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# Cytochrome P450 CYP307A1/Spook: A regulator for ecdysone synthesis in insects

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**Abstract**

The prothoracic gland (PG) has essential roles in synthesizing and secreting a steroid hormone called ecdysone that is critical for molting and metamorphosis of insects. However, little is known about the genes controlling ecdysteroidogenesis in the PG. To identify genes functioning in the PG of the silkworm, *Bombyx mori*, we used differential display PCR and focused on a cytochrome P450 gene designated *Cyp307a1*. Its expression level positively correlates with a change in the hemolymph ecdysteroid titer. In addition, *Drosophila Cyp307a1* is encoded in the *spook* locus, one of the Halloween mutant family members showing a low ecdysone titer in vivo, suggesting that *Cyp307a1* is involved in ecdysone synthesis. While *Drosophila Cyp307a1* is expressed in the early embryos and adult ovaries, the expression is not observed in the PGs of embryos or third instar larvae. These results suggest a difference in the ecdysone synthesis pathways during larval development in these insects.


*Keywords:* *Bombyx mori*; *Cyp307a1*; Cytochrome P450; *Drosophila melanogaster*; Ecdysone; Halloween mutant; Prothoracic gland; *Spook*; Steroidogenesis

Insect development is controlled by a critical titer of ecdysteroids including ecdysone [1,2]. During larval and pupal development, ecdysone is synthesized in and released from the prothoracic gland (PG). The biosynthesis of ecdysone in the PGs is regulated by neuropeptides such as prothoracicotropic hormone (PTTH) [2–5], prothoracicostatic peptide (PTSP) [6], and myosuppressin [7]. Stimulation of PGs by PTTH and the subsequent secretion of ecdysteroids involves several signaling cascades, such as the cAMP second messenger cascade and a classical MAP kinase pathway [2,4].

Recent studies using the fruit fly *Drosophila melanogaster* have revealed three Halloween P450 genes, designated *Cyp306a1/phantom* (*phm*), *Cyp302a1/disembodied* (*dib*), and *Cyp315a1/shadow* (*sad*), that are essential for each of the terminal hydroxylation steps in ecdysteroid biosynthesis in the PG, namely 5β-ketodiol to ecdysone [4,8–11]. The mRNA expression levels of all three genes and their orthologs positively correlate with changes in the ecdysteroid titer during postembryonic development in *Drosophila* and the silkworm *Bombyx mori* [9–12]. However, much less is known about the other steps of ecdysteroid biosynthesis pathway in the PG at molecular level.

In this study, we used fluorescent differential display (FDD) PCR techniques to identify genes exhibiting stage-specific expression in PGs from the fifth instar lar-

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vae to pupae of *Bombyx*. In the course of this study, we focused on one of the clones, designated the *Cyp307a1* gene, which encodes a member of the cytochrome P450 monooxygenases. *Bombyx Cyp307a1* is exclusively expressed in the PG, and its transcriptional upsurge occurs in accordance with the increase in ecdysone secretion. We also isolated the *Drosophila* homolog of *Cyp307a1* and showed that *Cyp307a1* is identical to the *spook* locus, one of the other loci of the Halloween mutants [13]. However, *Drosophila Cyp307a1* expression is observed in the early embryo and ovary but not in the PG during either the embryonic or larval stages. These results shed light on the possibility that the ecdysteroid biosynthesis pathways in larval development might be different between these two insects.

## Materials and methods

*Animal strains and culture.* Silkworms, *B. mori* (KINSYU x SHOWA F1 hybrid), were reared on an artificial diet (Silkmate, Nihon-Nosan-Kogyo, Japan) at 25 °C under 15-h light/9-h dark cycles. Wandering occurred on day 6 of the fifth larval instar and pupation occurred 3 days thereafter. The first days corresponding to the developmental stages of the fourth to fifth larval ecdysis, wandering, and pupation were designated as V0, W0, and P0, respectively. *D. melanogaster* flies were reared on a standard agar cornmeal medium at 17 or 25 °C under a 12-h light/12-h dark photoperiod. *yw* was used as wild type. $spo^1$ [13] flies were obtained from the *Drosophila* Genetic Resource Center, Kyoto Institute for Technology. *Actin5c-GAL4/TM3* (originally established by Yasushi Hiromi in National Institute for Genetics, Japan) was provided by the Bloomington stock center.

*Preparation of differential display. Bombyx* prothoracic glands (PG) from stages V2, V4, V6, W0, W3, and P0 were dissected. Total RNA (2 μg) extracted from each stage of PG was treated with RNase-free DNase I (Invitrogen, Carlsbad, CA) and then reverse-transcribed with SuperScript II RT (Invitrogen). The differential display method was performed using a Fluorescence Differential Display kit (TaKaRa, Shiga, Japan) with the combinations of three types of downstream primers and nine types of upstream primers as described [14]. We conducted two sets of PCR for each stage of cDNA template, loaded samples on a PAGE gel next to each other, and performed electrophoresis.

