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# Identification, characterization and developmental expression of Halloween genes encoding P450 enzymes mediating ecdysone biosynthesis in the tobacco hornworm, *Manduca sexta*

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**Abstract**

The insect molting hormone 20-hydroxyecdysone (20E) plays a central role in regulating gene expression during development and metamorphosis. In many Lepidoptera, the pro-hormone 3-dehydroecdysone (3DE), synthesized from cholesterol in the prothoracic gland, is rapidly converted to ecdysone (E) by a hemolymph reductase, and E is subsequently converted to 20E in various peripheral target tissues. Recently, four *Drosophila melanogaster* P450 enzymes, encoded by specific Halloween genes, were cloned and functionally characterized as mediating the last hydroxylation steps leading to 20E. We extended this work to the tobacco hornworm *Manduca sexta*, an established model for endocrinological and developmental studies. cDNA clones were obtained for three *Manduca* orthologs of *CYP306A1* (*phantom*; *phm*, the 25-hydroxylase), *CYP302A1* (*disembodied*; *dib*, the 22-hydroxylase) and *CYP315A1* (*shadow*; *sad*, the 2-hydroxylase), expressed predominantly in the prothoracic gland during the fifth (final) larval instar and during pupal–adult development, with fifth instar mRNA levels closely paralleling the hemolymph ecdysteroid titer. The data indicate that transcriptional regulation of *phm*, *dib* and *sad* plays a role in the developmentally varying steroidogenic capacities of the prothoracic glands during the fifth instar. The consistent expression of the Halloween genes confirms the importance of the prothoracic glands in pupal–adult development. These studies establish *Manduca* as an excellent model for examining the regulation of the Halloween genes.


*Keywords:* Real time PCR analysis; Phantom; Disembodied; Shadow; Ecdysteroids; Prothoracic gland; Development; Steroid hydroxylation

## 1. Introduction

One of the major challenges to insects is growth beyond the physical limitation of the rigid exoskeleton (cuticle) and this is met by periodic molts. In holometabolous insects this includes larval–larval molts and metamorphic molts to the pupa and then to the adult. These processes are coordinated and controlled by a polyhydroxylated steroid, 20-hydroxyecdysone (20E), the precursor of which is ecdysone (E) (see Gilbert et al., 2002). In pre-adult insects, the prothoracic glands are the principal source of ecdysteroids (Sakurai and Gilbert, 1990), synthesized from dietary cholesterol or phytosterols. This series of sterol modifications concludes with several hydroxylations catalyzed by cytochrome P450 enzymes (see Lafont et al., 2005; Gilbert and Warren, 2005). P450s comprise a diverse family of heme-thiolate enzymes known for their monooxygenase (hydroxylase) activity (see Feyereisen, 2005).

The complete pathway for ecdysteroid biosynthesis in the endocrine glands of insects is not known, but the overall process resembles vertebrate steroidogenesis in that the action of several P450 enzymes and the movement of precursors between intracellular compartments are required (Gilbert et al., 2002). The first step is the dehydrogenation of cholesterol to 7-dehydrocholesterol (7dC) in the endoplasmic reticulum (ER), likely catalyzed by a P450 enzyme (Grieneisen et al., 1993; Warren et al., 1995; Warren and Gilbert, 1996). Not understood are the subsequent, and possibly rate-limiting, oxidative

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transformations of 7dC that result in the formation of the characteristic 5$\beta$[H]-6-keto-7-ene-14$\alpha$-ol ecdysteroid structure (see Lafont et al., 2005). In the fruit fly *Drosophila melanogaster*, the three terminal reactions leading to the biosynthesis and secretion of E from the ring gland have recently been identified as being catalyzed by P450 enzymes. The hydroxylation at $C_{25}$ is mediated by CYP306A1 (Phantom: Phm) located in the ER, followed by hydroxylations at $C_{22}$ and $C_2$ carried out in the mitochondria by CYP302A1 (Disembodied: Dib) and CYP315A1 (Shadow: Sad), respectively (see Gilbert and Warren, 2005). Final conversion of E to 20E is catalyzed by a fourth hydroxylase, CYP314A1 (Shade: Shd, the ecdysone 20-monooxygenase: Petryk et al., 2003), variously located in the ER and the mitochondria of peripheral target tissues. These P450s are encoded by members of the Halloween gene family that were identified in *Drosophila* based on phenotypes associated with mutations disrupting embryogenesis (see Gilbert and Warren, 2005; Lafont et al., 2005).

