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Insect Biochemistry and Molecular Biology 36 (2006) 366–374
Insect Biochemistry and Molecular Biology
www.elsevier.com/locate/ibmb

Comparative genomics of insect juvenile hormone biosynthesis ☆

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Abstract

The biosynthesis of insect juvenile hormone (JH) and its neuroendocrine control are attractive targets for chemical control of insect pests and vectors of disease. To facilitate the molecular study of JH biosynthesis, we analyzed ESTs from the glands producing JH, the *corpora allata* (CA) in the cockroach *Diploptera punctata*, an insect long used as a physiological model species and compared them with ESTs from the CA of the mosquitoes *Aedes aegypti* and *Anopheles albimanus*. The predicted genes were analyzed according to their probable functions with the Gene Ontology classification, and compared to *Drosophila* and *Anopheles gambiae* genes. A large number of reciprocal matches in the cDNA libraries of cockroach and mosquito CA were found. These matches defined known and suspected enzymes of the JH biosynthetic pathway, but also several proteins associated with signal transduction that might play a role in the modulation of JH synthesis by neuropeptides. The identification in both cockroach and mosquito CA of homologs of the small ligand binding proteins from insects, Takeout/JH binding protein and retinol-binding protein highlights a hitherto unsuspected complexity of metabolite trafficking, perhaps JH precursor trafficking, in these endocrine glands. Furthermore, many reciprocal matches for genes of unknown function may provide a fertile ground for an in-depth study of allatal-specific cell physiology.

Keywords: Juvenile hormone (JH); Corpora allata (CA)

1. Introduction

The growth, development, metamorphosis and reproduction of insects are under control of juvenile hormones (JHs) and ecdysteroids, or molting hormones, secreted by specific endocrine glands, the corpora allata (CA) and prothoracic glands (Gilbert et al., 2000; Stay, 2000). The receptors for these two major groups of insect hormones have each become targets for non-neurotoxic, insect-selective pesticides. The development of these "biorational" insecticides, such as methoprene and tebufenozide, was based on classical bioassays measuring the agonist activity of these hormones (Dhadialla et al., 2005). It is likely that new classes of insecticides acting as inhibitors of insect hormone biosynthesis will emerge as well, with a biological effect based on hormone deficit rather than hormone excess. However, little is known on the molecular aspects of hormone biosynthesis and regulation, so that high-throughput screens of such endocrine targets cannot be deployed. Recently, several genes coding for enzymes of the ecdysteroid biosynthetic pathway in prothoracic glands have been identified in *Drosophila melanogaster* through the study of Halloween mutants, and in *Bombyx mori* (Warren et al., 2002, 2004; Niwa et al., 2004). Fewer molecular tools are available for the analysis of JH biosynthesis even though the early steps of JH biosynthesis follow the mevalonate pathway from acetyl-CoA to farnesyl pyrophosphate (Bellés et al., 2005).

☆Data deposition: ESTs are deposited in GenBank under the accession numbers DV017592-DV018447 (*Diploptera punctata*) DR746432-DR747949 (*Aedes aegypti*) and DR747950-DR748310 (*Anopheles albimanus*).

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doi:10.1016/j.ibmb.2006.01.013

The cockroach, *Diploptera punctata*, has been extensively used as a model species for the study of JH biosynthesis and its regulation, because of its favorable life history traits (viviparity ensuring well-timed cycles of JH synthesis in the adult female) and because of its high levels of hormone production (Stay, 2000). It is important to extend and validate the knowledge obtained in this model species to species of greater biomedical relevance, such as mosquitoes. In order to facilitate the study of mosquito endocrinology, it was felt that a transfer of molecular tools specifically associated with the endocrine function and regulation of the CA from the model species to mosquitoes would be desirable. In the absence of a cockroach genome project, we chose a comparative EST approach.

We present here the analysis of ESTs from the CA of *D. punctata* compared to a similar effort from *Aedes aegypti* and *Anopheles albimanus*. Reciprocal matches between the cockroach and the mosquito ESTs, as well as reference to the *Anopheles gambiae* and *Drosophila* genomes in particular allowed us to assign probable function to the majority of genes coding for enzymes of the JH biosynthetic pathway. This has already been validated by the functional identification of three cockroach ESTs as representing methyl farnesoate epoxidase, the last enzyme in JH biosynthesis (Helvig et al., 2004). In addition, we identified a number of gene products coding for potential elements of the signaling pathways in the CA. Our study provides a basis for the systematic and comprehensive analysis of molecules that may play an active role in the function and regulation of this endocrine gland.

2. Materials and methods

2.1. Insects

D. punctata were reared as previously described (Unnithan and Feyereisen, 1995). *A. aegypti* and white-striped pupal phenotype of *An. albimanus* were obtained from the insectary of the Instituto Nacional de Salud Pública (INSP, Cuernavaca, Mexico). Adult mosquitoes were reared under a photoperiod cycle of 12 h light:12 h dark, 27–28 °C and 70–80% relative humidity, and were fed ad libitum with 5% sugar. All mosquitoes used in this study were two- and three-day-old mated females.