*Preparation and analysis of a cDNA microarray.* A total of 338 candidate bands identified using the differential display method were selected and gel excised. The cDNAs were re-amplified, subcloned into a pGEM-T vector (Promega, Madison, WI), and transfected into *Escherichia coli* DH5α (TaKaRa). Six transformants per band were picked and cultured in LB broth. Plasmid DNA was extracted from each broth using a PI-50α (Kurabo), and 5 μl of each plasmid solution was diluted 1% and used as a template for polymerase chain reaction (PCR). cDNA inserts were amplified by PCR using the T7 (5′-CGCCAGGGTTTTCCCAGTCACG-3′) and SP6 (5′-TCACACAGGAAACAGCTATGAG-3′) primers. The PCR conditions were: (94 °C for 30 s, 50 °C for 30 s, and 72 °C for 1.5 min) × 40 cycles. A portion of the resulting PCR products was subjected to 1.0% agarose gel electrophoresis to confirm re-amplification. The remaining samples were precipitated with isopropanol and the pellets were washed with 70% ethanol. The samples were then dissolved in 20 μl D.W. Thirty-five micrograms each of total RNA was prepared from V2 PG, V2 SG, W3 PG, and W3 SG using TRIzol reagent (Invitrogen). Spotting, fluorescent labeling, hybridization, detection, and analysis were conducted by Bio Matrix Research (Kashiwa, Japan).

*Molecular cloning.* cDNAs (2)–(5) (Fig. S1E) exhibited high expression in the W3 PG but lacked the 5′ portion of the full-length cDNA. The 5′ ends of the cDNAs were obtained by a modified 5′ RACE method using a GeneRacer kit (Invitrogen). The first-strand cDNA template was synthesized from the total RNA extracted from the PGs of eighth-day fifth instar *Bombyx* larvae. Primers were designed based on the sequences of the cDNA fragments cloned in Differential Display-PCR. The first 5′ RACE product was amplified with a gene-specific primer and an upper universal primer supplied by the manufacturer. We then performed nested PCR using initial PCR products diluted 1:50 with another gene-specific primer for nested PCR and another universal primer. The products were subcloned into pGEM-T and sequenced. Based on the subcloned sequence, new primers were designed. We repeated 5′ RACE until we reached the 5′ end of the gene. The positions and sequences of the gene-specific primers are shown in Fig. S1E and its legend. The DNA sequence data of *Cyp307a1-Bm* was deposited in GenBank (AB206555).

*Reverse transcription (RT)-PCR.* Extraction of total RNA from tissues of second-day wandering (W1) fifth instar *Bombyx* larvae and synthesis of single-stranded cDNAs were previously described [11,12]. Specific primers for *Cyp307a1-Bm* (Forward, 5′-GTACTGGTCCGAGCCGGA GAAATTCG-3′; Reverse, 5′-CACGTTCTCTTACCAATGCTGAAGG GTATG-3′) and *ribosomal protein L3* (*rpL3*) [15] were used, yielding 319- and 219-bp fragments, respectively. Twenty-seven cycles (95 °C for 10 s, 68 °C for 20 s) were carried out for amplification of these genes. In order to quantify the transcripts of *Cyp307a1-Bm*, we performed a quantitative RT-PCR as previously described [12,16]. PCR conditions for Fig. 2D and E were: (94 °C for 10 s; 68 °C for 20 s) × 40 cycles for *rpL3* and *Cyp307a1-Bm*. Serial dilutions of plasmids containing cDNAs of *Cyp307a1-Bm* and *rpL3* were used as standards. Transcript levels of each P450 were normalized with *rpL3* transcript levels in the same samples.

*In vitro culture of PG.* Recombinant PTTH (rPTTH) was prepared as previously described [17]. V4 silkworms were anesthetized by water submersion for 5 min. The PGs were dissected rapidly in sterile saline and preincubated in 100 μl of Grace's Insect Medium (Sigma, St. Louis, MO). After 20 min, each single PG was transferred into 100 μl medium in the presence or absence of 10 nM rPTTH, 100 ng/ml of α-ecdysone (Sigma) or 200 ng/ml of 20-hydroxyecdysone (Sigma). At 10 nM rPTTH, ecdysone release from the PGs is saturated [7,18]. After incubation for 30 min, 2, 4 or 6 h, each PG was removed, frozen at −80 °C, and analyzed by quantitative RT-PCR as described [12].

*Northern and in situ hybridization.* Digoxigenin (DIG)-labeled sense and antisense RNA probes were synthesized using the DIG RNA labeling kit (Roche, Indianapolis, IN) and SP6, T3 or T7 RNA polymerase (Invitrogen) according to the manufacturer's instructions. For *Cyp307a1-Bm*, one cDNA (1) from the FDD bands, corresponding to nucleotides 1218–1701 of the full-length *Cyp307a1-Bm* (Figs. S1A and E), was used as the template for the RNA probe. For *Cyp307a1-Dm*, the EST clone RE13908 (Open Biosystems, Huntsville, AL) was used as the template. A probe for *Cyp306a1/phm-Dm* was previously described [11]. Northern blots using DIG probes were performed as described previously [19], and UltraHyb (Ambion, Austin, TX) was used as a hybridization buffer. In situ hybridization on embryos, ovaries, and brain-ring gland complexes of *Drosophila* were performed as described [11,20,21].

*Observation of embryos.* Definition of embryonic stages of *Drosophila* as well as cuticle preparation were previously described [22].

