CYP306A1, a Cytochrome P450 Enzyme, Is Essential for Ecdysteroid Biosynthesis in the Prothoracic Glands of Bombyx and Drosophila*†§

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Ecdysteroids mediate a wide variety of developmental and physiological events in insects. In the postembryonic development of insects, ecdysone is synthesized in the prothoracic gland (PG). Although many studies have revealed the biochemical and physiological properties of the enzymes for ecdysteroid biosynthesis, most of the molecular identities of these enzymes have not been elucidated. Here we describe an uncharacterized cytochrome P450 gene, designated Cyp306a1, that is essential for ecdysteroid biosynthesis in the PGs of the silkworm Bombyx mori and fruit fly Drosophila melanogaster. Using the microarray technique for analyzing gene expression profiles in PG cells during Bombyx development, we identified two PG-specific P450 genes whose temporal expression patterns are correlated with changes in ecdysteroid titer during development. Amino acid sequence analysis showed that one of the Bombyx P450 genes belongs to the CYP306A1 subfamily. The temporal and spatial expression pattern of the Drosophila Cyp306a1 homolog is essentially the same as that of Bombyx Cyp306a1. We also found that Drosophila Cyp306a1 is disrupted in the phantom (phm) mutant, known also as the Halloween mutant. The morphological defects and decreased expression of ecdysone-inducible genes in phm suggest that this mutant cannot produce a high titer of ecdysone. Finally we demonstrate that S2 cells transfected with Cyp306a1 convert ketodiol to ketotriol via carbon 25 hydroxylation. These results strongly suggest that CYP306A1 functions as a carbon 25 hydroxylase and has an essential role in ecdysteroid biosynthesis during insect development.

Animal development consists of a complex schedule of stage-specific developmental events. In the ecdysozoa (1) including insects, developmental schedules are mediated by molting, metamorphosis, and diapause. It is well known that steroid hormones play pivotal roles in such developmental timing in ecdysozoan organisms (2–4). In insects, molting and metamorphosis are elicited by critical titer of ecdysone and 20-hydroxyecdysone. During postembryonic development, ecdysone is synthesized in and released from the prothoracic gland (PG)1 under the regulation of neuropeptides such as prothoraccicotropic hormone (3, 5, 6). Stimulation of the PG by neuropeptides involves several signaling cascades, including a cAMP-dependent pathway, a classical mitogen-activated protein kinase cascade, and an S6 kinase pathway (3, 5, 6). These pathways control synthesis of ecdysteroids from dietary cholesterol or phystosterols via a series of hydroxylation steps (3, 4, 6, 7). Although many intensive studies have shown the biochemical and physiological properties of the enzymes in these steps, the molecular details of these enzymes and their coregulators for ecdysteroid biosynthesis are poorly understood.

To facilitate identification and characterization of components responsible for ecdysteroid biosynthesis in insects, we used both the silkworm Bombyx mori and the fruit fly Drosophila melanogaster. Bombyx has an advantage over Drosophila because its PG is larger, making it much easier to dissect and isolate larger amounts of tissue. In the course of cDNA microarray analysis of gene expression profiles derived from the PG of Bombyx, we focused on one of the cDNA clones named Cyp306a1 gene, which encodes a member of the cytochrome P450 monoxygenases. We also showed that both Bombyx Cyp306a1 and its Drosophila homolog were expressed predominantly in PGs.

Recent studies have shown that members of the Halloween gene family, including disembodied (dib), shadow (sad), and shade (shd), encode cytochrome P450s and catalyze several steps of the ecdysteroid biosynthetic pathway (5, 8–10) (see Fig. 5). Consistent with these studies, we confirmed that the Drosophila homolog of Cyp306a1 was disrupted in the phantom (phm) mutant, another member of the Halloween group (8, 11). Finally we demonstrated that products of the Bombyx Cyp306a1 gene have an enzymatic activity that introduces a carbon 25 (C-25) hydroxyl group of ecdysteroids under cell

