Precocious metamorphosis in transgenic silkworms overexpressing juvenile hormone esterase

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Insect growth and development are intricately regulated by the titers of juvenile hormones (JHs) and ecdysteroids (and/or their metabolites) in the insect hemolymph. Hydrolysis of the methyl ester of JH by a JH-specific esterase (JHE) is a key pathway for the degradation of JH. Here, we generate transgenic silkworm strains that overexpress JHE by using the binary GAL4/UAS system. Overexpression of JHE from the embryonic stage resulted in larval–pupal metamorphosis after the third stadium, two stadia earlier than that observed in wild-type insects. This precocious metamorphosis suggests that JHs are not critical for normal development of embryo or larva before the second molt in Lepidoptera (moths and butterflies). Our transgenic approach allowed us to dissect the function of key physiological events that occur from embryogenesis. Until now, these types of studies were possible only in later larval stadia by using physical techniques such as allatectomy or the application of JH analogues. We believe that our system will allow further pioneering studies in insect physiology.

Bombyx mori | juvenile hormones | transgenesis

Two major groups of nonpeptidic hormones, juvenile hormones (JHs) and ecdysteroids, play critical roles in the regulation of insect growth and development (1). The presence of JH during exposure to the ecdysteroids ensures a molt to a like stage, whereas the absence of JH during an ecdysteroid pulse allows metamorphosis. Concentrations of both hormones are regulated precisely by biosynthesis and degradation. Two definitive primary pathways for JH metabolism, hydrolysis of the methyl ester of JH by soluble esterases and hydration of the epoxide by microsomal epoxide hydrolases, have been described so far (2–5). In several insect orders, the key route of JH metabolism is by means of the hydrolysis of the methyl ester, and JH esterase (JHE) is considered the principal enzyme responsible for this function (2). Because of their importance in insect development, JHEs have been isolated and characterized from many insect orders such as Coleoptera (6), Diptera (7), and Lepidoptera (8–10), including the silkworm, *Bombyx mori* (11).

As the primary degradative enzyme of JH, JHE has been used as an attractive tool for the study of JH action. Inhibition of JHE activity by a variety of 3-substituted 1,1,1-trifluoropropanone sulfides reduces the rate of JH degradation, thus delaying metamorphosis and resulting in giant larvae of *Manduca sexta* (12). By contrast, recombinant baculoviruses expressing JHE can reduce JH levels in Lepidoptera (13). However, difficulties in establishing stable and prompt *in vivo* expression of JHE in Lepidoptera have halted further studies.

The mechanism of JH action in insects still remains a mystery despite a great deal of knowledge about its biological actions (5). Using *Drosophila* as a model for the study of JH action meets with difficulties because, in contrast to most insects, including those in Lepidoptera, *Drosophila* displays few morphogenetic actions that can be easily bioassayed (14). However, a highly efficient and easy-to-use transgenesis system using the transposon vector *P* element is available for *Drosophila*. Recently, transposons such as *piggyBac*, *Hermes*, *Minos*, *hobo*, and *mariner* have been identified and used as vectors for germ-line transfor-

mation in nondrosophilid insects (15). In *B. mori*, a system for germ-line transformation using a *piggyBac* transposon-derived vector has been established (16). On the basis of this method, the binary GAL4/upstream activating sequence (UAS) system for targeted gene expression in *B. mori* has been developed (17). The binary GAL4/UAS system relies on the generation of activator transgenic lines, which express the yeast transcriptional activator GAL4 and effector lines, which carry a gene cassette composed of the UAS GAL4-binding motif linked to the target gene. In the present study, we generated activator lines (GAL4 lines) in which GAL4 expression is under control of the *B. mori* cytoplasmic actin promoter (*BmA3*) (16) and effector lines (UAS lines) that carry the *B. mori* JHE gene (*BmJHE*) (11) linked to the UAS. The GAL4 and UAS lines also expressed the marker genes, DsRed2 (human codon optimized red fluorescent protein) and ECFP (enhanced cyan fluorescent protein) (18), under the control of the artificial eye-specific 3xP3 promoter, which allows for the identification of transgenic animals and further investigation from the embryonic stage (19, 20). JHE was expressed at massively high levels in the progeny of the cross between the UAS and GAL4 lines. Remarkably, these animals underwent precocious metamorphosis only after the third larval stadium, indicating that JH is not necessary for development during the embryonic and early larval stages.

Materials and Methods

***B. mori* Strains and Chemical Treatment.** The *B. mori* nondiapause strain, pnd-w1, was used for germ-line transformation. After injection of DNA into preblastoderm eggs, the embryos were incubated at 25°C in humidified chambers for 11–14 days until hatching. The larvae were reared on an artificial diet (Nihon Nosanko, Yokohama, Japan) under standard conditions (16). To evaluate the effect of nonmetabolizable JH mimic on JHE overexpression, the transgenic larvae were topically treated on the dorsal surface with acetone dilutions of methoprene (10 µg per larva) at day 0 of the third stadium.