2.2. Corpora allata ESTs

CA + corpora cardiaca (CC) from *A. aegypti* (80 pairs) and *An. albimanus* (40 pairs) were dissected and rinsed in PBS and stored in 50 μl of RNAlater (Ambion). Mosquito mRNA was isolated using the Micro-FastTrack mRNA isolation kit (Invitrogen, San Diego, CA, USA). The PCR-based cDNA library was made following the instructions for the SMART cDNA library construction kit (Clontech, Palo Alto, CA, USA) exactly as described (Francischetti et al., 2002). Samples were prepared and sequenced on a CEQ 2000 DNA sequencing instrument (Beckman Coulter Inc.) (10). ESTs from the CA of adult mated female *D. punctata* aged 3–5 days were obtained as described (Helvig et al., 2004).

2.3. Bioinformatic tools

ESTs were trimmed of primer and vector sequences, assembled, and compared to other databases (such as GO, PFAM, KOG, SMART, *Drosophila melanogaster* PROT, *An. gambiae* PROT, etc.), as described before (Valenzuela et al., 2003). The BLAST tool (Altschul et al., 1990) and CAP3 assembler were used (Huang and Madan, 1999).

2.4. Searches for known sequence similarities and known protein domains of the cDNA sequences

To obtain information on the possible role of the cDNA sequences, the assembled FASTA file was blasted against the GenBank nonredundant protein database (NR) from the National Center for Biotechnology Information (NCBI) using the standalone BlastX (Altschul et al., 1990). Databases were organized in spreadsheets as shown in AnoXcel (Ribeiro et al., 2004) and are available from the authors upon request.

2.5. Organization of an A. aegypti cDNA database

144,672 ESTs were downloaded from the TIGR site http://www.tigr.org/tigr-scripts/tgi/cat_download.pl?db=aaest. These were clusterized in 19,282 contigs and singletons as indicated above, and organized in a spreadsheet format similar to AnoXcel (Ribeiro et al., 2004). This assembled database of ~19,000 contigs and singletons were compared to the assembled EST database from *Aedes* CA to obtain full-length or near-full-length putative transcripts of interest.

The database can be accessed from AnoBase at http://www.anobase.org/AnoBase/Genes/AegyXcel.html.

3. Results

3.1. Corpora allata ESTs

The 5′ end of randomly selected cDNA clones was sequenced to establish the EST libraries. Following clusterization and assembly, the resulting contigs (consensus sequence resulting from more than one sequence) or singletons (sequences not containing enough similarity to other sequences in the database to be grouped into a cluster of related sequences) were analyzed by BLAST against the NR and other databases. For the *D. punctata* ESTs, 778 high-quality sequences were retained for analysis from an initial set of 1152 clones, and 478 independent clusters of contigs and singletons were organized. The most abundant ESTs coded for the mitochondrially encoded NADH dehydrogenase (159 ESTs) and cytochrome c oxidase (28

ESTs). For the *A. aegypti* ESTs, 1491 sequences were organized in 821 independent contigs and singletons. The most abundant ESTs were 60 S ribosomal protein (185 EST) and 40 S ribosomal protein (99 EST). Cytochrome c oxidase (61 EST) and NADH dehydrogenase (31 EST) were also very abundant. For *An. albimanus* ESTs, 356 sequences were organized in 260 contigs and singletons. The most abundant ESTs were an 18K mitochondrial protein (30 EST) and cytochrome c oxidase (24 EST). The 60 S ribosomal protein (14 EST) and 40 S ribosomal protein (13 EST) were also very abundant. Because in Diptera the tissue that comprises the CA cells also encompasses the CC cells, the major neurosecretory tissue of insects, it followed that genes encoding neurohormone precursors would be represented in the EST collection, and indeed we found the adipokinetic hormone (AKH) preprohormone in *A. aegypti* (18 ESTs) and in *An. albimanus* (10 ESTs) and the cardioacceleratory peptide (CCAP) preprohormone in *A. aegypti* (1 EST).