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1 The abbreviations used are: PG, prothoracic gland; dib, disembodied; MS, mass spectrometry; EI-MS, electron impact mass spectrometry; EST, expression sequence tag; HA, hemagglutinin; ORF, open reading frame; phm, phantom; RACE, rapid amplification of cDNA ends; RT, reverse phase; HPLC, high pressure liquid chromatography; sad, shadow; shd, shade; Bom, B. mori; Dm, D. melanogaster; PBS, phosphate-buffered saline; GFP, green fluorescent protein.
type five genome DNA as a template and the following primers: IMP-1 down (5'-TGCTGCACTCTTGTACGCAACTGGG-3'), IMP-1 up (5'-GAGCTGCACTGCTTTGCGTGG-3'), IMP-1-L1 down (5'-GCATTGTGTCCTGTGGTCCGTCC-3'), and IMP-1-L1 up (5'-GCTATTGGGACTGTAGACATTACCGG-3'). Then each PCR product was subcloned into pBluescript SKI- and used as the template for a RNA probe. UltraHyb (Ambion) was used as a hybridization buffer. Northern blot using digoxigenin probes was performed as described previously (21). For in situ hybridization, embryos were fixed and treated with Proteinase K as described previously (22). Brains and ring glands were dissected in PBS, fixed for 30–60 min in PBS containing 4% paraformaldehyde at 4°C, and treated with 5 μg/ml Proteinase K for 1 min 15 s. Fixation, hybridization, and detection for the above probes were carried out as described previously (23). In situ hybridization was done on the ovaries as described before (23).

Identification of Mutation on phm Chromosome—phm+/Y embryos were distinguished from phm+/phm (FM7) (19) embryos by their presence or absence of GFP signals (12) and by their morphological defects at stages 15–17. Collected phm+/Y embryos were homogenized, and their genomic DNA was extracted. The genomic DNA was amplified by PCR using four-set primers whose products spanned the entire Cyp306a1-Dm-phm gene locus. Primer sets used are as follows: Up1st, 5'-CAGTAACTGCCAGGCTTCGCGGACG-3' and Down1st, 5'-GCCGCGAGCTTTAGAGGATCTGC-3'; Up2nd, 5'-CGCGCTCTACCCACCCGCTCTAGTGG-3' and Down2nd, 5'-GATGGAGCCGGCGCGGAACCTGCTACATTCC-3'; Up3rd, 5'-CTGGTTCCTGCTCTACTTTGGCCCGCGAACG-3' and Down3rd, 5'-CGGATACAATAAGATGCACTCAGGATGCAC-3'. Sequences of these fragments were determined by ABI3100 (Applied Biosystems).

Observation of Embryos—Cuticle preparations have been described previously (24). For immunostaining, embryos were fixed with 3.7% formaldehyde in PBS. The samples were blocked in PBS containing 0.1% Triton X-100 and 2% bovine serum albumin. The mouse anti-Anti-actin antibody (Chemicon) was applied at 1:200 dilution in PBS at room temperature for overnight. The signal was visualized using Alexa568-coupled secondary antibody (Molecular Probes). Embryos were viewed under a laser scanning confocal microscope LSM510 (Zeiss).

UAS Vector Construction—Overexpression of the gene was carried out using a GALA/UAS system (25). The construct for overexpression of each P450 without tag was generated by ligation of a NotI/XhoI fragment, isolated from a pBluescript clone containing full-length cDNA of each P450, into a NotI/XhoI site of the pUAST vector. For generating HA-tagged protein of interest, a BglII site at the 5' end and a NotI site at the 3' end of the cDNA fragment was ligated together, coding each entire ORF, were introduced by PCR. The primers for constructing tagged P450 were as follows: cyp306a1-Dm (5'-ATGATCTAAAGTGGGTTGATGAC-3'), cyp306a1-Bm (5'-GCGGGCGCATGTTGGTCAATGGT-3'), cyp306a1-Dm-3 (5'-AGATCTTATGCAGGCGGACATCGTGACG-3'), and cyp306a1-Dm-3 (5'-GCCGCGCCAGCTCCTGACAGTGCCTCG-3'). Each ORF region was digested with BgIII/NotI, and the fragment was ligated into pUAST vector with sequences coding three tandem HA tags at the 5' end (26).

