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Author(s) :Anuradha Roy, Sakiko Shimizu, Taketoshi Kiya, Kazuei Mita and Masafumi Iwami
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Identification of 20-hydroxyecdysone-inducible Genes from Larval Brain of the Silkworm, *Bombyx mori*, and Their Expression Analysis

Anuradha Roy[1†], Sakiko Shimizu[2†], Taketoshi Kiya[2], Kazuei Mita[3], and Masafumi Iwami[1,2*]

[1]*Division of Life Sciences, Graduate school of Natural Science and Technology, Kanazawa University, Kakuma-machi, Kanazawa 920-1192, Japan*
[2]*Division of Biological Sciences, Graduate school of Natural Science and Technology, Kanazawa University, Kakuma-machi, Kanazawa 920-1192, Japan*
[3]*National Institute of Agrobiological Sciences, Tsukuba 305-8634, Japan*

The insect brain secretes prothoracicotropic hormone (PTTH), which stimulates the prothoracic gland to synthesize ecdysone. The active metabolite of ecdysone, 20-hydroxyecdysone (20E), works through ecdysone receptor (EcR) and ultraspiracle (USP) to initiate molting and metamorphosis by regulating downstream genes. Previously, we found that EcR was expressed in the PTTH-producing neurosecretory cells (PTPCs) in larval brain of the silkworm *Bombyx mori*, suggesting that PTPCs function as the master cells of development under the regulation of 20E. To gain a better understanding of the molecular mechanism of the 20E control of PTPCs, we performed a comprehensive screening of genes induced by 20E using DNA microarray with brains of day-2 fifth instar silkworm larvae. Forty-one genes showed greater than twofold changes caused by artificial application of 20E. A subsequent semiquantitative screening identified ten genes upregulated by 20E, four of which were novel or not previously identified as 20E-response genes. Developmental profiling determined that two genes, *UP4* and *UP5*, were correlated with the endogenous ecdysteroid titer. Whole-mount in situ hybridization showed exclusive expression of these two genes in two pairs of cells in the larval brain in response to 20E-induction, suggesting that the cells are PTPCs. BLAST searches revealed that *UP4* and *UP5* are *Bombyx* homologs of *vrille* and *tarsal-less*, respectively. The present study identifies 20E-induced genes that may be involved in the ecdysone signal hierarchies underlying pupal–adult development and/or the 20E regulation of PTPCs.

Key words: 20-hydroxyecdysone, brain, insect, in situ hybridization, metamorphosis, microarray, prothoracicotropic hormone, silkworm

INTRODUCTION

The brain of the silkworm, *Bombyx mori*, produces prothoracicotropic hormone (PTTH), the primary stimulator of temporal transition, in two pairs of lateral neurosecretory cells (LNCs) (Agui et al., 1979; Kawakami et al., 1990; Mizoguchi et al., 1990). PTTH signals the prothoracic glands (PGs) to trigger production and release of ecdysone, the precursor of its active metabolite 20-hydroxyecdysone (20E) (Kawakami et al., 1990; Gilbert et al., 2002). 20E governs morphological changes of the brain (Champlin and Truman, 1998; Kraft et al., 1998) by regulating gene expression cascades associated with ecdysteroidogenesis and metamorphosis (Beckstead et al., 2005).

* Corresponding author. Tel. : +81-76-264-6251;
Fax : +81-76-264-6215;
E-mail: masafumi@staff.kanazawa-u.ac.jp
† These two authors contributed equally to this work.
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A heterodimeric receptor complex composed of ecdysone receptor (EcR) and ultraspiracle (USP) binds to 20E (Koelle et al., 1991; Yao et al., 1992; Thomas et al., 1993; Kamimura et al., 1996) to elicit ecdysone signal pathway (Riddiford, 1993; Riddiford et al., 2003). This nuclear complex directly induces the transcription of some primary response early genes (Segraves and Hogness, 1990; Thummel et al., 1990; Dibello et al., 1991; Thummel, 1995) through the positive transcription cascade. The early gene products then regulate 20E signal by expressing the secondary response late genes (White et al., 1997; Jiang et al., 2000), enabling appropriate developmental responses to 20E.

We previously reported that EcR is expressed in two pairs of PTTH-producing neurosecretory cells (PTPCs) (Hossain et al., 2006) in larval brain of the silkworm, suggesting that they function as the master cells of development. However, the molecular mechanism of PTTH signaling by 20E remains largely unexplored.