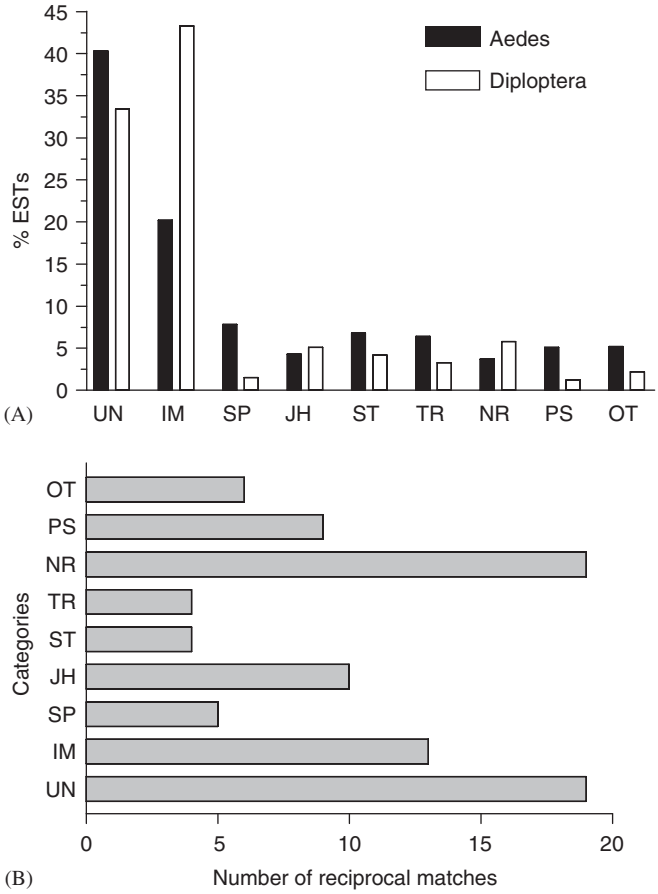

Fig. 1. (A) Distribution of ESTs from *Aedes aegypti* and *Diploptera punctata* corpora allata according to categories of function. The categories are: UN: unknown function; IM: Intermediate metabolism enzymes; SP: structural proteins; JH: enzymes linked to JH synthesis; ST: signal transduction; TR: transport; NR: nuclear regulation and transcriptional control; PS: protein synthesis; OT: others (proteases, immune system, apoptosis, and transposable elements). (B) Reciprocal matches between the EST collections from the cockroach and *A. aegypti* corpora allata classified by function as in panel A.

Annotation of the contigs by Gene Ontology (Fig. 1A) revealed slight differences between the *A. aegypti* and *D. punctata* contigs, with a greater portion of transcripts involved in protein synthesis and cytoskeleton (included in the structural protein Gene Ontology category) in the mosquito, and a greater portion of transcripts involved in intermediate metabolism in the cockroach. These differences may be related to the identity of the cell types represented in our collections, with the cockroach being more specifically representative of the CA, and the mosquito collection including transcripts from the CC, a neurosecretory organ, and probably contamination from other cell types as well (Li et al., 2003). The cockroach transcripts had a large proportion of transcripts related to transposable elements. As opposed to the mosquito sequences that are significantly related to *An. gambiae* or *Drosophila* sequences as expected (i.e. two-third of the contigs had close matches), the cockroach transcripts had a significant number of sequences of unknown function or "no hits" at stringent *e* values. About one-third of the *D. punctata* contigs had matches to the *An. gambiae* and *Drosophila* genomes. This lower overall measure of homology simply reflects the evolutionary distance from cockroaches to Diptera. However, the 89 reciprocal matches between the mosquito and the cockroach contigs represented a large proportion of the EST collection (Fig. 1B).

3.2. ESTs that represent enzymes of the JH biosynthetic pathway

Fig. 2 shows a proposed pathway for the synthesis of JH III. Four categories of ESTs were considered:

(1) ESTs representing enzymes of the classical isoprenoid pathway from acetyl-CoA to farnesyl pyrophosphate. These enzymes were predicted to be active in the CA, either because of precursor incorporation studies or because of partial biochemical characterization (see Bellés et al., 2005 for review). Those enzymes with corresponding ESTs include: hydroxymethylglutaryl-CoA synthase, that condenses acetoacetyl-CoA and acetyl-CoA to form HMG-CoA; mevalonate kinase, a phosphotransferase that phosphorylates mevalonic acid to 5-phosphomevalonic acid; isopentenyl-diphosphate delta-isomerase, that catalyzes the interconversion of isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP), farnesyl pyrophosphate synthetase, a prenyltransferase that catalyzes the condensation of isopentenyl pyrophosphate with geranylpyrophosphate to farnesyl pyrophosphate, which is the last product of the classical terpenoid pathway in the CA. An additional three sequences were amplified by PCR from the *A. aegypti* cDNA library, representing HMG-CoA reductase, mevalonate kinase, and phosphomevalonate kinase. Mevalonate kinase was represented by six ESTs in the *D. punctata* collection (Table 1).