Generation of Transgenic Strains and Rescue Crosses—Drosophila transformatants were obtained and rescued on standard medium. For rescue experiments, we established FM7/Kr (GFP); Actin1c-GAL4 UAS-Cyp306a1-Bm-HA/Cyo and FM7/Kr (GFP); Actin1c-GAL4 UAS-Cyp306a1-Dm-HA/Cyo by chromosome recombination. We crossed males of these strains with phm+/phm females and rear the offspring at 25°C and 17°C and 60%--70% humidity, and 13°C. Each strain was injected into S2 cells with heat-inactivated fetal calf serum and penicillin-streptomycin solution (10,000 U/ml). All strains were transfected using modified calcium phosphate transfection reagent (Qiagen) as described by Monsh and Crews (www1.qiagen.com/literature/qiagenews/0499/499effe.pdf).

Immunostaining for S2 Cells—S2 cells were co-transfected with HA-tagged vector and mSpI-GFP vector. Two days after transfection,
cells were collected from dishes and were placed on concanavalin A-coated Lab-Tek II slides (28). After 4 h, the cells were washed with PBS once, fixed with 4% paraformaldehyde, and permeabilized in PBS containing 0.5% Triton X-100. HA-tagged proteins were detected by using anti-HA monoclonal antibody 16B12 (Babco, 1:200 dilution) and antiamouse Alexa568-coupled secondary antibody (1:200 dilution). Cells were mounted in FluoroSave (Chemicon) and observed by confocal microscopy.

Steroidal Substrates for Incubation—[3α-2H]3β,14α-Dihydroxy-5β-chol-7-en-6-one (ketodiol) was prepared from [3α-2H]3β-acetoxy-14α-hydroxy-5α-chol-7-en-6-one by alkaline (NaOH-MeOH/tetrahydrofuran) treatment. 3β,14α,25-Trihydroxy-5β-chol-7-en-6-one (ketodiol, or 2,22-dideoxyecdysone) was synthesized as described previously (29). [3α-2H]3β-Hydroxy-5β-chol-7-en-6-one (ketol) was synthesized as described before (30). These samples were purified by reverse phase (RP)-HPLC prior to use.

Incubation of Transfected S2 Cells with Ecdysteroid Intermediates—48 h after transfection of S2 cells, the old medium was replaced with fresh medium (2 ml) containing MeSO (1%), Tween 80 (0.001%), and each ecdysteroid intermediate (10, 50, or 125 μg) as described previously with modification (9). After the incubation at 25 °C for 8 h, medium was collected and was mixed with 1-butanol. The pooled solvents were desiccated, the residues were dissolved in MeOH, and aliquots (1/50 volume) were analyzed by HPLC.

HPLC, MS, and NMR Analysis—A Shimadzu LC-6A apparatus equipped with an SPD-6A UV detector and a 4.6-mm inner diameter, Capcell Pak C18 (Shiseido) were used for HPLC. Major metabolites were analyzed by RP-HPLC, and electron impact mass spectrometry (EI-MS) (70 eV) spectra were recorded on a JEOL JMS-700 spectrometer. NMR was recorded with a Bruker DRX-500 (1H, 500 MHz) spectrometer in CDCl3.