*UAS vector construction.* Overexpression of the genes was carried out using a GAL4/UAS system [23]. To generate HA-tagged proteins of interest, a *Bgl*II site at the 5′ end and a *Not*I site at the 3′ end of the cDNA fragments, encoding the *Cyp307a1-Bm* and *Cyp307a1-Dm* ORFs, respectively, were introduced by PCR. The primers used to construct tagged P450 were as follows: Cyp307a1-Bm-5 (5′-CCCAGATCTATGAGTTC GTTAATCATTGTATTATTCG-3′), Cyp307a1-Bm-3 (5′-ATAAGAAT GCGGCCGCCCTTCCTAGGTATCAAATGCATTTTG-3′), Cyp307a1-Dm-5 (5′-GGGAGATCTATGCTGGCTGCTT TGATTTACAC-3′), and Cyp307a1-Dm-3 (5′-ATAAGAATGCGGCCGCCTAGTGGTCCGATCT TTTCCCTG-3′). Each ORF region was digested with *Bgl*II/*Not*I, and the fragment was ligated into pUAST vector with sequences encoding three tandem HA tags at the 3′ region [24].

*Transfection and immunostaining for S2 cells.* UAS-mSpitz-GFP (mSpi-GFP, a gift from Ben-Zion Shilo, Weizmann Institute of Science, Israel) was used as an endoplasmic reticulum marker as described previously [25]. The UAS vectors were transfected with the *Actin5c-GAL4*

construct (a gift from Y. Hiromi). Transfection of S2 cells with these vectors and immunostaining with anti-HA antibody 16B12 (BAbCO, Richmond, CA) were described previously [11].

*Identification of mutations on the spo mutant chromosome.* Collected *spo*$^{1}$ embryos were homogenized and their genomic DNA was extracted. The genomic DNA was amplified by PCR using five-set primers whose products spanned the entire *Cyp307a1-Dm* gene locus. Primer sets used were as follows: Forward1st (5′-GACTTTCAGTCCGTTTAATA ATGTG-3′) and Reverse1st (5′-GCAAAGGGACTATCCCTGTATCG-3′), Forward2nd (5′-GAGTGTTCTGGCCACGTCCTAC-3′) and Reverse2nd (5′-CCTCAACGAACACATGTACTGAC-3′), Forward3rd (5′-GATTGGTTGCGAGGAAATGG-3′) and Reverse3rd (5′-CATCGT GTAGGGCATAGCATTC-3′), Forward4th (5′-GCGGTTGGAAATC TA GTAATGCTAG-3′) and Reverse4th (5′-CTTTTCCCTGGGTGTCA A GAC-3′), and Forward5th (5′-GATTAAGATCAGTCCGGAGAGTT T G-3′) and Reverse5th (5′-AGAAGGTCGTTTTCGATTTATTCAG-3′). Sequences of these fragments were determined by ABI3100 (Applied Biosystems).

*Generation of transgenic strains and rescue crosses. Drosophila* transformants were obtained using standard protocols. For rescue experiments, *yw;UAS-Cyp307a1-Bm-HA spo*$^{1}$*/TM3[y+]* and *yw;UAS-Cyp307a1-Dm-HA spo*$^{1}$*/TM3[y+]* were established by chromosomal recombination. Females of these strains were crossed with *yw;Actin5c-GAL4 spo*$^{1}$*/TM3[y+]* males that were also made by chromosomal recombination, and offspring were reared at 25 °C. The viable adult flies of *yw;UAS-Cyp307a1-Bm-HA spo*$^{1}$*/Actin5c-GAL4 spo*$^{1}$ or *yw;UAS-Cyp307a1-Dm-HA spo*$^{1}$*/Actin5c-GAL4 spo*$^{1}$ were then counted.

## Results

### *Screening of genes expressed preferentially in the Bombyx PG using cDNA microarray*

To investigate the genetic regulation of ecdysteroidogenesis in the *Bombyx* PG, FDD was performed to detect genes whose expressions change along with silkworm development. Total RNAs were extracted from *Bombyx* PG at six developmental stages, and FDD was conducted with 27 primer combinations. Of a total of 2450 bands, the densities of 338 varied among the six *Bombyx* developmental stages, as shown in Fig. S1A. Six independent subclones derived from each band (a total of 2028 clones were derived from the 338 candidate bands) were isolated and printed onto a single slide glass [14]. This cDNA microarray was used to search among the clones for genes that are specifically expressed in the PG and that are also expressed in a stage-specific manner. Three pairs of samples were compared using microarray methods as shown in Figs. S1B–D. Ultimately, 46 cDNAs were identified as PG-selective candidate genes in the W3 stages (Figs. S1C and D).

In the 46 candidate clones for both PG- and W3-selective genes, four clones encoded a protein identical to *Bombyx Cyp306a1/phm* [10,11], and 28 clones (cDNAs (1)–(5) in Fig. S1A) encoded a protein that had the highest sequence similarity (53% identical amino acids) to the *Drosophila* CYP307A1 (CG10594) protein.

