The regulatory mechanisms of ecdysteroidogenic P450 gene expression were investigated in the silkworm, *Bombyx mori*. Bommo-FMRFamide (BRFa), a neural suppressor of prothoracic gland (PG) activity, was found to suppress the expression of several P450 genes induced by prothoracicotropic hormone (PTTH) in the PG. A transcription inhibitor suppressed PTTH-induced expression of the P450 genes and the opposing effects of BRFa, while their short-term effects on ecdysteroidogenesis remained unchanged. This result suggests that the effects of these factors on the P450 gene transcripts become obvious on a longer time scale. Moreover, spontaneous expression of a P450 gene was observed in long-term PG culture, and was repressed by juvenile hormone. These results explain well the developmental fluctuation patterns of the P450 gene transcripts in the PG, indicating that multiple factors coordinate to regulate basal PG activity during insect development.

Key words: ecdysteroid; P450; prothoracicotropic hormone; FMRFamide; juvenile hormone

Insect molting and metamorphosis are regulated by steroid hormones named ecdysteroids, synthesized primarily in the prothoracic gland (PG). The hemolymph ecdysteroid level changes greatly in line with the ecdysteroidogenic capacity of the PG during insect development, suggesting that this fluctuation in basal PG activity (i.e., the ecdysterogenic activity of the gland, which can be measured by short-term in vitro PG culture in the absence of prothoracicotropic or prothoracico-static factors) is critical to the timing of molting and metamorphosis. While the short-term effects of prothoracicotropic or prothoracico-static factors are not reflected in basal PG activity, expression regulation of key molecules in the PG should control the basal PG activity on a longer time scale during development. The PG regulators with such long-term effects, however, remain largely unknown.

Recent identification of the ecdysteroidogenic P450 enzymes and several other molecules involved in ecdysteroidogenesis has opened a door to a better understanding of the molecular basis of the PG regulatory pathways. The abundance of transcripts of the P450 genes has been shown generally to correlate well with basal PG activity and thus with the hemolymph ecdysteroid titer during larval development in a few insects. These reports clearly suggest that the transcript levels of these ecdysteroidogenic enzymes are the critical determinants of basal PG activity, and that the regulators of P450 gene expression are key players in the regulation of insect development.

Prothoracicotropic hormone (PTTH) is a principal candidate for such regulators. PTTH has been extensively studied for its short-term prothoracicotropic activity, which requires translation of new proteins, as evidenced by the ability of translation inhibitors to block PTTH-stimulated ecdysteroid synthesis. It is also clear that PTTH stimulates transcription, but the target genes of this long-term trophic effect of PTTH have been elusive until recently. In 2005, Niwa *et al.* reported that PTTH stimulates the transcription of an ecdysteroidogenic P450 gene in the PG, showing for the first time that steroidogenic enzymes are the principal target of the trophic effect of a steroidogenic hormone in insects, as in vertebrates.

In response to this, it is important to investigate the effects of other PG regulators on the expression of ecdysteroidogenic P450 genes in the PG. Among these...
regulators is Bommo-FMRFamide (BRFa), a neural regulator of ecdysteroidogenesis that counteracts the prothoracicotropic activity of PTTH in the silkworm, *Bombyx mori*. Juvenile hormone (JH) has also been reported to suppress the gland during the early stages of the last instar larvae in *B. mori*.

In this study, we found that BRFa and JH also modulate the expression of certain P450 genes in the PG of *B. mori*. Our results suggest that multiple factors differentially regulate the expression of the P450 genes, achieving a complex developmental fluctuation of basal PG activity.

### Materials and Methods

**Experimental animals.** *B. mori* racial hybrid (Kinsnate × Showa) larvae were fed on the artificial diet Silkmate (Nihon Nosan Kogyo, Yokohama, Japan) at 25 °C under a 16 h light/8 h dark photoperiod, and staged after the final (fourth) larval ecdysis. Most larvae started wandering behavior on day 6 of the fifth instar and pupated on day 10.

**Synthetic and recombinant peptides.** Four BRFa peptides were custom synthesized and further purified by HPLC. Recombinant PTTH was prepared as described previously.