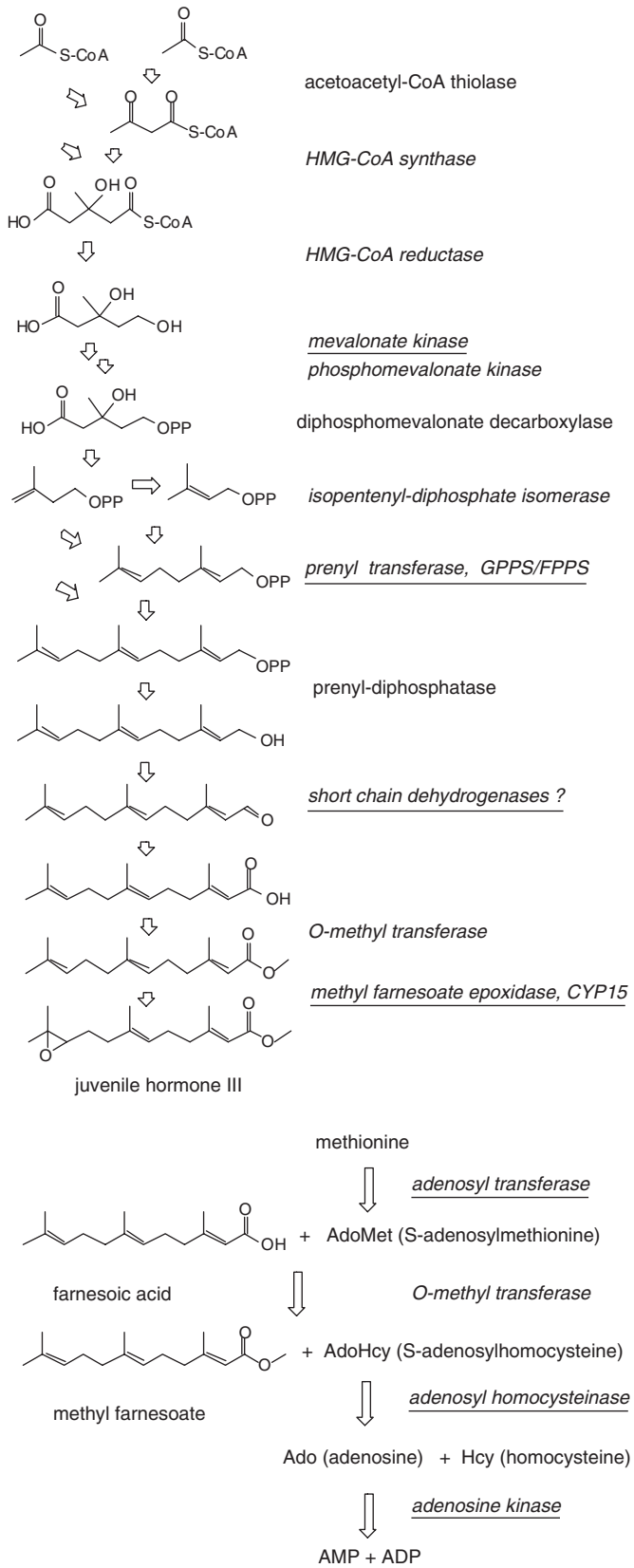

Fig. 2. Scheme of juvenile hormone biosynthesis from acetyl-CoA (top) and details of the methyl transfer portion of the pathway (bottom). Enzymes for which ESTs have been identified in both cockroach and mosquito are underlined in italics. Enzymes marked in italics were represented by ESTs in just one of the two insects.

(2) ESTs representing JH-specific steps starting from farnesyl pyrophosphate. Little is known on these enzymes and even their biochemical nature is in doubt. Two short-chain dehydrogenase homologs were found, and are potential candidates for the NAD^+-dependent farnesol/al dehydrogenases, that catalyze the oxidation of farnesol into farnesal or farnesal to farnesoic acid (Baker et al., 1983). However, this oxidation may instead be catalyzed by a specific alcohol oxidase (Sperry and Sen, 2001). Strong staining for farnesol dehydrogenase activity and farnesal oxidase activity has been observed in the CA portion of larval ring glands of a Drosophila mutant lacking alcohol dehydrogenase (Madhavan et al., 1973). In the Aedes EST collection, we found an ortholog of the *B. mori* JH acid methyl transferase (Shinoda and Itoyama, 2003), and, interestingly, for an homolog of the sequence described as a farnesoic acid *O*-methyltransferase in Crustacea (Gunawardene et al., 2002) as well. The *B. mori* JH acid methyl transferase homolog and short-chain dehydrogenase were also present in the *An. albimanus* EST collection. Finally, three ESTs coding for a new cytochrome P450 were found in *D. punctata* and shown previously to encode methyl farnesoate epoxidase (Helvig et al., 2004). This P450, CYP15A1, catalyzes the oxidation of methyl farnesoate into JH III. Its ortholog was found in the *A. aegypti* EST collection.

(3) ESTs representing enzymes of S-adenosylmethionine (AdoMet) metabolism (Fig. 2). AdoMet is the second substrate of farnesoic acid *O*-methyl transferase (OMT), and AdoHcy is its second product. Significantly, we found ESTs in both cockroach and the two mosquito libraries for the enzyme synthesizing AdoMet, adenosyl transferase, on one hand, and for adenosine homocysteinase on the other hand. Adenosyl homocysteinase catalyzes the degradation of AdoHcy into homocysteine and adenosine and thus removes AdoHcy, a known inhibitor of the methylation reaction (Feyereisen and Farnsworth, 1987a). Further, ESTs for adenosine kinase were also identified in both cockroach and the two mosquito libraries.