RESULTS

Identification of Bombyx Cyp306a1—To compare gene expression profiles of PGs between 1st-day fifth (final) instar (V0) and 2nd-day wandering (W1) Bombyx larvae, we took an approach using cDNA microarray on which 5780 nonredundant cDNA clones from various tissues of Bombyx were spotted. Microarray data obtained from two experiments yielded 1883 cDNAs showing at least 2-fold increased expression, while 457 cDNAs showed at least 2-fold lower expression in the PGs at W1 compared with those at V0 in both experiments (data not shown). Among these genes, we focused on two cDNA clones, named prgv0240 and prgv0649, because each contained a partial ORF with a homology to cytochrome P450 monoxygenases (see below). According to the signal intensity of the microarray spots, prgv0240 in the PGs of W1 larvae showed an 8-fold higher expression (average of two spots) than that of V0 larvae, while prgv0649 in the PGs of W1 larvae showed 2.5-fold higher expression than that of V0 larvae. Consistent with the microarray data, Northern blot analysis revealed that changes in mRNA expression level of these genes were positively correlated with the changes of hemolymph ecdysteroid titers during Bombyx development (Fig. 1A) (31–33). Maximal expression levels of prgv0240 and prgv0649 were observed at the V5-V6 and W1 stages, respectively (Fig. 1A), indicating that expression of these genes reached their maximum before the peak of the ecdysone titer (W2-W3) (31, 33). Expression of the two genes in various tissues of the W1 stage of fifth instar larva was examined by reverse transcription-PCR. Both prgv0240 and prgv0649 transcripts were detected exclusively in the PG-derived cDNA (Fig. 1B). These results suggested that prgv0240 and prgv0649 were prime candidates as functional elements in temporal regulation of ecdysteroid biosynthesis in the PG.

The full-length nucleotide sequence of each of the two cDNAs was obtained by modified 5’ RACE. We found that the predicted ORFs for the full-length clones of prgv0240 and prgv0649 encoded proteins that were 538 and 513 amino acids in length, respectively. A BLAST search for the ORFs revealed that the predicted protein encoded members of the cytochrome P450 family. When comparing the sequences of all predicted P450 proteins in D. melanogaster and Anopheles gambiae genomes, the full-length clone of prgv0240 was most similar to CYP306A1 family proteins (Fig. 1C), and the clone of prgv0649 was most similar to CYP315A1 family proteins, which is also known as Shadow (Sad), C-2 hydroxylase of ecdysteroids in Drosophila (9). We focused on the uncharacterized P450 CYP306A1 below. In this article, we have designated the Bombyx and Drosophila Cyp306a1 (CG6578) genes as Cyp306a1-Bm and Cyp306a1-Dm, respectively.
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**Fig. 2. In situ expression pattern of Cyp306a1-Dm.** A–L, embryonic expression. Lateral views (A–H) and dorsal views (I–L) are shown. A, preblastoderm stage embryo. B, cellular blastoderm stage embryo. Initial expression of Cyp306a1-Dm mRNA was ubiquitous (B) but then occurred primarily on the ventral side (C). D, mRNA was also enriched at stage 7. E, germ band extension stage 9; staining appeared in the epidermal cells. F, stage 11 at the beginning of germ band retraction, showing expression in segregated cells. G, at stage 12, expression disappeared in all tissues but then resumed in ring gland primordial cells occurring primarily on the ventral side. H, at stage 12, expression was detected specifically in the ring gland at stage 13. I, mRNA was also enriched at stage 7. J, at stage 11, expression was detected in follicle cells. K, at stage 12, expression was detected in allatom (arrowhead) and corpus cardiacum (arrow). L, wild type. M–O, cellular blastoderm stage embryo. N, lateral views (M–O). O', higher magnification of O. Expression was detected only in the region of prothoracic gland cells but not in the corpus allatum (arrowhead) or corpus cardium (arrow). P, ovarian expression was detected in follicle cells (arrow). No staining was obtained for the sense RNA probes in embryos, larvae, and ovaries (data not shown).

In Situ Expression of Drosophila Homolog of Cyp306a1—To analyze the expression pattern of Cyp306a1-Dm during *Drosophila* development, *in situ* RNA hybridizations were carried out on embryos, larvae, and adult ovaries. There was no development.