### *Characterization of the Cyp307a1-Bm*

The full-length nucleotide sequence of the *Cyp307a1*-like gene was obtained by modified 5′ RACE. The cDNA contained an ORF of 536 amino acid residues, which also had the highest sequence similarity to the entire region of *Drosophila* CYP307A1 protein (Fig. 1A). Both *Bombyx* and *Drosophila* CYP307A1 have the same structural motifs common to microsomal cytochrome P450s, including a signal-anchor sequence, a proline-rich region, a putative hydrogen-binding sequence (helix-K), an aromatic region, and a heme-binding region [26]. Consistent with the prediction, an epitope-tagged CYP307A1-Bm protein localized with a microsomal marker in S2 cells (Figs. 1C–E). After comparing the sequences of all the predicted P450 proteins in the *Drosophila* and *Anopheles gambiae* genomes, the predicted protein was confirmed to be a member of the *Cyp307a1* family of proteins (Fig. 1B). In this article, the *Bombyx* and *Drosophila Cyp307a1* genes have been designated as *Cyp307a1-Bm* and *Cyp307a1-Dm*, respectively.

### *Expression profile of Cyp307a1-Bm in the Bombyx PG*

We examined *Cyp307a1-Bm* expression in various tissues from the W1 stage of the fifth instar larvae by using RT-PCR. The *Cyp307a1-Bm* transcript was detected exclusively in PG derived cDNA (Fig. 2A). The gene expression profile during development, as shown by Northern blot and its densitometric analyses, demonstrated that changes in *Cyp307a1-Bm* mRNA expression levels positively correlated with the hemolymph ecdysteroid titers during development (Fig. 2B). *Cyp307a1* expression is almost completely repressed in the early stage of the fifth instar larvae (V0–V2; Figs. 2B and C) when the hemolymph ecdysone titer is very low [27,28]. Maximal expression levels of *Cyp307a1-Bm* were observed at the fourth instar stage day 3 and the fifth instar W2, respectively, indicating that expression of the gene culminates when the ecdysone titer peaks (IV3–IV4, W2–W3) [27].

These results could be explained by the hypothesis that *Cyp307a1* is involved in temporal regulation of ecdysteroid biosynthesis in *Bombyx* PG. However, an alternative interpretation is also possible. Since the ecdysteroid titers are elevated in the *Bombyx* hemolymph during the V6–W0 period [27–29], it is possible that the *Cyp307a1-Bm* upregulation in the PG may be a consequence of the increased levels of ecdysteroid. To test the second possibility, *Bombyx* V4 PGs were cultured in medium with α-ecdysone or 20-hydroxyecdysone (20E), and the *Cyp307a1-Bm* expression levels were examined (Fig. 2D). Neither α-ecdysone nor 20E stimulation induced the *Cyp307a1-Bm* expression 6 h after treatment in this assay (Fig. 2D), suggesting that *Cyp307a1-Bm* expression in the PG is not regulated by ecdysteroids.

In addition to *Cyp307a1-Bm*, *dib-Bm* transcript levels also remarkably increase at V6–W0 (Fig. 2C) [12]. It has recently been shown that the *dib-Bm* expression is rapidly induced by treatment with recombinant PTTH (rPTTH) in cultured PGs from V4 [12], the stage when the hemolymph PTTH titer is first elevated [27,28]. We observed that rPTTH stimulation also increased *Cyp307a1-Bm* expression in the cultured V4 PGs (Fig. 2E). The induction was significant after the