**Short-term PG culture.** Day 4 fifth instar larvae were anesthetized in water for 10–20 min. The PGs were dissected rapidly in sterile saline (0.85% NaCl, w/v) and pre-incubated in 100 μl of Grace’s Insect Medium (Sigma-Aldrich, St. Louis, MO). After 30 min, the glands were transferred into 100 μl of medium with or without 1 nM PTTH and/or 10 μM BRFa (mixture of BRFa-1-4, 2.5 μM each), and further incubated for the designated periods. For treatment with transcription inhibitor actinomycin D (Wako, Osaka, Japan), 10 μM of the drug was added to the pre-incubation medium 15 min before the beginning of incubation as well as to the incubation medium. At the end of incubation, the glands were removed and were kept frozen at −80 °C until cDNA preparation, while the remaining medium was diluted with the assay buffer (0.5% BSA and 0.05% sodium azide in 50 mM borate buffer, pH 8.4), and analyzed using the ecdysteroid radioimmunoassay as described previously. Although 15 glands were incubated for each preparation in order to prepare a large enough amount of cDNA, the incubation medium of nine glands randomly selected was analyzed by ecdysteroid radioimmunoassay.

**Long-term PG culture.** Small pieces of thoracic body wall with the PG attached were dissected from day 0 fifth instar larvae and cultured as described previously. The culture medium was renewed every 24 h, and the collected medium was analyzed for amounts of secreted ecdysteroids by radioimmunoassay, as described previously. After designated periods of time, the body wall-PG complexes were removed from the medium and the PGs were carefully dissected. The dissected PGs were stored at −80 °C until cDNA preparation.

**Quantitative RT-PCR.** Single-stranded cDNA synthesis was performed as described previously. For short-term-cultured PGs, three batches of total RNAs for each preparation were prepared independently from five glands, each of which was then converted into cDNA. For long-term-cultured PGs, 5–7 batches of total RNAs for each preparation were prepared independently from a single gland, each of which was then converted into cDNA. For analysis of the developmental expression profiles, three batches of total RNAs for each preparation were prepared independently from the five glands, each of which was then converted into cDNA. Quantification of transcripts of the P450 genes were performed by the Smart Cycler System (Cepheid, Sunnyvale, CA), essentially as described previously. RpL3 was chosen as a reference gene. After 1 min at 95 °C, 40 cycles (95 °C for 10 s and 68 °C for 20 s) were carried out for the amplification of each gene. Specific primers used to amplify *spo* (*spo*), *phantom* (*phm*), *disembodied* (*dib*), and *RpL3* were as previously described, while specific primers for *shadow* (*sad*) were as follows: forward primer, 5'-TCGAGGAAGGGACTCCAGTAA-TGC-3'; reverse primer, 5'-CAATTTGCAGTGATGGCAGATGTAC-3'. Serial dilutions of plasmids containing cDNAs of *spo*, *phm*, *dib*, *sad*, and *RpL3* were used as standards, and the transcript levels of the P450 genes were normalized with *RpL3* levels in the same samples.

### Results and Discussion

**BRFa repressed PTTH-induced transcription of P450 genes in the PG**

Since BRFa inhibits ecdysteroidogenesis in the presence of PTTH, it was important to determine the opposing effects of these two factors on the expression of ecdysteroidogenic P450 enzymes in the PG. As described previously, PTTH significantly upregulated the transcript levels of *spo*, *phm*, and *dib* in the short-term PG culture, with the effect on *dib* most prominent (Fig. 1). When the mixture of four BRFa peptides was added to the incubation medium in the presence of PTTH, the expression levels of *spo*, *phm*, and *dib* were significantly repressed under some of the experimental conditions (Fig. 1). Especially, the repression of *phm* was high enough, to the extent that the trophic effect of PTTH was canceled almost completely in the presence of BRFa. BRFa also suppressed the basal transcript amounts of *spo* and *phm*, though to a lesser extent, corresponding to its weak prothoracicostatic effect in the absence of PTTH.
Transcriptional inhibition of P450 genes did not block the prothoracicotropic or prothoracicostatic effect of PTTH or BRFa

In vertebrates, the steroidogenic effects of peptide hormones can be clearly divided into two phases: tropic effects, occurring within minutes, mainly mediated by the facilitated intracellular mobilization of substrate cholesterol, and slower trophic effects, occurring on the order of several hours, which involve increased gene transcription. On the other hand, in our research, transcriptional regulation of the P450 genes by PTTH and BRFa has occurred on the same time scale as stimulation or inhibition of ecdysteroidogenesis (within minutes to hours; Fig. 1). Hence, it is important to check whether the prothoracicotropic and prothoracicostatic effects of these neuropeptides are indeed coupled with their effects on the expression levels of P450 genes.