Plasmid Construction. The activator and effector plasmid constructs (Fig. 1) were obtained as described below.

***pBac{A3-GAL4-3xp3-DsRed2}* (GAL4 lines).** The initial plasmid, *pBac{A3-GAL4}*, was constructed in a previous study (17). To introduce the transformation marker gene DsRed2 into *pBac{A3-GAL4}*, the 3xp3-DsRed2-SV40 terminator fragment was PCR-amplified from *pBac{3xp3-DsRed}* (21) by using the following primers: 5'-CAAGATCTAATTCGAGCTCGC-CCGGGGATCTATTC-3' (forward) and 5'-TAGCAGATCTGTACGCGTATCGATAAGCTTTAAG-3' (reverse). Both

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Abbreviations: JH, juvenile hormone; JHE, JH esterase; ECFP, enhanced cyan fluorescent protein; DsRed2, human codon optimized red fluorescent protein; UAS, upstream activating sequence.

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Fig. 1. Physical map of activator and effector constructs. (Upper) The activator constructs of the vector *pBac*{A3-GAL4-3xp3-DsRed2}. The vector contains the full ORF of the yeast transcriptional factor GAL4 under the control of BmA3 promoter and the marker gene, DsRed2, under the control of the 3xp3 promoter. (Lower) The effector constructs of the vector *pBac*{UAS-JHE-3xp3-ECFP}. The vector contains the DNA-binding motif (UAS) for GAL4, linked to the ORF of the targeted gene, BmJHE, and the marker gene, ECFP, under the control of the 3xp3 promoter. Arrow, *piggyBac* inverted terminal repeat; box, target sequence, TTAA.

primers contain BglII sites at their 5' ends. The PCR product was digested by BglII and inserted into the BglII site of *pBac*{A3-GAL4} to generate *pBac*{A3-GAL4-3xp3-DsRed2}.

***pBac*{UAS-JHE-3xp3-ECFP} (UAS lines).** A vector containing the BmJHE linked to the UAS and coupled with the transformation marker gene of ECFP was constructed initially from *pBac*{UAS-GFP} as described in ref. 17. The 3xp3-ECFP-SV40 terminator fragments were PCR-amplified from *pBac*{3xp3-ECFPafm} by using the following primers: 5'-ACCGGTAATTCGAGC-TGCCCCGGGGATCTA-3' (forward) and 5'-ACCGGTG-TACGCGTATCGATAAGCTTTAAG-3' (reverse). Both primers contain AgeI sites at their 5' ends. The PCR product was digested by AgeI and inserted into the AgeI site of *pBac*{UAS-GFP} to generate *pBac*{UAS-GFP-3xp3-ECFP}. The target gene, BmJHE, was PCR-amplified from a larval whole-body *B. mori* cDNA library (11), by using the primers 5'-GCGGGATC-CCTCAGTGCTGCGCCAGTTGT-3' (forward) and 5'-GCGGCGGCCGTTTACTTAAGTCTGCTTTC-3' (reverse). These primers contain BamHI and NotI sites at their 5' ends, respectively. The 1710-bp-long PCR product was digested with BamHI and NotI and used to replace the GFP cDNA between the BamHI and NotI sites of *pBac*{UAS-GFP-3xp3-ECFP}.

The sequences of the PCR products and constructed plasmids were confirmed by using a BigDye termination DNA sequence kit (Applied Biosystems) on an ABI310 sequencer. The DNA vector and helper plasmid were injected into silkworm eggs at a concentration of 0.2 $\mu\text{g}/\mu\text{l}$ for each plasmid.

Inverse PCR and Junction Sequences. Genomic DNA was extracted from the silk glands of G₁ larvae of the UAS lines by standard SDS lysis–phenol treatment after incubation with proteinase K, followed by RNase treatment and purification. Inverse PCRs were carried out by using an *in vitro* Cloning Kit (Takara Bio, Tokyo) according to the manufacturer's instructions. The amplified DNAs were digested with Sau3AI and circularized by ligation for 30 min at 16°C. The 5' junction sequences were amplified by PCR with primers designed from the left-hand regions of the vector as follows: 5'-CGCGGTCGTTATAGT-TCAAATCAGTG-3' for the first PCR and 5'-TCCAAGCG-GCGACTGAGATGTCCTAAA-3' for the second PCR. PCR-amplified fragments were gel-purified after 2% agarose gel electrophoresis and directly sequenced.