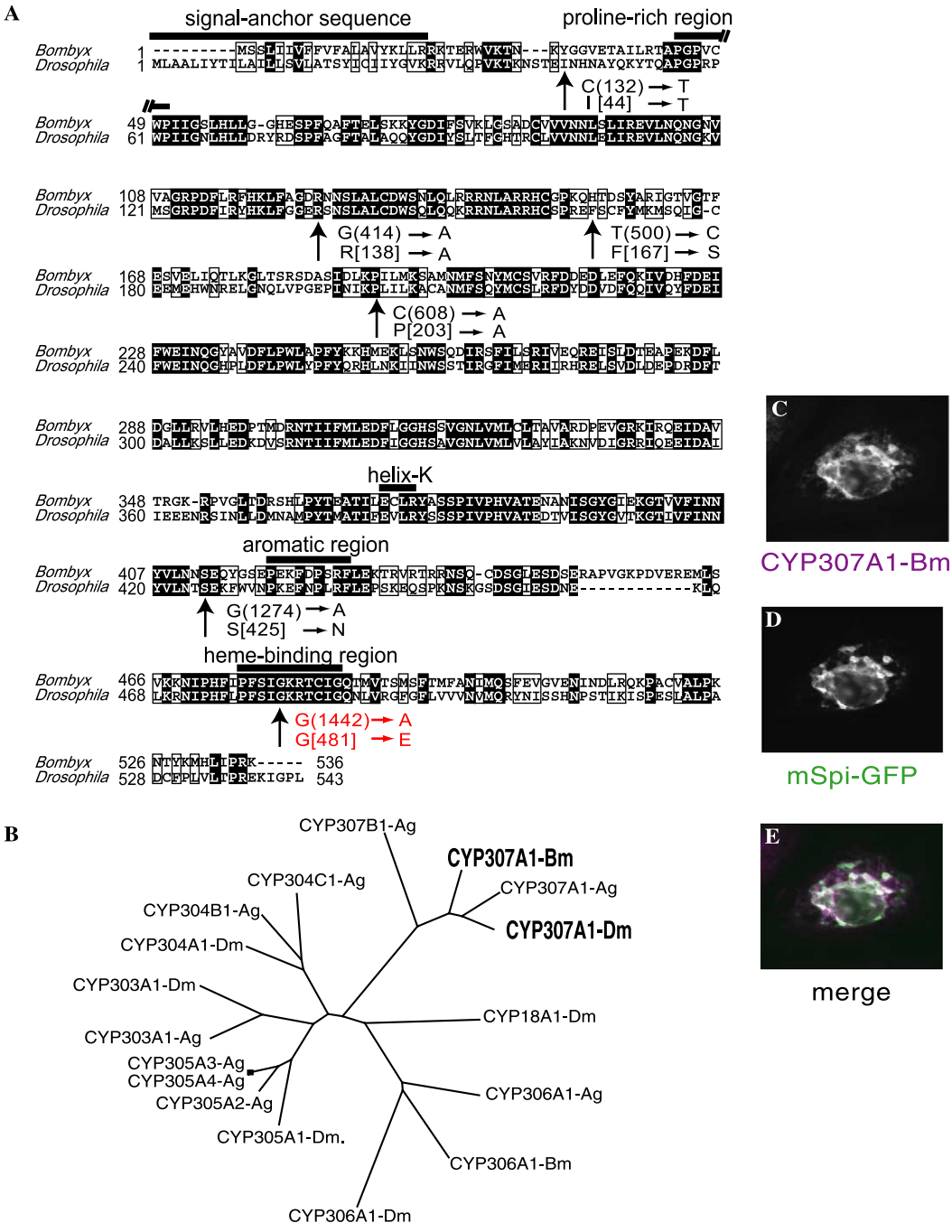


Fig. 1. *Cyp307a1* amino acid sequences and phylogenetic analysis. (A) Sequence alignment of *Cyp307a1-Bm* with *Cyp307a1-Dm*. Alignment of the predicted sequences was achieved with the ClustalW program (http://www.ddbj.nig.ac.jp/E-mail/clustalw-j.html). Hyphens indicate gaps inserted to optimize the alignment. Identical residues are white on black and similar residues are boxed. Conserved structural motifs common to most CYP enzymes are shown [26], i.e., a proline-rich region following a string of about 20 N-terminal hydrophobic and basic residues downstream of the initiating methionine residues, helix-K (ExxR; a putative hydrogen-binding sequence), an aromatic region (known also as "PERF"; PxxFxPExF), and a heme-binding region (PFxxGxRxCxG). Sequences typical of helix-C (WxxxR; a heme-interacting region) or helix-I (AGxxT; a putative oxygen-binding pocket) were unable to be identified between the proline-rich region and helix-K [26]. Arrows indicate nucleic acid substitutions that were found in the *spo*$^{l}$ mutant genome. Round and square brackets indicate the nucleic acid substitutions and predicted changes of amino acids, respectively. Red letters show the mutation point supposed to hinder the function of the heme-binding domain. (B) Phylogenetic tree showing the relationship of CYP307A1-Bm to other insect P450s. Endoplasmic reticulum P450s were selected according to ref [31]. The amino acid sequences were obtained from the INRA insect P450 database (http://p450.antibes.inra.fr). The phylogenetic tree was generated based on the entire amino acid sequences using ClustalW and the tree-drawing software TreeView [41]. Bm, *Bombyx mori*; Dm, *Drosophila melanogaster*; Ag, *Anopheles gambiae*. (C–E) Immunolocalization of the HA-tagged CYP307A1-Bm protein and an endoplasmic reticulum marker (mSpi-GFP) in transfected S2 cells. HA immunoreactivity (C), GFP signal from the ER marker (D), and a merged image (E) were obtained using confocal microscopy.