Cyp306a1-Dm mRNA in unfertilized eggs, suggesting that there is no maternal contribution of Cyp306a1-Dm (Fig. 2A) as was previously noted for dib and sad (8, 9). Zygotic Cyp306a1-Dm mRNA was found in cellular blastoderm embryos at stage 4 (Fig. 2B). Cyp306a1-Dm mRNA was then enriched in the ventral side of the embryo by segmental stripe pattern (Fig. 2, C and D). By the time of complete germ band extension, Cyp306a1-Dm was expressed in a subpopulation of epidermal cells (Fig. 2E) and then in the neuronal progenitor cells (Fig. 2F). After stage 12, expression of

![Graphical representation](image_url)


**CYP306A1 Acts as a Carbon 25 Hydroxylase of Ecdysteroid**

**Table I**

|                        | First instar larvae | Adults |          |          |
|------------------------|---------------------|--------|----------|----------|
|                        | phmE7 (without GFP signal) | Others (with GFP signal) | phmE7/Y | FM7/Y   |
| Actin5c-GAL4           | 0                   | 196    | 0        | 421      |
| Actin5c-GAL4 UAS-Cyp306a1-Dm | ND*                | ND     | 303      | 262      |
| Actin5c-GAL4 UAS-Cyp306a1-Bm | 44                 | 143    | 7        | 102      |

* ND, not determined.

**Cyp306a1-Dm** was greatly decreased in all tissues and then resumed in primordia of the ring gland, which contains Drosophila PG cells (Fig. 2, G–I). The signal was detected in the first segment of the thorax (prothorax) (Fig. 2, G and H). Expression in the ring gland continued through the remainder of embryogenesis (Fig. 2, J–L).

In addition to late embryogenesis, Cyp306a1-Dm mRNA also was expressed specifically in larval PG cells but not in the corpus allatum or corpus cardiacum cells of the ring gland (Fig. 2, O and O'). In the case of dib, sad, and Start1 (Drosophila homolog of Start) (34), the transcriptional activity of these genes correlates with the ecdisyoid titer changes during the larval molting cycle (35). This also was the case for Cyp306a1-Dm. In situ hybridization revealed that Cyp306a1-Dm mRNA was also expressed strongly in the PG of late second and third instar larvae (Fig. 2, M and O). Just after ecdisis to the third instar, Cyp306a1-Dm mRNA expression was slightly but significantly down-regulated (Fig. 2N). The wave-like diurnal pattern of Cyp306a1-Dm expression was similar to that of the expression of dib, sad, and Start1 (8, 34). It should be noted that significant expression of Cyp306a1-Dm was observed in the PG of larvae just after ecdisis, although previous studies have shown that expression levels of dib, sad, and Start1 are almost undetectable at this stage (8, 34). Besides the PG cells, no other larval cells showed Cyp306a1-Dm mRNA signals (data not shown).

In adult females, Cyp306a1-Dm mRNA was expressed in the posterior follicle cells (Fig. 2P), which are known to be another site of ecdisyoid biosynthesis (3, 8, 36). As with dib (8) yet unlike sad, Start1 and dare (Drosophila homolog of adrenodoxin reductase) (9, 34, 37), we did not observe Cyp306a1-Dm expression in the nurse cells.

We also examined the expression pattern of Drosophila Cyp18a1 (Cyp18a1-Dm, CG6816). It encodes the second most closely related protein to CYP306A1-Bm in the Drosophila genome (Fig. 1C) and is located next to Cyp306a1-Dm on the X chromosome (38) (Fig. 3A). We did not observe preferential expression of Cyp18a1-Dm in the PG during either embryogenesis or larval development (data not shown). We concluded that the Drosophila ortholog of Cyp306a1-Bm was Cyp306a1-Dm, but not Cyp18a1-Dm, based on sequence similarities and in situ expression patterns.

**Confirmation of phm as Cyp306a1 and Phenotype of phm Mutant**—Previous studies have shown that dib, sad, and shd, which code for essential components of ecdisyoid biosynthesis, belong to the Halloween group of mutants characterized by embryonic lethality and by similar cuticular patterning (8–10). One uncharacterized Halloween mutant, phm, was mapped to the 17C5-D2 cytological interval of the X chromosome (11), which was in the vicinity of the Cyp306a1-Dm gene located at 17D1 (Fig. 3A) (38, 39). To confirm whether phm corresponds to Cyp306a1-Dm, genomic DNA around the Cyp306a1-Dm region was amplified by PCR from the phm/Y mutant and then sequenced. We found that phmE7 (11) has a stop codon at position 286 of the predicted CYP306A1-Dm protein (a change in the first base from C to T, Fig. 3A), indicating that Cyp306a1-Dm is the product of the phm gene. Since this nonsense mutation eliminates the C-terminally positioned heme-binding site that is critical for catalytic activity of all cytochrome P450 enzymes (40), we assume that phmE7 is a null allele.