(4) ESTs that may provide a glimpse into the biochemistry of precursor supply for the JH pathway. In this category, we identified in *Diploptera* a dodecenoyl-CoA isomerase, possibly involved in fatty acid β-oxidation, and in *Aedes* a pantothenate kinase involved in CoA biosynthesis and also a mitochondrial citrate synthase. In addition, in *Aedes* and *Diploptera* we found a tricarboxylate transport protein that exports citrate from the mitochondria to the cytosol where it is converted to acetyl-CoA by ATP-citrate synthase, as well as ESTs for porphobilinogen synthase, also known as 5-aminolevulinate dehydratase represents the heme and vitamin B12 biosynthetic pathway.

Table 1
Corpora allata ESTs representing genes involved in the juvenile hormone biosynthetic pathway

Enzyme	Function	*D. punctata*	*A. aegypti*	*Anopheles* ortholog	*Drosophila* ortholog	*Aedes* TIGR
Acetoacetyl CoA thiolase EC 2.3.1.9	Condenses 2 molecules of Acetyl-CoA	—	—	XP_321828	NP_612094	TC65400 TC40994
Hydroxymethylglutaryl-CoA synthase EC 2.3.3.10	Condenses Acetoacetyl-CoA + acetyl-CoA	1	—	XP_315872	NP_725570	—
Hydroxymethylglutaryl-CoA reductase EC 1.1.1.34	Reduces HMG-CoA to mevalonate	—	PCR	XP_307890	NP_996271	TC37800
Mevalonate kinase EC 2.7.1.36	Phosphorylates mevalonate	6	PCR	XM_319701	NM_176158	TC38755
Phosphomevalonate kinase EC 2.7.4.2	Phosphorylates phosphomevalonate	—	PCR	XP_310779	Q9VIT2	TC38722
Diphosphomevalonate decarboxylase EC 4.1.1.33	Decarboxylates MPP to IPP	—	—	—	—	TC36231
Isopentenyl-diphosphate delta-isomerase EC 5.3.3.2	Isomerization of IPP into DMAPP	—	1	XM_321388	NP_650962	TC37152
Farnesyl diphosphate synthetase (prenyltransferase) EC 2.5.1.1/10	Sequential condensation of IPP with DMAPP and then with GPP to form FPP	1	1	XP_308653	NP_477380	TC37496
Prenyl-diphosphatase EC 3.1.7.1	Hydrolysis of FPP to farnesol	—	—	EAA01914	—	—
Short-chain dehydrogenase	Oxidation of farnesol to farnesal?	1	1	XM_556135	NM_132467	TC37281
Short-chain dehydrogenase	Oxidation of farnesal to farnesoic acid?	1	—	XM_310515	NM_132238	TC43233
Crustacean "Farnesoic acid O-methyltransferase" homolog	Function unclear	—	2	XP_318631	NP_611544	TC41847
Bombyx JHA methyl transferase ortholog	Transfers methyl group from AdoMet to farnesoic acid	—	11	XP_314173	NP_609793	TC46414
Methyl farnesoate epoxidase (CYP15)	Oxidation of MF into JH III	3	1	XP_315675	NP_649151	TC45811
Citrate (*si*)-synthase EC 2.3.3.1	Synthesis of citrate in the mitochondria	—	1	XM_320478	—	TC46756
Mitochondrial citrate transport protein	Transports citrate from mitochondria to cytosol	1	22	XP_308964	NP_727450	TC17106
ATP citrate lyase EC 2.3.3.8	Synthesis of cytosolic acetyl-CoA from citrate	—	—	XM_319323	—	TC45530
Methionine adenosyl transferase EC 2.5.1.6	Synthesis of S-adenosyl-L-methionine (AdoMet)	3	1	XM_307861	NM_164362	TC36191 TC35749
Adenosylhomocysteinase EC 3.3.1.1	Hydrolysis of S-adenosyl-L-homocysteine (AdoHcy)	5	2	XM_311257	NM_078609	TC42030
Adenosine kinase EC 2.7.1.20	Phosphorylates adenosine	1	1	XM_307001	NM_168532	TC35765

The columns show the number of ESTs present in each collection (*D. punctata* and *A. aegypti*). PCR: indicates sequences that were amplified by polymerase chain reaction using as template the *A. aegypti* library.

ESTs potentially representing CA signal transduction pathways. The CA are under humoral and nervous control and although some neuropeptide effectors have been identified, little is known on their mode of action. A homolog of the *Drosophila* Nmda1 gene coding for an *N*-methyl-D-aspartate receptor (NMDAr) associated protein was found in the *D. punctata* EST collection. It has been suggested that NMDAr has a role in regulating JH synthesis (Chiang et al., 2002a). Additional examples of ESTs representing elements of signal transduction pathways were an AMP-activated protein kinase α2 (AMPK) homolog and a GTP-binding protein of the Rho subfamily in the *D. punctata* library. A presenilin homolog was found in the three CA libraries, and a calmodulin EST was found in *A. aegypti* and *An. albimanus*.