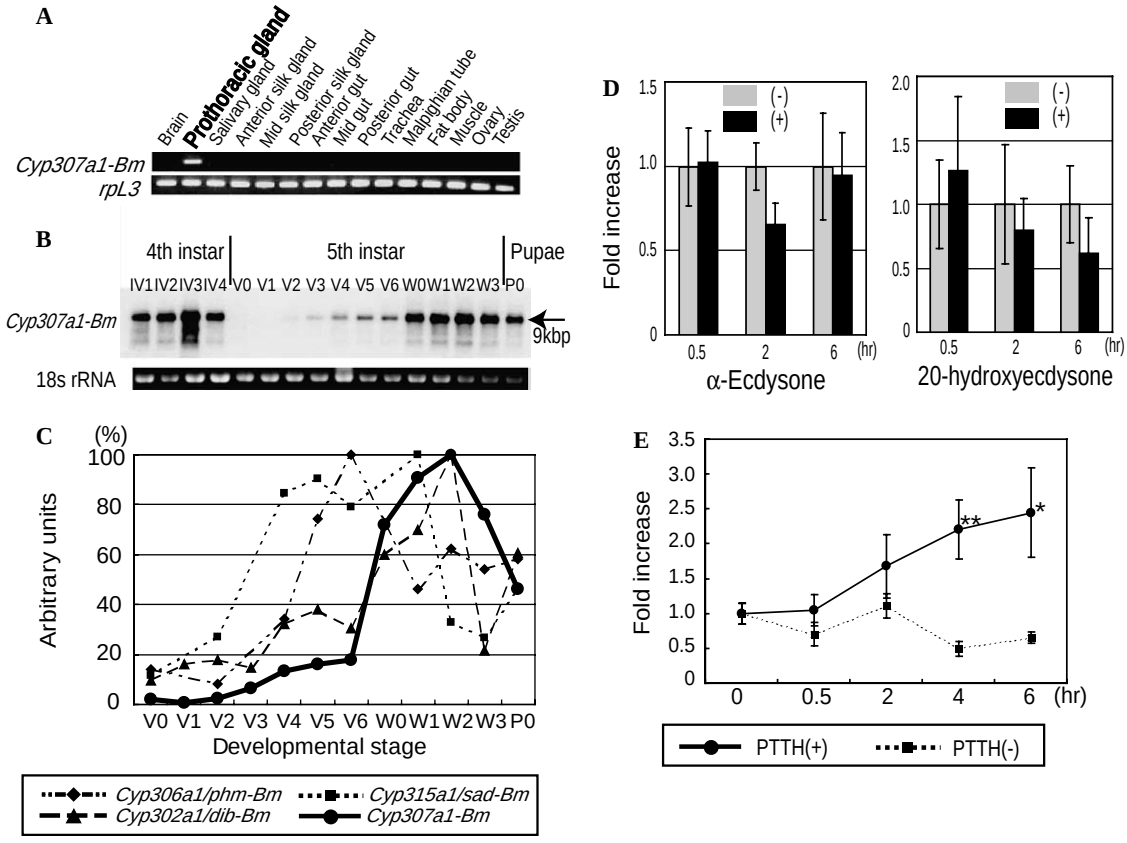


Fig. 2. Characterization of *Cyp307a1-Bm* expression. (A) Reverse transcription-PCR analysis of *Cyp307a1-Bm* tissue expression (above) and a control gene, *rpL3* (below), in the W1 fifth instar larvae. (B) Northern blot analysis showing the temporal expression profiles of *Cyp307a1-Bm*. (C) Comparison of the temporal expression profiles of *phm-Bm*, *dib-Bm*, *sad-Bm*, and *Cyp307a1-Bm*. The data for densitometric scans were derived from (B) of this article (for *Cyp307a1-Bm*), Fig. 1A of ref [11] (for *phm-Bm* and *sad-Bm*), and Fig. 2B of [12] (for *dib-Bm*). Hybridization intensity is presented as a percentage of the maximum level for each gene during the fifth instar larval period. Note the remarkable suppression of *Cyp307a1-Bm* expression during the early stages of the *Bombyx* fifth instar larvae. (D) Neither α-ecdysone (100 ng/ml) nor 20-hydroxyecdysone (20E; 200 ng/ml) induces *Cyp307a1-Bm* transcription in cultured prothoracic glands (PGs) from V4 larvae of *Bombyx*. Closed black bars and gray bars represent the *Cyp307a1-Bm* mRNA amounts (±SE; $n = 4$) in the treatment with or without each ecdysteroid, respectively. The gene expression level of each sample in the absence of the reagent is represented as 1 on the vertical axis. (E) mRNA expression levels of *Cyp307a1-Bm* in cultured V4 PGs in the presence (circle dots and solid lines) or absence (square dots and dashed lines) of 10 nM recombinant prothoracicotropic hormone (rPTTH). Each horizontal axis represents the time-points for incubation periods of the PGs in the presence or absence of stimulants. Each vertical axis indicates the fold increase of the mRNA expression level compared to each mRNA amount at the incubation time 0 h. Each value is an average of the fold increase ± SE ($n = 4$). Asterisks (*) and double asterisks (**) indicate statistical significance from Student's *t* test: * for $P < 0.05$ and ** for $P < 0.01$.

4- and 6-h treatment (Fig. 2E), suggesting that *Cyp307a1-Bm* expression might be under the control of PTTH.

### *Cyp307a1-Dm is encoded in the spo locus*

Previous studies have shown that *phm*, *dib*, *sad*, and *Cyp314a1/shade*, which code for essential components of ecdysteroid biosynthesis in *Drosophila*, belong to the Halloween group of mutants characterized by embryonic lethality and by cuticular patterning defects [8–11,30]. One uncharacterized Halloween mutant family member, *spo*, was mapped to the 64E cytological interval of the third chromosome [13], in the vicinity of the *Cyp307a1-Dm* gene at 64D5 [31]. Like other Halloween mutants, the $spo^1$ mutant [13] did not produce differentiated cuticle structure (Figs. 3A and B) [8]. To confirm whether *spo* corresponds to *Cyp307a1-Dm*, genomic DNA around the *Cyp307a1-Dm* region was amplified by PCR from the $spo^1$ mutant and then sequenced. We found that $spo^1$ mutant genome has several nonsynonymous nucleotide substitutions that change amino acids of the predicted CYP307A1-Dm protein (Fig. 1A). One of the mis-sense mutations changes an evolutionally conserved Glycine at position 481 within the heme-binding domain (Fig. 1A), which is one of pivotal domains of all cytochrome P450 enzymes [26]. Furthermore, *Cyp307a1* is likely the product of the *spo* gene, since the $spo^1$ mutant lethality can be rescued by *Cyp307a1-Dm* cDNA or *Cyp307a1-Bm* cDNA expression using a widely expressed *Actin5c-GAL4* driver (Table 1). These results also suggest that CYP307A1-Dm and CYP307A1-Bm proteins metabolize the same ecdysteroid intermediate.