In contrast to *Drosophila* where E is the primary pro duct of the prothoracic gland cells of the ring gland, 3-dehydroecdysone (3DE) is produced by the prothoracic glands of the lepidopteran, *Manduca sexta*, and many other insects (Warren et al., 1988; Kiriishi et al., 1990; see Gilbert et al., 1996). This difference is thought to be simply the result of an earlier reduction of a universal upstream 3-dehydro sterol intermediate to the 3$\beta$-alcohol rather than a fundamentally different pathway (Rees, 1989; Dauphin-Villemant et al., 1997). Thus, in *Manduca*, after secretion into the hemolymph, 3DE is rapidly reduced to E by a 3$\beta$-reductase prior to its conversion into 20E (see Lafont et al., 2005). In Lepidoptera, ecdysteroid production by the prothoracic glands is under control of the prothoracicotropic hormone (PTTH), a neuropeptide, released from neurosecretory cells in the brain (see Rybczynski, 2005).

Recent work by Parvy et al. (2005) indicates a general correlation between the steroidogenic capacity of the *Drosophila* ring gland and the abundance of transcripts of Halloween genes during larval development. In the silkworm *Bombyx mori*, *phm* expression in the prothoracic gland is correlated with the hemolymph ecdysteroid level (Warren et al., 2004), but a subsequent study by Niwa et al. (2005) indicates a somewhat unique temporal expression pattern of each Halloween gene in the prothoracic gland. However, a detailed quantitative analysis of the developmental expression of the characterized *Drosophila* Halloween genes is not available, since the rapid development, small size and life history (larvae burrowing into food) makes this model genetic insect less than optimal as an endocrinological model. In contrast, *Manduca* qualifies as an excellent insect model organism for such studies e.g. the large size offers a considerably greater amount of tissue for experimentation, the slower growth rate and life cycle makes precise staging relatively easy, and the hemolymph ecdysteroid and juvenile hormone titers during critical developmental stages are known and are readily quantified (Bollenbacher et al., 1981; Warren and Gilbert, 1986; see Gilbert et al., 1996; see Riddiford, 1996).

Taking advantage of these attributes of *Manduca*, we demonstrate here that the *Manduca phm*, *dib and sad* gene products are orthologs of, and functionally identical to, the *Drosophila* counterparts. Further, quantitative real time PCR (qPCR) analyses revealed that these genes are co-ordinately expressed in the prothoracic glands.

## 2. Materials and methods

### 2.1. Insects

*Manduca* larvae were group-reared on an artificial diet at 26 °C under high humidity conditions and a 16:8 light: dark cycle (Rybczynski and Gilbert, 1994). With this regimen, the fifth (final) larval instar lasts approximately 9 days (indicated by $V_1$–$V_9$); and pupal–adult development takes approximately 21 days (indicated by $P_0$–$P_{21}$). Tissues were extirpated under insect saline (Weevers, 1966) and rinsed quickly in Grace's medium before being flash-frozen and stored at −80 °C.

### 2.2. Identification of Manduca Halloween P450s

For the cloning of *Manduca* Halloween genes, total RNA was extracted from day 4 fifth instar ($V_4$) larva1 prothoracic glands using the RNA microprep kit (Stratagene) according to the kit protocol, and quantified spectrophotometrically. First strand cDNA was reverse transcribed from total RNA using M-MuLV reverse transcriptase (New England Biolabs), an oligo$(dT)_{17}$ or an oligo$(dT)_{17}$ primer containing an anchor sequence (Danielson and Fogleman, 1997), in the presence of RNasin ribonuclease inhibitor (Promega). *Drosophila* and *Bombyx* Halloween P450 sequences were used as templates to design degenerate primers against conserved regions of: *phm* (*Drosophila Cyp306a1*, GenBank accession no. AF484413; *Bombyx CYP306A1*, GenBank accession no. AB124840); *dib* (*Drosophila Cyp302a1*, GenBank accession no. AF237560; *Bombyx CYP302A*, GenBank accession no. AB198340); and *sad* (*Drosophila Cyp315a1*, GenBank accession no. AY079170; *Bombyx CYP315A*, GenBank accession no. AB167737). Pairs of degenerate primers (Table 1) were used to amplify *Manduca dib* and *sad* products by RT–PCR from prothoracic gland cDNA, and a *Manduca phm* product was generated in a 3′-RACE–PCR reaction with a degenerate sense primer and an antisense primer matching the anchor sequence as described by Danielson and Fogleman (1997). First strand cDNA was amplified using Hotstart *Taq* polymerase (Qiagen). Samples were heated to 95 °C for 15 min followed by 35 amplification cycles (94 °C for 30 s, 50 °C or 45 °C for 30 s, and 72 °C for 1.5 min), and a final 7 min extension period at 72 °C. Amplification products were separated on an agarose gel, purified using the Qiaquick gel extraction kit (Qiagen), and cloned into the pGEM-T vector (Promega).