In order to determine this, we examined the effect of the transcription inhibitor actinomycin D. When actinomycin D was added to the culture medium, induction of spo, phm, and dib by PTTH and the opposing effect of BRFa was completely suppressed after 3 h of incubation (Fig. 2B–D; the expression level of dib somewhat decreased in the presence of PTTH, for unknown reasons). The effects of PTTH and BRFa on the amount of secreted ecdysteroids, however, were not inhibited significantly on this time scale (Fig. 2A). This result clearly suggests that the transcriptional induction of P450 genes by PTTH and its inhibition by BRFa are independent of their prothoracicotropic or prothoracicostatic effects, which are perhaps mediated by increased translation and/or phosphorylation of key molecules, as in vertebrates.

Expression profiles of some P450 genes correlated well with the fluctuation of PTTH and BRFa

The above results indicate that PTTH and BRFa not only counteract each other in the short-term stimulation and inhibition of ecdysteroidogenesis, but also inversely regulate the expression of several P450 genes, thereby
sculpting the complex expression pattern of the ecdysteroidogenic enzymes in vivo. In order to compare the expression profiles of the P450 genes with the fluctuation patterns of hemolymph PTTH titer and BRFa neuron firing, quantitative RT-PCR was performed throughout the last instar. The expression profiles of these genes corresponded well with those reported previously (Fig. 3).12,19,21,23)

When the expression profiles of phm and dib were compared, the effects of PTTH and BRFa on these gene

Fig. 2. Effects of Actinomycin D on P450 Gene Expression Regulated by PTTH and BRFa.
Secreted ecdysteroid amounts after 3 h of incubation are shown in A, while expression levels of the three PTTH-inducible P450 genes are shown in B–D. The presence or absence of actinomycin D (ActD, 10^{-6} M), PTTH (10^{-9} M), and BRFa (10^{-5} M) is indicated at the bottom. The expression levels of each P450 gene were normalized against that of the control sample; n = 9 for A and n = 3 for B–D. Each value represents the average ± SE. Statistically significant differences from Student’s t-test are indicated by asterisks: *P < 0.05; **P < 0.01.

Fig. 3. Developmental Expression Profiles of the Four P450 Genes in the PG in Relation to the Regulatory Factors.
A. Schematic illustrations of the PTTH titer in hemolymph (solid line), the BRFa neuron firing frequency (dashed line), and the ecdysteroid titer (gray area) in hemolymph (based on refs. 10 and 13). B–E, Developmental expression profiles of the four ecdysteroidogenic P450 genes in the PG; n = 3. Vertical bars represent SE. The expression levels of each P450 gene were normalized against that of the highest expression level of the same gene. IV-HCS, fourth instar head capsule slippage; V–0–5, fifth instar days 0–5; W–0–3, wandering days 0–3; P–0, pupal day 0.
transcripts became obvious. The abundance of dib transcript was gradually upregulated during the late stages of the last instar, when the PTTH titer in the hemolymph was high. In contrast, the expression of phm increased long before the rise in the PTTH titer, and was repressed after the onset of wandering, possibly reflecting the sensitivity of this gene to BRFa. In fact, the overall expression profile of phm is a mirror image of the firing profile of BRFa neurons, suggesting the importance of neural input in the regulation of phm expression.

On the other hand, the expression profile of spo cannot be explained well by these two factors alone. The upregulation of the spo transcript level was more than 500-fold on day 2 of the wandering stage as compared to that on fifth instar day 0 (Fig. 3B), which is hardly explained only by the modest effect of PTTH (Fig. 1B). Together with the expression profile of sad, this clearly suggests the existence of other mechanisms involved in the regulation of P450 gene expression in the PG.

spo expression was increased autonomously, but repressed by JH

One of the possible candidates for these mechanisms is the autonomous activation of the PG, which can be seen in long-term PG culture. This autonomous activation might be mediated by the positive feedback effects of ecdysteroids on the PG, although the existence of unknown autocrine factors has also been indicated.