### *In situ expression of Cyp307a1-Dm*

To analyze the expression pattern of *Cyp307a1-Dm* during *Drosophila* development, whole mount in situ RNA

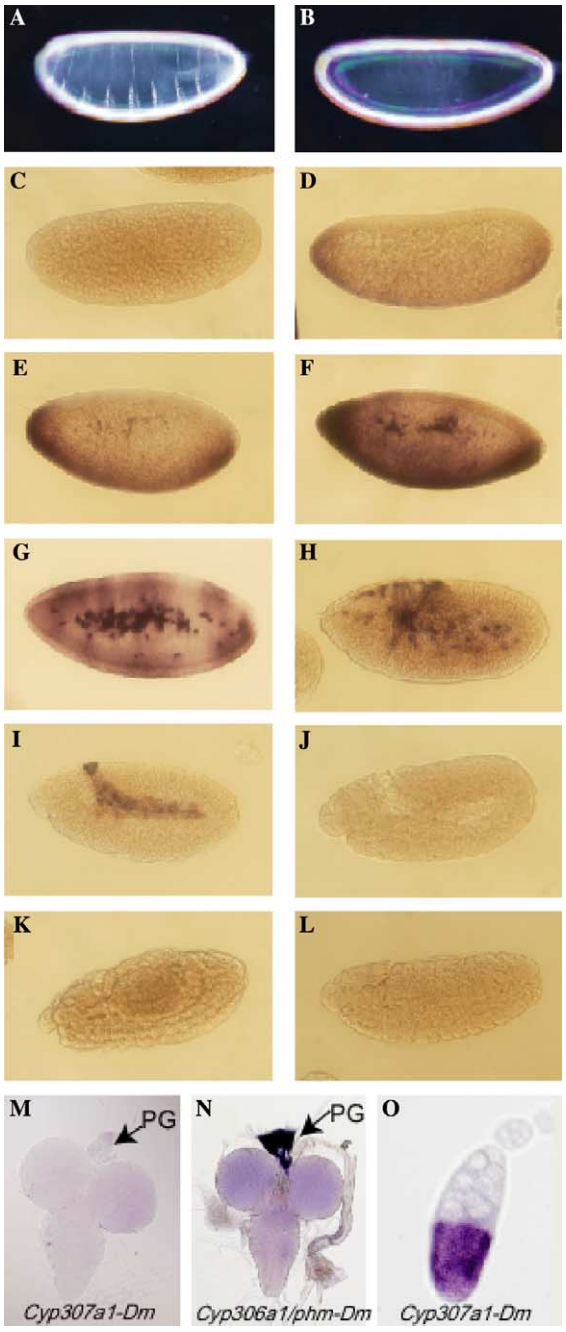


Fig. 3. In situ expression pattern of *Cyp307a1-Dm*. (A–B) Cuticular preparations. (A) wildtype cuticle, and (B) $spo^{1}$ cuticle. The $spo^{1}$ mutation resulted in the production of embryos with undifferentiated cuticle. (C–L) Embryonic expression of *Cyp307a1-Dm*. (C) Preblastoderm stage embryo. (D) cellular blastoderm stage embryo. Initial expression of *Cyp307a1-Dm* mRNA was located on the ventral side (D) and then progressed towards the middle of the embryo (E,F). (G) Stage 6. mRNA was also enriched in yolk nuclei and epithelial cells. (H) Stage 9. (I) Stage 10. Staining appeared in and around the amnioserosa region. (J–L) Stages 11–16. No expression was observed in any of the tissues. (M and N) Brain, ventral nerve cord, and ring gland complex from the wandering stage of third instar larvae. No *Cyp307a1-Dm* expression was observed in the PG cells (M), although *Cyp306a1/phm-Dm* is clearly expressed in the PG cells of the ring gland (N). (O) *Cyp307a1-Dm* ovarian expression was detected in follicle cells just prior to egg maturation. No staining was obtained for the sense RNA probes in the embryos, larvae, or ovaries (data not shown). PG, prothoracic gland cells.

hybridizations were carried out on embryos, larvae, and adult ovaries. No signal was detected in unfertilized eggs, suggesting that there is no maternal contribution of *Cyp307a1-Dm* (Fig. 3C) as was previously noted for *dib*, *sad*, and *phm* [8–11]. Zygotic *Cyp307a1-Dm* mRNA was observed on the ventral side of stage 3 cellular blastoderm embryos (Fig. 3D). After stage 5, *Cyp307a1-Dm* mRNA became more abundant and enriched in epithelial cells with a segmental stripe pattern and yolk nuclei in the middle of the embryo (Figs. 3E–G). At the time of complete germ band extension, *Cyp307a1-Dm* was expressed in the amnioserosa, a major ecdysone synthesis region in the early embryonic stages (Figs. 3H and I) [32–34]. After stage 11 in embryogenesis, *Cyp307a1-Dm* expression was drastically decreased in all tissues. In adult females, *Cyp307a1-Dm* mRNA was strongly expressed in the mature follicle cells (Fig. 3O), which are known to be another site of ecdysteroid biosynthesis. During *Drosophila* embryogenesis, ecdysteroid levels begin to rise at around stage 6–7, and peak during germ band movement (stage 10–11) [35,36]. The temporal expression levels of *spo*, as well as *phm*, *dib*, and *sad* [8–11], correlated well with the change in ecdysteroid titers during *Drosophila* embryogenesis.