Like other Halloween mutants (8–10), the phm mutant did not produce a differentiated cuticle structure (Fig. 3, B and C) (11). Immunostaining with anti-Actin antibody (to visualize the overall morphology of the embryos) revealed that early phm mutant embryos were morphologically normal until approximately stage 14 (Fig. 3, D and E). At stages 15–16, abnormal morpogenic movements that involve the failure of head involution and defects in dorsal closure became apparent (Fig. 3, F–I). This phenotype was very similar to that of dib, sad, and shd (8–10). As with other Halloween mutants (8–10), the epidermal expression of both IMP-E1 and IMP-L1 (19, 20, 41), which are ecdysone-inducible genes, was greatly reduced or absent in stage 14 embryos of the phm mutant (Fig. 3, J–M). The phenotypic result of reduced IMP-E1 expression in the phm mutant was in agreement with the previous reports (8).

These results were consistent with the notion that the phm mutant is defective in ecdisyoid biosynthesis.

The lethality and phenotype of phmE7 were due to loss of phm function as shown by the fact that lethality was recovered by Cyp306a1-Dm cDNA expression using a widely expressed GAL4 driver, Actin5c-GAL4 (Table I). Furthermore the overexpression of Cyp306a1-Bm cDNA by Actin5c-GAL4 rescued phmE7 lethality in first instar larvae and adults (Table I), although the rescue penetrance of adult viability was somewhat lower than that of Cyp306a1-Dm. These results suggested that CYP306A1-Bm and CYP306A1-Dm proteins metabolize the same ecdisyoid intermediate in PGs.

**Bombyx CYP306A1 Acts as a C-25 Hydroxylase—CYP306A1 family proteins are predicted to be members of the microsomal class of P450s (39), which contain uncharged amino acid residues at the N terminus (40) and a microsomal type proline-rich consensus sequence (PDX(P/V)XP) (42). Consistent with this prediction, both CYP306A1-Bm and CYP306A1-Dm proteins with epitope tags were colocalized with a microsomal marker in S2 cells (Fig. 1, D–F; data not shown). Since it is well known that a C-25 hydroxylating enzyme, which converts ketotriol to ketotriol in the PG, is a microsomal enzyme (6, 7, 36, 43), we examined whether CYP306A1-Bm could introduce the C-25 hydroxyl group to ecdisyoids. Two days after transfection with Cyp306a1-Bm, S2 cells were incubated with ketotriol for 8 h, and the medium was then extracted and analyzed by RP-HPLC. The data revealed that S2 cells transfected with Cyp306a1-Bm produced a new metabolite (Fig. 4A, arrow). This peak showed the UV property of an ecdisyoid because the
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In this study, a combined approach using both Bombyx and Drosophila led to identification of the Cyp306a1 genes from B. mori and D. melanogaster that encode C-25 hydroxylase responsible for catalyzing the conversion of ketodiol to ketotriol (Fig. 5). By using microarray analysis, two Bombyx P450 genes were cloned. The temporal expression levels of these genes appear to cycle in concert with changes in ecdysone titer during Bombyx development. Both P450s are expressed specifically in the PG. The amino acid sequence of one of the P450s is similar to Sad, which is known as ecdysteroid C-2 hydroxylase; the other is similar to the CYP306A1 subfamily. In Drosophila, we showed that the spatiotemporal expression pattern of Cyp306a1-Dm was essentially the same as that of Cyp306a1-Bm. We also found that Cyp306a1-Dm was disrupted in the phm mutant that is known as the Halloween mutant. The morphological defects and the decreased expression of ecdysone-inducible genes in the phm mutant suggested that the phm mutant failed to produce a high enough titer of ecdysone. Finally we demonstrated that S2 cells transfected with Cyp306a1-Bm converted ketodiol to ketotriol. CYP306A1-Bm could also introduce the hydroxyl residue on C-25 of ketol, which is consistent with properties of the putative C-25 hydroxylase reported previously.