Real-Time Quantitative PCR. Total RNA was extracted from embryos and larval whole bodies of each stadium by using Isogen reagent (Nippon Gene) and treated with RNase-free DNase I (Promega). Subsequently, cDNAs were synthesized by using a T-primed first-strand kit with an oligo(dT)₁₈ primer (Amersham Pharmacia Biosciences). Real-time PCRs were performed in

96-well plates by using a SYBR green PCR Master Kit (Applied Biosystems). The sequences of primer pairs for JHE were 5'-TAGATGCCACCGAGGAAGGT-3' (forward) and 5'-ATTGGCGTAGATGCATGCC-3' (reverse). Quantitative measurements were performed in triplicate and normalized to an internal control of *B. mori* *rp49* mRNA for each sample.

JHE Activity Determination. JHE activity in embryos and larval whole body homogenates was determined by the partition assay method of Hammock and Sparks (22), using ³H-labeled JH III as a substrate. JHE activity in the larval hemolymph was assayed by the method of McCutchen *et al.* (23) by using a spectrophotometric assay with methyl heptylthioacetothioate as a JH-mimic substrate. The experimental conditions were optimized for *B. mori* as reported in ref. 24.

Western Blot Analysis. The BmJHE antiserum was prepared in ref. 11. Larval hemolymph collected from different stadia was resolved by SDS/10% PAGE and transferred to a poly(vinylidene difluoride) membrane. The BmJHE protein on the membrane was detected with anti-BmJHE antiserum (1:10,000 dilution) as the primary antibody and alkaline phosphatase-conjugated goat anti-rabbit IgG (1:20,000 dilution; Kirkegaard & Perry Laboratories) as the secondary antibody.

Results and Discussion

To obtain the stable binary GAL4/UAS transgenic strains, we first generated the activator and effector lines by injection of the activator and effector vectors (Fig. 1), respectively, into preblastoderm silkworm embryos mixed with the transposase carrying helper plasmid pHA3PIG (16). After sib selection according to the markers, the G₁ moths of the UAS line were crossed with the G₁ moths of the GAL4 line to generate JHE-overexpressing GAL4/UAS lines. The progeny of this crossing showed four different phenotypes in terms of eye color: (i) neither ECFP-positive nor DsRed2-positive, wild type, [E(–)D(–)]; (ii) only DsRed2-positive, GAL4 lines, [E(–)D(+)]; (iii) only ECFP-positive, UAS lines, [E(+)D(–)]; and (iv) both ECFP-positive and DsRed2-positive, GAL4/UAS lines, [E(+)D(+)]. The segregation ratio of the G₁ progeny of this cross was nearly 1:1:1:1 as expected (Table 1), indicating that the transgenes were stably inherited in a Mendelian manner.

The insertion of the transgenes into the genome of the UAS line was confirmed by inverse PCR using DNA isolated from the silk gland of ECFP-positive G₁ larvae. Three different genomic junction sequences of 645, 369, and 139 bp flanking the 5' *piggyBac* inverted terminal repeat sequences were determined in three different transgenic lines (Table 2). A search of the Kaikoblast database (<http://kaikoblast.dna.affrc.go.jp/>) con-

Fig. 2. The expression of JHE was dramatically higher in the GAL4/UAS lines. Total RNA was extracted from embryos or larval whole-body homogenates. The expression of JHE was determined by real-time PCR and was normalized to *B. mori* *rp49* expression. Representative data (mean $\pm$ SEM) from at least three independent experiments are shown. E(-)D(-), wild-type lines; E(-)D(+), GAL4 lines; E(+D(-), UAS lines; E(+D(+), GAL4/UAS lines. E6, day 6 of the embryonic stage; L1D3, day 3 of the larval first stadium; L2D3, day 3 of the larval second stadium; L3D3, day 3 of the larval third stadium.

KK-42 application or surgical allatectomy, additional time is needed to clear JH from the hemolymph (11, 28), resulting in the prolonged intermolt period. Considering that in early stadia larvae, chemical allatectomy (e.g., KK-42 application) is lethal and surgical allatectomy is highly difficult or impossible to perform, the present transgenic system is exceptionally useful as a form of “genetic allatectomy.”

The remarkable phenotype of the third stadium JHE-overexpressing *B. mori* raises the question of why precocious pupation occurs at this stage. Because the final stadium is apparently different from the penultimate stadium in *B. mori*, we speculate that larval stadia before the penultimate stadium, from the first to the third stadia in Lepidoptera, share similar molt mechanisms. Here, we propose that the early stadia (first and second) are JH independent. Furthermore, we proposed that there is a fundamental difference between the early stadia and later stadia (third through final) such that JH is not a critical factor for normal development from embryogenesis through the larval second stadium. Although JH secretion from the corpora allata can be detected from the embryonic stage, the JH-signaling pathways may not be activated until the third stadium. Because the ecdysteroid-coordinated molting process is unaffected by the presence or absence of JH (5), we hypothesize that the first and second molts that occur in the absence of JH result in the same morphology, and no metamorphosis. When the animal enters the JH-dependent phase from the third stadium, JH-regulated genes and endocrine pathways are induced. JH deprivation (by means of JHE overexpression) then leads to a failure in JH signaling initiation, whereas the ecdysone pulse in the absence of JH eventually results in developmental transitions from larva to pupa. In agreement with this hypothesis, the application of methoprene prevented the JHE-mediated induction of precocious metamorphosis and demonstrated that the precocious metamorphosis is the result of the lack of JH. The appearance of larval-pupal intermediates may be the result of interindividual variation of JHE expression level (i.e., JH titer in these individuals did not decline to the critical level for complete pupation). Quantitative determination of JH levels in transgenic larvae is still needed to determine the minimal concentration of JH necessary for normal development.

