E. In addition, the 5'-untranslated region of the LAP mRNA shares similarity with elements conferring negative translational control by steroids. The LAP gene is controlled by dual promoters, one of which is dominant during enhanced expression. The identification of putative regulatory elements should help in determining the regulation of the gene during both housekeeping and enhanced expression.

INTRODUCTION

The vitellogenic cycle of the anautogenous mosquito *Aedes aegypti* can be divided into three distinct phases. First, the insect undergoes a preparatory phase in which the fat body becomes capable of intense synthesis of yolk protein precursors (YPs) and the ovary becomes competent to take-up and store the YPs. This process is thought to be under the control of juvenile hormone III and is followed by a state-of-arrest until blood-feeding takes place. After a blood meal, the insect enters the synthetic phase of vitellogenesis, during which the fat body produces YPs for subsequent uptake and storage in the yolk bodies of the eggs. Finally, in the termination phase, vitellogenesis is halted and the fat body cells are remodeled through a proliferation of lysosomes that selectively degrade the synthetic organelles responsible for YP production. 20-hydroxyecdysone (20E) is thought to be

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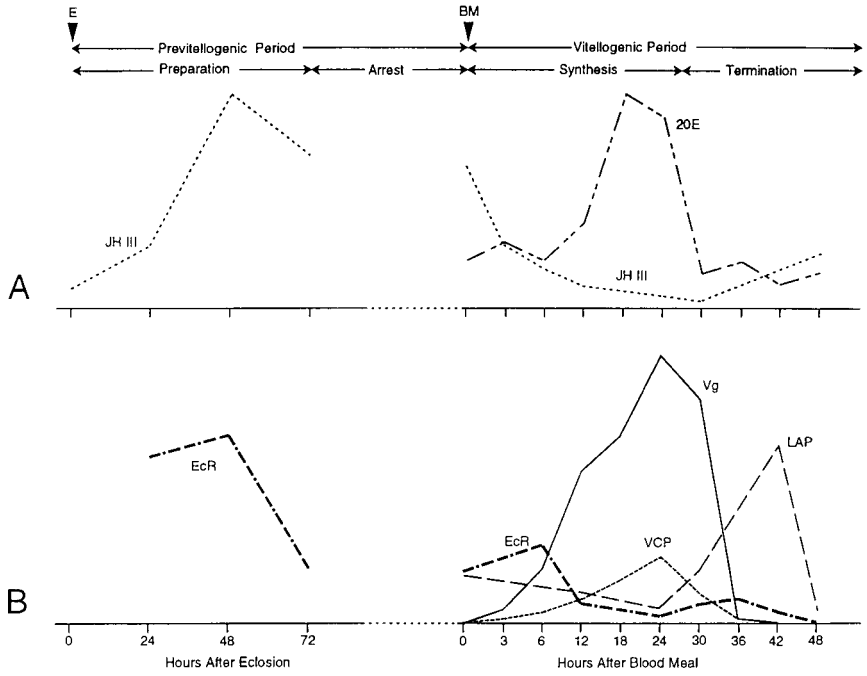

Fig. 1. Summary of events during the first vitellogenic cycle in the anautogenous mosquito, *Aedes aegypti*. A: hormonal events. JH III – juvenile hormone III (modified from Hagedorn 1985); 20E – 20-hydroxyecdysone (modified from Shapiro et al., 1986). B: events in the fat body. EcR – titer of mosquito ecdysteroid receptor mRNA (modified from Cho et al., 1994); VCP – rate of vitellogenic carboxypeptidase synthesis; Vg – rate of vitellogenin synthesis; LAP – enzymatic activity of lysosomal aspartic protease. (From Raikhel, 1992.)

involved in the control of these phases (Raikhel, 1992; Dhadialla & Raikhel, 1994). The events associated with this cycle are summarized in Fig. 1.

During the synthetic phase, the fat body transcribes large quantities of YP mRNA, and the corresponding proteins are produced and transported to the developing oocytes. The titer of 20E is closely correlated with the rate of YP synthesis. 20-hydroxyecdysone titers begin to rise at 6 hr post blood meal, and reach their maximum level at 18–24 hr post blood meal (Hagedorn, 1983, 1985; Raikhel, 1992) (Fig. 1A). This observation led to the initial hypothesis that YP production is under the control of 20E. However, the exact nature of 20E action in the fat body was unclear.