However, unlike *phm*, *dib*, and *sad*, *Cyp307a1-Dm* mRNA was not detected in the primordia of the ring gland containing the *Drosophila* PG cells after stage 12 of embryogenesis (Figs. 3J–L). *Cyp307a1-Dm* expression was also not observed either in the PG cells in the third instar larvae (Fig. 3M); *phm*, *dib*, and *sad* are clearly expressed in PG cells at this developmental stage (Fig. 3N) [8–11]. These results suggest that *Cyp307a1-Dm*, unlike *Cyp307a1-Bm*, might not be necessary for ecdysone synthesis in the PG during larval development of *Drosophila*.

## Discussion

This report describes the use of FDD to find out genes involved in ecdysteroidogenesis in *Bombyx* PG and identified *Cyp307a1-Bm*, which is expressed specifically in the PG. *Cyp307a1-Bm* expression levels correlate with the changes in the ecdysone titer during *Bombyx* development, and the expression appears to be regulated by PTTH. It also describes the identification of the *Drosophila* ortholog of *Cyp307a1*. *Cyp307a1-Dm* expression is detected in embryonic amnioserosa and adult ovarian follicle, which are the pivotal ecdysone-producing organs in *Drosophila* [32–34,37]. Furthermore, *Cyp307a1-Dm* is shown to be encoded by the *spook* (*spo*) locus, which has been known to be critical for ecdysone production in the *Drosophila* embyo [8]. Taken together, these results suggest that *Cyp307a1* has an essential function in ecdysteroid biosynthesis in insects.

In early fifth instar larvae, *Cyp307a1-Bm* expression in the PG is undetectable, while a small but significant amount of the other P450 genes, about 10% of maximum expression level, are expressed during this stage. This observation implies that the repression of *Cyp307a1-Bm*

Table 1
The rescue of *spo*$^{1}$ lethality by overexpression of *Cyp307a1-Dm* or *Cyp307a1-Bm*

| Parental genotypes | F1 phenotypes | |
|---|---|---|
| | $y^+$ | *y* |
| *Actin5c-GAL4 spo*$^{1}$*/TM3* x *spo*$^{1}$*/TM3* | 110 | 0 |
| *UAS-Cyp307a1-Dm-HA spo*$^{1}$*/TM3* x *spo*$^{1}$*/TM3* | 142 | 0 |
| *UAS-Cyp307a1-Bm-HA spo*$^{1}$*/TM3* x *spo*$^{1}$*/TM3* | 112 | 0 |
| *Actin5c-GAL4 spo*$^{1}$*/TM3* x *UAS-Cyp307a1-Dm-HA spo*$^{1}$*/TM3* | 91 | 35 |
| *Actin5c-GAL4 spo*$^{1}$*/TM3* x *UAS-Cyp307a1-Bm-HA spo*$^{1}$*/TM3* | 132 | 27 |

Females of *yw;UAS-Cyp307a1 spo*$^{1}$*/TM3*[$y^+$] constructs were crossed with *yw;Actin5c-GAL4 spo*$^{1}$*/TM3*[$y^+$] males (see Materials and methods). Both *Actin5c-GAL4* and *UAS* constructs are located on the third chromosomes. The viable adults of *UAS-Cyp307a1 spo*$^{1}$*/Actin5c-GAL4 spo*$^{1}$ were distinguished by the absence of dominant marker ($y^+$) on *TM3*. When the lethality in the *spo*$^{1}$ mutant was rescued, the emergence of *y* adult flies was observed.

would cause the absence or very low titer of ecdysteroids during this period.

It is unexpected that *Cyp307a1-Dm* expression is not observed in *Drosophila* PG cells, because orthologs of each of the previously known Halloween P450s are expressed predominantly in the PGs of both *Drosophila* and *Bombyx* [4,10–12]. The different expression patterns of *Cyp307a1* in the two species may reflect differences in the ecdysone synthesis pathway between embryos and the third instar larvae, and/or between *Drosophila* and *Bombyx*. Further analysis of *Cyp307a1* might help us to understand the common and different features of ecdysteroid biosynthesis among insect taxa, which have not been elucidated so far.

Which step does CYP307A1 catalyze? There are at least two possibilities. The first possibility is that CYP307A1 functions in the conversion of cholesterol to 7-dehydrocholesterol. This conversion step is mediated by an unknown P450-class enzyme [38]. However, this possibility is doubtful, since cell lysates prepared from *Drosophila* larval PGs have enzymatic activity that converts cholesterol to 7-dehydrocholesterol in in vitro radiolabel incorporation studies [39], a finding that is inconsistent with the observation that *Cyp307a1-Dm* is not expressed in the organ. In order to test whether CYP307A1 can catalyze cholesterol and cholesterol derivatives (22-hydroxycholesterol and 25-hydroxycholesterol), an experiment was performed using the S2 cell system previously described in biochemical studies of Halloween P450s [9,11]. However, no metabolites have yet been detected (data not shown).

Alternatively, CYP307A1 could act in the ''Black Box'' (i.e., the reaction between 7-dehydrocholesterol and Δ-4-diketol), which is considered to be the rate-limiting step in ecdysteroid biosynthesis [2,40]. The ''Black Box'' reaction is also thought to be involved in an unknown P450 [2]. Elucidating the biochemical role of *Cyp307a1* will likely prove to be one of the key research areas in future studies of ecdysteroid biosynthesis.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2005.09.043.

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