3.3. Small ligand binding protein homologs

ESTs were found in both *D. punctata* and *A. aegypti* for a protein of the *takeout* family. The *D. punctata* CA *takeout*-like EST was fully sequenced. It encodes a protein of 250 aa most closely related to the *Drosophila takeout* paralog CG13618. RT-PCR indicates that this transcript is expressed in the CA, but not in the brain or fat body. The encoded protein has the two pairs of cysteine residues necessary for the JH binding protein (JHBP) function of its lepidopteran homologs (Wojtasek and Prestwich, 1995) (Fig. 3). Another reciprocal match between the *D. punctata* and *A. aegypti* ESTs was a homolog of the *Papilio xuthus* retinol-binding protein (Wakakuwa et al., 2003) (Fig. 4), a protein of the compound eyes of lepidoptera unrelated to other small ligand binding proteins.

4. Discussion

In an attempt to reveal the complexity of the insect CA transcriptome, several approaches can be considered. Differential display of mRNA can be useful when the tissues can be cleanly dissected. In the silkworm, mRNA differential display has been used to identify the OMT which is the last step of JH biosynthesis in lepidoptera (Shinoda and Itoyama, 2003). DNA microarray analysis is another potential tool. The transcriptome analysis using whole-genome microarrays gives a complete overview of those genes that are expressed significantly in certain tissues, when these tissues can be experimentally compared to "whole organism" transcriptome signatures. Such an approach has been elegantly described for the Malpighian tubules of *Drosophila* (Wang et al., 2004). Whole-genome arrays are not yet available for other insects, and their use with *Drosophila* ring glands which comprise both CA and prothoracic gland cells has not been attempted to our knowledge. In comparison, EST analysis is not dependent on prior knowledge of the whole genome, but relies on identification of the transcripts by homology. A careful analysis of ESTs from various tissues of the silkworm (Mita et al., 2003) indicates that about one-third or less of the ESTs found were specific for each tissue-derived library. In contrast, the silkworm prothoracic gland library yielded 49% library-specific ESTs, but a detailed description and analysis is not available.

In this study, an EST approach designed to identify a large number of cDNAs in the CA of a cockroach and two mosquito species has been employed. Highly expressed genes are likely to be represented in EST collections, but high expression is a criterion different from selective expression. We have identified highly expressed genes

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                21    *   *     *     :  . .    *          :*:     73
D.mel Takeout   PEDPKPCKYGD...GECIMKLCNTLFSENSAEGDPGLNL.MQLDPLKVDRMVI
H.vir JHBP      GVFFNPCYKSDIK...CLSNATETFLEKTCN.GYPNTEI.KAIDPLVIPELKV
M.sex JHBP      GALFEPCSTQDIA...CLSRATQQFLEKACR.GVPEYDI.RPIDPLIIPSLDV
M.sex JP29      AVHQKKCNSKD...SECLKNYFQEVVWEAAKNGLPEVGI.PILDPVEIKDYHV
M.sex Moling    PKKKVACALNDL...SCTTLTADPLFKSIMR.GRPDLNV.PASEPLREAEISG
D.mel CG13618   PEGFPKCKR.DANFDKCLVDAVNVAI.QQLKAGNREFGI.PPLEPLTVKKLVI
D.pun CA-EST    PDTFLKCKRTDY.ENVCLKHAVEKAL.KAMKNGIPSLQL.LPIDPLAVTKISI
A.aeg CA-EST    PAFIKTCRFSDADFVACSTESVQGLFDKLVT.GIEGLEHVGTIDPMKISKIRI
                       S ————————S
```

Fig. 3. Partial sequence alignment of the *D. punctata* and *A. aegypti* ESTs homologous to *Drosophila* Takeout protein and lepidopteran JH binding proteins (JHBPs). This N-terminal portion of the protein known to bind JH includes a conserved pair of cysteine residues (disulfide bridge is shown under the alignment) which are necessary for JH binding activity (22). D. mel: *Drosophila melanogaster*; H. vir: *Heliothis virescens*; M. sex: *Manduca sexta*. Numbering on top is from the Takeout protein. Asterisks indicate consensus residues.