Most of the early research into YP production in the mosquito focused on the major YP, vitellogenin (Vg). In addition to Vg, a second YP has been described called vitellogenic carboxypeptidase (VCP) (Hays & Raikhel, 1990; Cho et al., 1991a). In vivo and in vitro experiments indicate that this protein is synthesized in the same sex-, stage- and tissue-specific manner as Vg. Recently, cDNAs encoding both Vg and VCP have been cloned and sequenced (Cho et al., 1991a; Chen et al., 1994), providing molecular probes for investigating the role of 20E in the control of these two genes.

Ashburner et al. (1974) proposed a model for the mode of action of 20E in *Drosophila* larval salivary glands. They hypothesized that 20E, complexed with its receptor, directly regulates two classes of genes: a small class of early genes that are transcribed, and a large class of late genes that are repressed. The early genes encode transcription factors that repress early gene transcription and induce late gene transcription. Recent research at the molecular level has revealed many genes in *Drosophila* whose regulation is consistent with this model (Segraves & Hogness, 1990; Burtis et al., 1990; Koelle et al., 1991). Thus, 20E is thought to operate through a cascade or hierarchy of genes initially induced by the 20E/receptor complex, leading ultimately to expression of numerous downstream target genes. A similar cascade may be involved in the control of YP synthesis in the vitellogenic fat body of the mosquito. The following is a summary of recent research done by the authors of this paper at the molecular level concerning the hormonal control by 20E of the vitellogenic events in the fat body of the mosquito, *Aedes aegypti*.

INDIRECT CONTROL OF 20E ON THE YP GENES

The yolk protein genes are hormonally regulated and stringently controlled in a sex-, stage- and tissue-specific manner. They are not expressed in fat bodies of previtellogenic female mosquitoes, however, when isolated abdomens with adhering fat bodies (hereafter referred to as the fat body) from previtellogenic females are cultured in vitro, 20E initiates transcription of the YP genes encoding Vg and VCP. This 20E stimulation is dose dependent, with maximal levels of mRNA observed at 10^{-6} and 10^{-5} M 20E in the culture medium (Deitsch et al., in press). If the Ashburner model applies to 20E action in the vitellogenic fat body of mosquitoes, then inhibition of protein synthesis should block the cascade and eliminate induction of Vg and VCP gene expression. Alternatively, if the action of 20E on the YP genes is direct, then inhibition of protein synthesis should not effect Vg and VCP mRNA levels. This question was addressed using the protein synthesis inhibitor cycloheximide (Chx). A dose response curve showed that a 10^{-4} M concentration of Chx inhibited > 98% of protein synthesis in cultured vitellogenic fat bodies (Deitsch et al., in press).

The effect of Chx on induction of the YP genes in response to 20E was tested by culturing previtellogenic female fat bodies in media containing 10^{-4}M Chx for 1 hr, then in media containing 10^{-5}M 20E and 10^{-4}M Chx for 6 hr. Cycloheximide virtually eliminated induction of the Vg and VCP genes in response to 20E. At the same time, Chx appeared to have little effect on the mRNA levels of the housekeeping actin gene (Table 1). Removal of Chx from the culture media enabled the cultured tissue to recover and respond to 20E by increasing the levels of Vg and VCP mRNA, indicating that the inhibition of Vg and VCP by Chx was not simply the result of cell death (Deitsch et al., in press). Thus, these experiments indicate that inhibition of protein synthesis abolishes the ability of 20E to induce YP expression and the effect of the hormone is therefore indirect.

In *Xenopus laevis,* the liver produces high levels of Vg in response to estrogen. Experiments using Chx showed that the effect of estrogen on Vg mRNA production is direct because inhibition of liver protein synthesis does not prevent activation of Vg gene transcription (Hayward et al., 1982). The action of 20E on the YP genes in flies appears to be different. In *Drosophila*, Bownes et al. (1987) used Chx to determine the nature of the effect of 20E on YP transcription. Their results indicated that protein synthesis is required

for 20E to initiate and maintain YP expression. However, these studies were done in vivo and secondary effects of Chx on the insect cannot be ruled out. By using a well defined in vitro system, the relevant tissue can be isolated and a more controlled experiment done. Our studies indicate that in the mosquito 20E is acting on the genes for Vg and VCP indirectly. 20E induces these genes in a dose dependent manner, yet protein synthesis inhibition completely eliminates this induction. Thus, a 20E response cascade is likely induced by a rising titer of the hormone, with the genes for Vg and VCP somewhere downstream in the hierarchy.