**Fig. 4. CYP306A1-Bm acts as a C-25 hydroxylase of ecdysteroids.** A and B, RP-HPLC analysis after Cyp306a1-Bm- (upper) and GFP (lower)-transfected S2 cell incubations with 125 µg of [3α-2H]ketodiol in 2 ml of medium. Conditions were as follows: solvent, MeOH-H2O (4:1); flow rate, 1 ml/min; detection, UV absorption at 243 nm. Cyp306a1-Bm- but not GFP-transfected S2 cells produced a new metabolite (arrow). Ketodiol is indicated with the arrowhead in A. B, Rp-HPLC analysis of metabolites by CYP306A1-Bm with (right) or without (left) standard ketodiol. The metabolite by CYP306A1-Bm with ketodiol was co-eluted with standard ketodiol (arrows). Conditions were as follows: solvent, MeOH-H2O (4.5:1); flow rate, 1 ml/min; detection, UV absorption at 243 nm. C, Rp-HPLC analysis of standard ketotriol and of the metabolite by CYP306A1-Bm with [3α-H]ketodiol. The ion peaks at m/z 433 and 432 correspond to the molecular ions of [2H-labeled and non-labeled ketotriol, respectively. D, Rp-HPLC analysis after Cyp306a1-Bm- or GFP-transfected S2 cell incubations with [3α-2H]ketol. CYP306A1-Bm, but not GFP, converted ketol to 25-hydroxyketol. Conditions were as follows: solvent, CH3CN-H2O (5:1); flow rate, 1 ml/min.

maximum absorption was at 243 nm (data not shown). No such activity was observed in GFP (control), dib-, and sad-transfected cells (Fig. 4A; data not shown). RP-HPLC and EI-MS analysis identified this product as ketotriol. First, the metabolite was co-eluted with synthetic ketotriol standard on RP-HPLC (Fig. 4B). Second, EI-MS analysis revealed that the fragmentation pattern of the metabolite was essentially identical to that of ketotriol (Fig. 4C). Furthermore an intense fragment ion at m/z 59, [(CH3)2C-OH]+, in the MS spectrum supported C-25 hydroxylation (Supplemental Fig. 1). Over the 8-h period, ~2.5 µg of ketotriol was recovered from the 10 µg of ketodiol added to the incubation mixture, a yield of ~25% at a substrate concentration of ~12 µM. CYP306A1-Bm-expressing cells did not provide any other metabolite from ketodiol and did not show any conversion activity on ketotriol (data not shown). These results indicated that CYP306A1 converts the ketodiol substrate solely into ketotriol and has a critical role for ecdysteroid biosynthesis in the PG.

Previous *in vitro* studies using subcellular fractions of the PG have shown that putative C-25 hydroxylase in the PG also introduces a hydroxyl residue on C-25 of ketol (44, 45). Consistent with these studies, S2 cells expressing Cyp306a1-Bm, but not cells expressing GFP alone, produced a new metabolite when incubated with a ketol substrate (Fig. 4D, arrow). The result from EI-MS analysis was consistent with the notion that the new metabolite was 25-hydroxyketol (Supplemental Fig. 2). Furthermore the position of the hydroxylation was determined by 1H NMR analysis, which exhibited the 26- and 27-methyl at 1.22 (s, 6H), indicating that CYP306A1 was a C-25 hydroxylase and was consistent with the property of the putative C-25 hydroxylase reported previously (44, 45).