In order to test this hypothesis, the long-term PG culture system was used. Every 2d of culture, 5–7 glands were removed from each group, and the expression levels of the ecdysteroidogenic P450 genes were determined (Fig. 4). As reported previously, basal PG activity increased spontaneously within several days of culture, as evidenced by the secreted amounts of ecdysteroids in the medium (Fig. 4A). Remarkably, the expression level of spo also increased autonomously, in line with the change in basal PG activity (Fig. 4B). In this culture system, the addition of corpus allatum (CA) to the incubation medium has been reported to repress autonomous activation of the PG. This probably mimics the in vivo effect of JH, which has been reported to act on the PG and to suppress its function just after the fourth larval ecdysis. The JH titer in the hemolymph rapidly declines in the first few days of the fifth instar, inducing physiological events leading to pupal metamorphosis.

When day-0 fifth larval PG was cultured with the CA of the same stage, spontaneous activation of the PG was inhibited, as described above (Fig. 4A). Implantation of the CA inhibited the secretory activity of the PG in the CA-implanted cultures, whereas the PGs in CA-removed cultures started ecdysteroid secretion (Fig. 4A). Importantly, the transcript level of spo was also repressed in the presence of CA, suggesting that JH secreted from the CA inhibits the spontaneous expression of spo.

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Fig. 4. P450 Gene Expression in Long Term-Cultured PGs.

Secrested ecdysteroid amounts in the medium, renewed every 24 h, are shown in A, while expression profiles of the P450 genes are shown in B–E. Incubation with or without CA is indicated at the bottom. Since 5–7 glands were removed from each group every 2d, each datum point of A was calculated from 6–24 cultures, while each datum point of B–E was calculated from the expression levels of the 5–7 glands. Vertical bars represent SE. The expression levels of each P450 gene were normalized against that of the highest expression level of the same gene.
The expression of the spontaneous expression of spo was also observed when a JH analog was added to the culture medium (specifically, an increase in the spo transcript from day 2 to day 3 of 8.8 fold was suppressed completely to 0.4 fold, by the addition of 25 nM from day 2 to day 3 of 8.8 fold was suppressed by PTTH, BRFa, and JH.

The suppressive effect of JH on the autonomous expression of spo was also observed when a JH analog was added to the culture medium (specifically, an increase in the spo transcript from day 2 to day 3 of 8.8 fold was suppressed completely to 0.4 fold, by the addition of 25 nM methoprene throughout the incubation period).

**Differential regulation of P450 gene expression by multiple factors**

The differential regulation of P450 gene expression by PTTH, BRFa, and JH is summarized in Fig. 5. These regulators of PG activity modulate the expression of the ecdysteroidogenic enzymes, which in turn changes the basal activity of the PG during larval development.

The suppressive effect of JH on the autonomous expression of spo is one of the most significant findings of this study. Considering the possible function of spo in ecdysteroidogenesis,\(^1\)\(^2\)\(^3\) it is tempting to assume that JH directly inhibits the expression of spo in the PG, thus achieving very low basal PG activity during the early periods of the last instar. It is also possible, however, to hypothesize that JH suppresses the basal PG activity via unknown pathways, thus inhibiting the positive feedback cycle of the PG, which leads to upregulation of spo. Further analysis of the expression regulation of spo should answer these questions.

It is interesting that the three factors play different roles in the transcriptional regulation of the P450 genes. Specifically, PTTH, BRFa, and JH act mainly on dib, phm, and spo respectively, making each P450 gene a transducer of each regulatory factor (Fig. 5). Based on this idea, identification of the possible regulators of sad expression and of the other genes involved in ecdysteroidogenesis should lead to the elucidation of as yet unknown mechanisms of PG regulation. It is also interesting to note that the overall expression profiles of the four P450 enzymes did not always correspond well with those for another well-studied lepidopteran insect, Manduca sexta.\(^7\) Investigation of the species difference and of the underlying molecular mechanisms of the differential regulation of P450 genes is necessary for a deeper understanding of the regulation of insect development.

**Acknowledgments**

We thank Dr. Honoo Satake for preparing synthetic peptides and Dr. Yoshiaki Tanaka for helpful discussion. N.Y. and R.N. are recipients of a research fellowship from the Japan Society for the Promotion of Science (JSPS). This work was supported by grants to H.K. from the Research for the Future Program of JSPS, a Grant-in-Aid for Scientific Research (B), and the Hamaguchi Foundation for the Advancement of Biochemistry.

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