TABLE 1. Effect of Chx on Vg, VCP and actin mRNA production. Previtellogenic fat bodies were incubated with or without Chx for 1 hr, then cultured for 6 hr in media (Dhadialla & Raikhel, 1990) containing different combinations of Chx and 20E. Fat bodies taken from previtellogenic sugar-fed females were used as a control. After culturing, total RNA was extracted and levels of Vg, VCP and actin mRNA measured by slot blot and hybridization to the appropriate radiolabeled cDNA probes. Values expressed are cpm (modified from Deitsch et al., 1995).

	Media	Media/Chx	Media/Chx/20E	Media/20E	Control
Vg	41.8 ± 9.0	48.2 ± 13.4	44.9 ± 1.7	451.0 ± 54.9	51.4 ± 1.2
VCP	16.6 ± 9.2	20.2 ± 4.1	16.6 ± 2.2	109.8 ± 7.1	18.8 ± 10.9
Actin	43.2 ± 6.3	48.9 ± 9.4	38.9 ± 1.0	62.8 ± 10.1	46.8 ± 2.8

STRUCTURE OF THE GENE ENCODING VITELLOGENIC CARBOXYPEPTIDASE

Indirect action of 20E is consistent with the recent finding that a putative response element exists upstream of the VCP gene, showing limited similarity to the consensus ecdysteroid receptor binding site (Deitsch & Raikhel, 1993). This palindromic sequence is tandemly repeated four times, with no spacing between half sites (Fig. 2A). In contrast, a

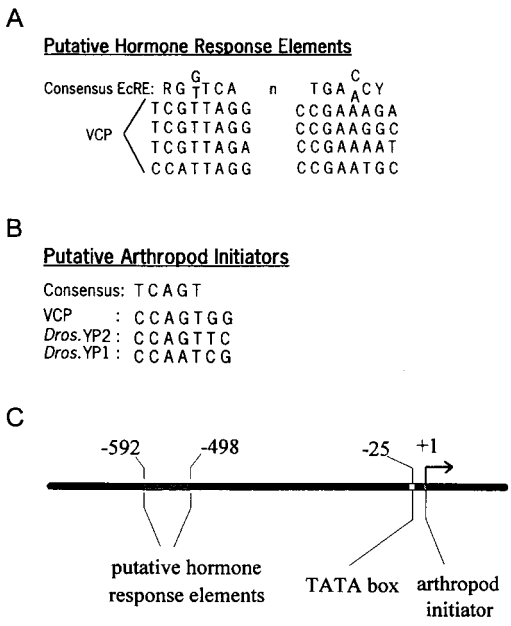

Fig. 2. Potential regulatory elements and their position upstream of the gene for VCP. A: A 16 bp imperfect palindrome found repeated four times with similarity to the consensus ecdysteroid response element. B: Comparison of a putative arthropod initiator sequence found at the start site of the VCP transcribed region with the consensus arthropod initiator and the start site sequences from the YP 1 (*Dros.* YP1) and 2 (*Dros.* YP2) genes from *Drosophila* (Garabedien et al., 1986). C: Diagrammatic representation of the structure of the VCP gene (modified from Deitsch & Raikhel, 1993).

A

	DNA Binding Domain (%)	Hormone Binding Domain (%)
Drosophila EcR	97	87
Chironomus EcR	98	77

B

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AaEcR    CLVCGDRASGYHYNALTCEGCKGFFRRSVTKN...
DmEcR    CLVCGDRASGYHYNALTCEGCKGFFRRSVTKS...
                          P-BOX

AaEcR    AVYCCKFGHACEMDMYMRRKCQECRLKKCLAVGM
DmEcR    AVYCCKFGRACEMDMYMRRKCQECRLKKCLAVGM
             D-BOX
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Fig. 3. Comparison of the mosquito ecdysteroid receptor with ecdysteroid receptors from *Drosophila* and *Chironomus*. A: Amino acid sequence identity in both the DNA binding domain and the hormone binding domain. B: Sequence comparison of the P-box and the D-box domains of the mosquito (AaEcR) and *Drosophila* (DmEcR) ecdysteroid receptors (modified from Cho et al., 1994).

one base pair spacer is necessary for binding of the ecdysteroid receptor in *Drosophila* (Antoniewski et al., 1993). Combined with the evidence showing indirect control of 20E induction of the YP genes, this suggests that the putative response element is bound by a transcription factor other than the 20E/receptor complex. Elucidation of this activating protein awaits further research. In addition, a sequence recently identified as an arthropod initiator (Cherbas & Cherbas, 1993) was found at the start site of VCP gene transcription (Fig. 2B). This sequence is thought to work in conjunction with upstream receptor binding sites. These elements, combined with a TATA box and a ribosomal binding site, potentially provide the control necessary for the hormone activation of this gene. The structure of the VCP gene is shown in Fig. 2C.