**DISCUSSION**

The mutant embryonic phenotype of *phm* closely resembles those of *dib*, *sad*, and *shd*, which is most likely a result of the low ecdysteroid titers in these embryos. In this study, however, we could not address whether ecdysone titer in the *phm* mutant is actually lower than that of the wild type. The overexpression of Cyp306a1-Bm, the Bombyx homolog of Cyp306a1-Dm, could rescue the embryonic lethality of the *Drosophila phm* mutant. This result strongly indicates that the *phm* mutant phenotype is caused by the low ecdysone titer. This result also suggests that CYP306A1-Dm is also C-25 hydroxylase as well as CYP306A1-Bm. Nevertheless we could not detect obvious en-
zymatic activity of CYP306A1-Dm under our experimental conditions (data not shown). It should be noted that the number of rescued phm adults by Cyp306a1-Bm was significantly fewer compared with the case of the Cyp306a1-Dm transgene (Table I). It is possible that CYP306A1-Bm enzymatic activity is regulated differently from CYP306A1-Dm. Alternatively one CYP306A1, but not the other, may catalyze other ecdysoidal substrates besides ketodiol.

The expression pattern of Cyp306a1 during development is quite similar to that of dib and sad in the context of PG specificity and cyclic expression coupled to molting. As shown in Fig. 1, Cyp306a1-Bm and the putative Bombyx homolog of sad (sad-Bm) were expressed in the PG, and the temporal changes in expression correlated with hemolymph ecdysone titer levels. In Drosophila, ecdysenoid levels begin to rise during early embryogenesis around the onset of gastrulation (stages 6–7, 3 h after egg laying) and peak at stages 11–12 (7–9 h after egg laying) during germ band retraction (46). Changes in ecdysone titers correlate well with strong expression levels of phm, dib, and sad.

On the other hand, we also observed that temporal changes of both Cyp306a1-Bm and Cyp306a1-Dm expression are unique compared with other Halloween genes; that is, Cyp306a1 expression appears precociously relative to dib, sad, and shd. First, in Bombyx fifth instar development, the expression peak of Cyp306a1-Bm was at V5-V6, but that of sad-Bm was at W1 (Fig. 1A). Second, while dib and sad expression in PG progenitors was detected at stage 15 in embryogenesis (8, 9), Cyp306a1-Dm/phm expression in the ring gland appeared at stage 12 in the PG primordial on epidermal walls (Fig. 2G). Third, while dib and sad are completely down-regulated immediately after ecysis (8, 9), the Cyp306a1-Dm/phm transcript was present at that time (Fig. 2N). A previous report has also pointed out that the expression of dib and sad precedes that of shd (10). These data suggest that the timing of Halloween gene expression correlates with the order of hydroxylation steps during ecdysenoidogenesis in the PG (Fig. 5). As Start1 expression in the PG appears to depend on ecdysone in a positive feedback manner (34), it is possible that the Halloween genes could be regulated by changes in ecdysenoid titer. Understanding both the temporal and tissue-specific regulation of the expression of these genes should help to elucidate the control of ecdysenoid biosynthesis.

*In situ* hybridization experiments also shed light on one aspect of PG development. A previous histological study argued that the progenitors of the “large cells” of the ring gland, which may correspond to PG cells, were derived from the dorsal wall of the invaginating stomodeum at 9–10 h after egg laying (stages 12–13) (47). However, our data clearly showed that the prothoracic gland primordial cells appeared from epidermal walls in the prothorax. Because Cyp306a1-Dm is expressed at an earlier stage than previously known PG-specific genes, Cyp306a1-Dm may help us to analyze PG development, which is largely unknown thus far.

This study demonstrates the advantage of using both Bombyx and Drosophila for identifying genes functioning in the PG in conjunction with Drosophila mutant analysis (5, 37, 48) and enhancer-trap strategy (49). For example, we already succeeded in isolating another novel P450 that may be encoded at the spook locus, which is one of the uncharacterized genes in the Halloween group (5, 9). Furthermore we also have identified genes other than the P450 genes that are preferentially and temporally expressed in the PGs of Bombyx and Drosophila. Further analysis will identify and characterize new components involved in the biosynthetic pathway of ecdysteroids. Because all insects require ecdysteroids for normal development, these studies may be useful to develop novel strategies for the screening of insecticides that can be used for control of insect growth. This combined strategy would be useful and powerful to identify and characterize the genes functioning in other small endocrine organs such as corpus allatum and corpus cardiacum.

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2 R. Niwa, T. Matsuda, T. Yoshiyama, T. Namiki, K. Mita, Y. Fujimoto, and H. Kataoka, unpublished observations.