The minimal effects of a low JH titer on embryonic and early larval stages in transgenic silkworms are consistent with the

Fig. 3. The activity and protein expression of JHE were dramatically higher in the GAL4/UAS lines. (A) JHE activity in embryos and larval whole bodies was determined by using [3 H]JH III as a substrate. (B) JHE activity in the hemolymph was measured with the spectrophotometric JH-mimic substrate methyl heptylthioacetothioate. (C) Western blot analyses were performed on the hemolymph by using antibody against BmJHE. E(-)D(-), wild-type lines; E(-)D(+), GAL4 lines; E(+)D(-), UAS lines; E(+)D(+), GAL4/UAS lines. E6, day 6 of the embryonic stage; L1D3, day 3 of the larval first stadium; L2D3, day 3 of the larval second stadium; L3D3, day 3 of the larval third stadium; L5D4, day 4 of the larval fifth stadium. * at L5D4, larvae of the GAL4/UAS lines halted development at the third stadium.

hypothesis that the ancestral developmental role of JH is the regulation of embryogenesis (33). Exogenous JH has striking effects on the embryogenesis of ametabolous insects (34), but only minor effects in the embryogenesis of holometabolous insects (26, 27). In eggs of *Locusta migratoria migratoroides*, the topical application of the anti-allatin substance, precocene III (which does not function in Lepidoptera), results in prothetelic morphogenetic disturbances in second stadium larvae (35, 36), one stadium earlier than our results in *B. mori*. These investigations suggest that postembryonic effects of JH are initiated later in holometabolous insects than in ametabolous insects.

Fig. 4. Transgenic silkworms of the GAL4/UAS lines pupated at the end of the larval third stadium without progressing through the fourth and fifth stadia. On the left is an intermediate between larva and pupa, in the center is a precocious pupa, and on the right is a wild-type late pupa (i.e., normal pupa after metamorphosis at the end of the larval fifth stadium). (Scale bar: 1 cm.)

Current knowledge of how JH acts at the molecular level is largely dependent on the studies of ecdysteroid-regulated genes because the role of JH is to direct the action of ecdysone and 20-hydroxyecdysone (20E). A number of ecdysteroid-regulated transcription factors are involved in the JH endocrine pathways (5). Numerous attempts have been made to identify JH receptors. For example, Ultraspiracle (USP), a homologue of the vertebrate retinoid X receptor (RXR), has been considered a candidate JH receptor gene (37, 38) in

Drosophila, although subsequent studies do not support this view (39, 40). Methoprene-tolerant gene (*Met*) identified from methoprene-resistant *D. melanogaster* (41) shows potential as a JH-dependent transcription factor (42). However, *Met* is not an essential gene, and *Met*-null mutant flies do not show morphogenetic abnormalities; thus, its involvement in JH signal cascade remains to be seen. By contrast, because JHE is a critical JH-degradative enzyme, its substrate specificity and its functional role in the reduction of the JH titer have been proven (11, 43). Furthermore, in a lepidopteran, *Choristoneura fumiferana*, JHE expression is directly regulated by both JH and 20E in dose- and time-dependent manners (10), and a 30-bp-long JH-responsive region within the JHE promoter has been identified (44). Taking into consideration these previous approaches, the manipulation of JHE gene expression for JH titer regulation will provide a forceful tool for the better understanding of the JH signaling pathway. With the future availability of inducible promoters that are tissue- or developmental stage-specific in the binary GAL/UAS system, we will have more precise regulatory control of gene misexpression and will be able to introduce this system to other nondrosophilid insect orders.

This study presents the successful induction of premature metamorphosis in genetically engineered silkworm at the third larval stadium by using the binary GAL4/UAS system for a stable *in vivo* JHE overexpression. The present system to reduce JH titer will help resolve unidentified JH receptors because, analogous to 20E activation of ecdysone receptor, JH may stimulate the expression of JH receptors. A microarray analysis of JHE-overexpressing transgenic silkworms may characterize JH-dependent genes, allowing a further elucidation of JH action in insects.

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