MOSQUITO ECDYSTEROID RECEPTOR

A cDNA for a mosquito ecdysteroid receptor (AaEcR) has recently been cloned (Cho et al., 1994). This receptor has a DNA binding domain with two zinc fingers, and a ligand binding domain characteristic of members of the steroid hormone receptor superfamily. These domains share high levels of identity with the respective domains of the *Drosophila* and *Chironomus* EcRs (Fig 3A). The proximal (P) and distal (D) boxes in the zinc finger region of the DNA binding domain are important for the specific recognition of the particular hormone response element. The P-box of the AaEcR is 100% identical to that of the *Drosophila* EcR, while its D-box has only a single amino acid substitution (Fig. 3B). This indicates that the binding specificity of the AaEcR to DNA will likely depend on a one base pair spacer between the half sites of the response element, as has been shown for the *Drosophila* EcRE (Antoniewski et al., 1993).

There is a peak in AaEcR expression in previtellogenic females at 1 and 2 days after eclosion; the mRNA level then falls 3 fold at 3 days post-eclosion. Once vitellogenesis begins, there is a smaller peak at 6 hr post blood meal, and by 48 hr post blood meal, AaEcR mRNA levels are no longer detectable (Fig. 1C). These kinetics suggest that the AaEcR is produced in preparation for the major events of vitellogenesis in which 20E is

involved. The responsiveness of the fat body to 20E is therefore probably due to accumulation of the ecdysteroid receptor in this tissue during previtellogenesis.

REGULATION OF A LYSOSOMAL ASPARTIC PROTEASE BY 20E

In female mosquitoes, there is a dramatic increase in lysosomal activity in the fat body during termination of YP synthesis (Fig. 1B). This lysosomal activity is directed toward specific degradation of organelles involved in the biosynthesis and secretion of YPs (Raikhel, 1992). A mosquito lysosomal aspartic protease (LAP) with cathepsin D activity was purified and characterized (Cho et al., 1991b). A cDNA coding for LAP was cloned and sequenced. The amino acid sequence of the conserved domain of LAP is 92% and 81% similar to human cathepsin D and cathepsin E, respectively (Cho & Raikhel, 1992).

The LAP mRNA reaches its highest level 24 hr post blood-meal, 12 hr before peak protein accumulation and enzymatic activity. Between 12 and 24 hr post blood-meal, when the titer of 20E is high, the LAP protein titer is low (even though the LAP mRNA level is rising). As the 20E titer drops, LAP protein levels rise. We believe this represents negative translational regulation of LAP mRNA by 20E. In addition, the 5'-untranslated region of the LAP mRNA has sequences similar to elements that have been shown to confer negative translational control by steroids (Cho & Raikhel, 1992). This hypothesis was tested both in vivo and in vitro. Female mosquitoes were injected with 1 ng of 20E at 20, 30 and 36 hr post blood-meal, to keep the titer of 20E artificially high (Fig. 4A). Fat bodies were dissected at either 36 or 42 hr post blood-meal (when the level of LAP protein is normally at its peak)