```
                7                           35   137                                174
P.xut RetinolBP PKVWSEFE--RQADDGRILNFSIEDVPEDTW[…]IYDKYDVNAYLMGAGLSVTPEYRGLGIAVELLKARKAL
A.gam XP313828  PSVWHTFQA-KDTESDQVVTYRVQDLPEERF[…]VYERYGVDKYLGAMGLSVAPNYRGRGIATEILRARIPL
A.aeg CA-EST    PSVWHTFRA-KDVDSDQMVNYVVQDLPEERF[…]VFERYQVDQYLAAMGLSVVPKYRGRGLATEILRARIPL
D.pun CA-EST    PTVWRKCSGTKKMEDGTFSKFVIQDITEDTY[…]MFEHYGVNEYMTAMGLCVHPIYRGQGLGVEILKARICL
                *.**      :. :.. . .: ::*:.*: :   ::::* *: *: . **.* * *** *:..*:*:**  *
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Fig. 4. Partial sequence alignment of the *D. punctata* and *A. aegypti* ESTs homologous to lepidopteran retinol-binding protein. A. gam: *Anopheles gambiae*; P. xut: *Papilio xuthus*. Numbering on top is from the retinol-binding protein. Asterisks indicate consensus residues.

typical of metabolically active tissues (e.g. high metabolic rate seen with many mitochondrial ESTs), as well as CA-selective and specific genes. The ultrastructure of the *D. punctata* CA shows dramatic changes in the mitochondria, related to high levels of activity (Johnson et al., 1985).

Identification of the enzymes involved in the early steps of JH biosynthesis has taken advantage of the conservation of the mevalonate pathway between vertebrates and insects (Bellés et al., 2005), but this option was not available for the later, JH-specific steps. Regulatory pathways controlling JH biosynthesis are more difficult to decipher with classical homology inferences. The comparison of cockroach and mosquito ESTs was felt to be a useful tool to discriminate such CA-selective transcripts. Indeed, the CA of mosquitoes are minute (30–40 μm) and closely associated within other cell types; we dissected the CA–CC complex attached to a small piece of aorta (see Fig. 1 of Li et al., 2003). It is therefore more difficult to study the physiological control of JH biosynthesis in mosquitoes than in the model species *D. punctata*. In this species, the CA (CA cells plus neurosecretory and neuromodulatory terminals) are somewhat easier to dissect clean of contaminating tissues, the glands are larger. Most importantly, they produce far more JH than any other insect species studied to date, a thousand fold more than mosquito CA, heroic amounts that can only be compared to the prodigious amounts of JH produced by the CA of adult male Saturniidae, especially Cecropia. It is likely that the enzyme machinery is present in higher amounts, and we could expect a relatively high abundance of the most representative mRNAs. Indeed several ESTs were found for mevalonate kinase, and for the methyl farnesoate epoxidase, CYP15A1. A large number of contigs (70) with predicted Gene Ontology function had a reciprocal match in the cockroach and *A. aegypti* CA collections of ESTs. Interestingly, an additional 19 contigs of unknown function also had a reciprocal match, suggesting that a significant proportion of the CA transcriptome has so far completely eluded any functional understanding.

4.1. Enzymes of JH biosynthesis

Although ESTs with very high similarity to farnesyl diphosphate synthase (FPPS, EC 2.5.1.10) were found, we did not find a putative geranyl diphosphate synthase (GPPS, EC 2.5.1.11), suggesting that our prenyl transferase is a bifunctional enzyme, sequentially adding IPP to DMAPP and then to geranyl diphosphate. A separate GPPS is found in plants, but also in bark beetles (Keeling et al., 2004). The genomes of Diptera, *Drosophila* and *An. gambiae* carry just one prenyl transferase.

ESTs for HMG-CoA reductase were not identified in our survey, but the transcript was found by PCR in the *A. aegypti* cDNA library. The low abundance of this transcript may be physiologically relevant as a control mechanism, and it was shown in *D. punctata* CA that the half-life of the enzyme in active glands is longer than in inactive glands (Feyereisen and Farnsworth, 1987b).

The OMT case is of particular interest: the mosquito ESTs yielded representatives of both the silkworm OMT, and of a homolog of the putative crustacean OMT. The silkworm enzyme produced in *Escherichia coli* clearly produced methyl farnesoate from farnesoic acid and JHs from JH acids (Shinoda and Itoyama, 2003). In contrast, the heterologously expressed crustacean cDNA has only traces of OMT activity, and moreover, does not contain a characteristic AdoMet binding site (Gunawardene et al., 2002). Yet esterification of the farnesoic acid carboxylic group is clearly dependent in a stoichiometric manner on AdoMet (Feyereisen et al., 1981). The exact function of the "crustacean OMT" thus remains to be discovered. An EST with sequence homology to the "crustacean OMT" is also found in *Manduca sexta* antennae (Robertson et al., 1999), suggesting that the function is not specifically related to JH biosynthesis. ESTs for glutathione S-transferases and for Cu–Zn superoxide dismutases were found in *D. punctata* as well as in the mosquito, and may serve to protect the cell from oxidative stress related with fatty acid metabolism for acetyl-CoA generation and with CYP15 functioning.

DNA microarray analysis of genes downregulated by dietary cholesterol in mice (Soccio et al., 2002) revealed that only a subset of the isoprenoid pathway enzymes were affected significantly at the transcriptional level, namely HMG-CoA synthase, IPP isomerase and the prenyl transferase FPPS, of which we now have molecular probes. Similarly, DNA microarray analysis showed that mevalonate kinase is the enzyme most affected by sterol regulatory element binding proteins (Horton et al., 2003).