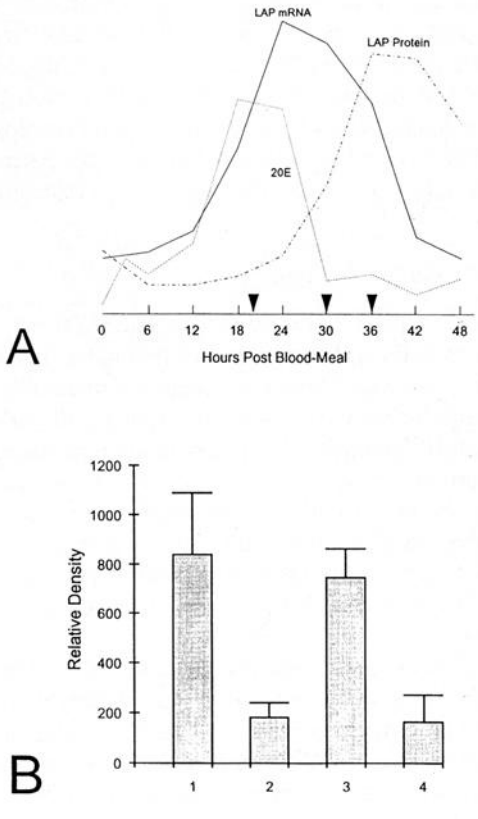

Fig. 4. Translational inhibition of LAP by 20E in vivo. A: Normal levels of 20E and of LAP mRNA and protein during vitellogenesis. 20E curve is the same as in Fig. 1. LAP mRNA and protein curves are taken from Cho & Raikhel (1992). Arrow heads indicate time of 20E injections. B: LAP protein levels in fat body tissue. LAP protein was detected using Western blot analysis utilizing anti-LAP antibodies and chemiluminescence. Quantification is by densitometric scanning (relative density). 1 – fat bodies dissected at 36 hr post blood-meal (control); 2 – fat bodies dissected at 36 hr post blood-meal after 20E injections at 20 and 30 hr post blood-meal; 3 – fat bodies dissected at 42 hr post blood-meal (control); 4 – fat bodies dissected at 42 hr post blood-meal after 20E injection at 20, 30 and 36 hr post blood-meal.

and protein levels were determined. This experiment showed that in vitellogenic mosquitoes injected with 20E, LAP protein levels were significantly lower than the intact controls (Fig. 4B). When fat bodies were dissected at 24 hr post blood-meal and cultured in vitro in the presence of 20E (10^{-4} M), LAP protein levels again remained low. Fat bodies cultured without 20E had LAP protein levels similar to that of the intact controls (data not shown). Thus we have identified a novel function of 20E, that of negative translational regulation. Elucidation of the molecular mechanism of this action presents a challenge for future investigations.

As a housekeeping gene, LAP is expressed ubiquitously in all cells at a basal level. Its increase in activity during vitellogenesis shows stage-, tissue- and sex-specific regulation. Primer extension analysis has identified two transcription start sites for the gene. Both transcripts are present in previtellogenic tissue, at approximately equal levels. However, during vitellogenesis, the smaller of the two transcripts is more highly expressed. To investigate the regulation of the LAP gene, in both its housekeeping and enhanced roles, a clone was isolated from a genomic library using the cDNA as a probe. The gene is divided into 4 or 5 exons (depending on the transcript expressed), ranging in size from 81–951 base pairs. Similar to VCP, a short sequence matching the arthropod initiator is located 2 base pairs downstream of the initiation site for the larger transcript. In addition, several sequences resembling tissue-specific enhancers and steroid response elements were found in the 5'-upstream region (Dittmer & Raikhel, unpublished data). These sequences may play a role in the regulation of the gene during either its housekeeping or enhanced expression.

CONCLUSIONS

During egg maturation, the mosquito fat body undergoes a cycle of massive YP production accompanied by stringently controlled proliferation and degradation of biosynthetic cellular machinery. Understanding this cycle at the molecular level offers great potential for developing novel methods of mosquito control. The steroid hormone 20E plays an important, if not dominant, role in the regulation of this developmental process, exerting its influence at both the transcriptional and translational levels.

The cloning and sequencing of the gene for VCP have provided a valuable tool for investigating the sex-, stage- and tissue-specific expression of the YP genes and their response to 20E. The fact that the effect of this hormone is indirect indicates that a hormone response cascade exists, and that the YP genes are controlled somewhere downstream in the hierarchy. By further investigating the putative control elements identified in the regulatory regions of the VCP gene, a more complete picture of how 20E acts to control YP production can be learned. The cDNA clone encoding a mosquito ecdysteroid receptor will be valuable in elucidating the components of the vitellogenesis hierarchy. The strong similarity of the AaEcR with the *Drosophila* EcR in the DNA binding domain indicates that it should recognize a nearly identical response element.

In vivo and in vitro experiments have provided evidence that 20E controls LAP gene expression through translational inhibition. The transcription of LAP is under the control of dual promoters, both of which are constitutively expressed at approximately equal levels. However, during enhanced expression, one of the promoters is dominant. Since this enhanced expression shows the same temporal pattern as Vg and VCP expression, 20E may also regulate LAP transcription.

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