4.2. Regulation of JH biosynthesis

Allatostatins that reversibly inhibit JH synthesis in *D. punctata* have been shown to affect a very early step of the biosynthetic pathway, specifically the export of citrate from the mitochondria and/or its cytosolic conversion to acetyl-CoA which then enters the classical terpenoid pathway (Sutherland and Feyereisen, 1996). The tricarboxylate carrier has now been identified as an EST. Receptors for peptides of the allatostatin family from *Drosophila*, *Periplaneta americana* and *B. mori* have been identified functionally (Auerswald et al., 2001; Secher et al., 2001). These GPCR receptors are closely related to members of the somatostatin/galanin opioid receptor family. The YXFGL-amides ("A-family" allatostatins) thus appear to be acting through a G-protein-dependent mechanism but it is not known whether this mode of action applies equally to the allatal and extra-allatal effects of these peptides, because the expression of these receptors in insect CA has not yet been demonstrated. Moreover, no such receptor-related EST has been found in this study. However, ESTs for homologs of SNF1A, a GPCR kinase, and of Rho1, a Ras-type GTPase have been found.

Activation of JH synthesis appears to involve ionotropic glutamate receptors such as NMDArs and kainate (or quisqualate) sensitive receptors through changes in Ca^{2+} levels (Chiang et al., 2002b). The *Drosophila* NMDAR1 and 2 genes are expressed in the CA portion of the ring gland, and a partial cDNA for a putative NMDAr was obtained by RT-PCR in the CA of *D. punctata* (Chiang et al., 2002a). Upon closer analysis, that sequence appears to represent a bacterial ABC transporter (tblastx *E* value: $1e^{-25}$) rather than a metabotropic receptor (blastx *E* value: 0.80) and may thus be a contaminant. Nonetheless, our finding of an EST for an NMDAr associated protein suggests that this transduction pathway is indeed present in *D. punctata* CA.

The role of calcium in regulating CA activity is well documented (Rachinsky and Tobe, 1996), and its importance is underscored by the identification of a calmodulin EST as well as by ESTs for homologs of presenilin in both cockroach and mosquito.

4.3. Takeout/JHBP homolog and retinol-binding protein homolog

The discovery of these ESTs in both cockroach and mosquito CA was quite unexpected. *Takeout* is a circadian clock-regulated output gene and has been proposed as a direct molecular link between the circadian clock and the feeding/starvation response in *Drosophila* (Sarov-Blat et al., 2000). There are 20 genes of the *takeout* family in the fruit fly (Dauwalder et al., 2002) and proteins of this family are related to hemolymph JHBPs of Lepidoptera and to the JP29 protein initially thought to represent a JH receptor in *M. sexta* (Palli et al., 1994). It has been proposed that Takeout participates in a circadian output pathway that conveys temporal and food status information to feeding-relevant metabolism and activities (Sarov-Blat et al., 2000). Transcription initiation of the *takeout* homolog *moling* in *M. sexta* requires both nutrient intake and decline in JH (Du et al., 2003). One possibility is that the *takeout* homologs expressed in the CA are also involved in feeding-related signals which are known to influence JH biosynthesis (Noriega, 2004). It is interesting that we have found a cDNA encoding for an AMPK $\alpha 2$ subunit; this is a catalytic subunit of the AMPK, an important energy-sensing enzyme that monitors the cellular energy status.

On the other hand, the homology to JHBP and the potential role of *takeout* homologs as small molecule binding proteins or carriers in antennae (Robertson et al., 1999; Bohbot and Vogt, 2005), and exocrine glands (Hojo et al., 2005), suggest that the CA proteins act as intracellular JH or JH precursor carrier proteins. This intriguing possibility calls for a new look at metabolite trafficking in these endocrine glands. The homolog of *Papilio xuthus* retinol-binding protein found in both insect CA libraries is also of interest in this regard, because this sequence appears to be insect-specific, with little homology to other proteins, and a known ligand of striking similarity with the JHs. The *A. aegypti* EST has close homologs in the *An. gambiae* genome (e.g. XP3138238, see Fig. 4), and except for the *Papilio xuthus* protein of 235 amino acids, no other member of this family of insect proteins has been functionally characterized to date.

The ESTs identified from CA libraries of two widely divergent insects, cockroaches and mosquitoes thus revealed the expected, enzymes of JH biosynthesis, the unexpected, small ligand binding proteins, and the unknown, reciprocal matches of unknown predicted function of which some at least have a high likelihood to have CA-specific functions. Our work opens the way for an in-depth study of allatal-specific cell physiology with specific molecular tools.

Acknowledgments

This work was supported by NIH grants AI 45545 to FGN and DK34549 to RF. This work was supported by the Intramural Research Program of the National Institute of Allergy and Infectious Diseases, National Institutes of Health.

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