To better understand the mechanism that underlies 20E

regulation of PTPCs, we carried out a comprehensive DNA microarray analysis of 20E-induced genes in brains of fifth instar day-2 silkworm larvae in the presence or absence of 20E in vitro. Forty-one genes showed greater than twofold changes after artificial 20E application. So far, twenty-six of them have been experimented for their reproducibility by semi-quantitative reverse transcription- (RT-) PCR and ten genes are actually upregulated by 20E. To focus on unknown genes induced by 20E, we conducted BLAST searches and found that four of these genes were novel or not previously identified as early or late genes in response to 20E (Ashburner et al., 1974; Ashburner, 1990). Given their 20E-responsiveness, we suspected that these genes respond to the hemolymph ecdysteroid titer. Therefore, to investigate the internal effect of endogenous 20E titer on these genes across developmental stages, temporal expression profiling was conducted by RT-PCR. The expression pattern showed that two genes, *UP4* and *UP5*, were correlated with natural hemolymph ecdysteroid titer during the last two instars. Cellular localization by in situ hybridization revealed that these genes are expressed in two pairs of LNCs of larval brains believed to be PTPCs (Hossain et al., 2006). Homology searches revealed that *UP4* and *UP5* are the *Bombyx* homologs of *vrille* and *tarsal-less* genes, respectively.

MATERIALS AND METHODS

Experimental animals and hormone

Bombyx eggs of a racial hybrid, Kinshu × Showa, were obtained from Ueda Sanshu (Ueda, Japan), and larvae were reared on an artificial diet (Silkmate II, Nihon Nousan Kogyo, Yokohama, Japan) under a 12 h light/12 h dark photoperiod at 25°C (Sakurai, 1984). Ages were counted in days, with a photophase followed by a scotophase. The 24 h photophase period following the scotophase during which the larvae molted was designated as day-0 of the next corresponding instar. Larvae of third instar day-0 (III0) through fifth instar day-8 (V8) were studied in the present research.

20E (Sigma-Aldrich, St. Louis, MO, USA) was dissolved and used for injection. The animals were injected with 1 μg 20E (+20E) or insect Ringer's solution (–20E) through the first proleg and dissected 2 h after injection.

Microarray analysis

DNA microarray, constructed with 16,000 expression sequence tag (EST) clusters from the silkworm EST project (Mita et al., 2003), was used to analyze RNA samples (20E-injected V2 × Ringer-injected V2) to identify the 20E-regulated genes in the larval brain. Total RNA (1 μg) was extracted from brains according to the acid guanidium thiocyanate phenol-chloroform method (Chomczynski and Sacchi, 1987) with minor modifications (Tsuzuki et al., 2001), treated with RNase-free DNase I (Promega, Madison, WI, USA), and used as RNA sample. 20E-injected and Ringer-injected RNA samples were both independently labeled with Cyanine 3-CTP (Cy3) and Cyanine 5-CTP (Cy5). Two array slides were used for each sample: one for 20E-injected Cy3 and Ringer-injected Cy5, and the other for 20E-injected Cy5 and Ringer-injected Cy3. RNA probe labeling and hybridization were performed using the Fluorescent Linear Amplification Kit and the In-situ Hybridization Kit Plus (Agilent Technologies, Santa Clara, CA, USA) according to the manufacturer's instructions. In the experiment, we compared the RNA samples by a two-color dye-swap protocol with two replicates. The arrayed series was then scanned by a microarray scanner (Agilent Technologies) and the signal level was quantified with the Feature Extraction software (Agilent Technologies) to identify genes with different expression levels. Differentially expressed genes in the two replicated samples in the microarray experiments were based on *P*-value. *P*-value for ≥ 1.36 fold changes was calculated as < 0.01 using the Feature Extraction software. We searched for homologous sequences in DDBJ (http://blast.ddbj.nig.ac.jp), NCBI (http://blast.ncbi.nlm.nih.gov) (Altschul et al., 1990), SILKBASE (http://silkbase.ab.a.u-tokyo.ac.jp) (Mita et al., 2003), and the Silkworm Genome Research Program (http://sgp.dna.affrc.go.jp//index.html). Microarray data were deposited in the Center for Information Biology Gene Expression Database (CIBEX, http://cibex.nig.ac.jp/index.jsp) under the accession number CBX249. The full-length cDNA sequences were retrieved from the silkworm database generated by University of Tokyo (http://pistil.ab.a.u-tokyo.ac.jp/kanzen/).

Template preparation for cDNA synthesis and PCR amplification

DNase I-treated RNA (1 μg) was used as a template for cDNA synthesis with ReverTra Ace (Toyobo, Osaka, Japan) and an oligo(dT) primer in a 20 μl reaction mixture. The product was diluted fivefold with TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA). The desired DNA fragments were amplified from the resulting cDNA using PCR in a 10 μl mixture containing GoTaq Green Master Mix (Promega) and gene-specific primers (Supplementary Table S1). The primers were designed using Primer3 software (http://frodo.wi.mit.edu/primer3/) with default criteria (Rozen and Skaletsky, 2000). Amplification was performed in a thermo-cycler (Mastercycler ep384, Eppendorf, Hamburg, Germany) with an initial DNA denaturation step at 94°C for 1 min, followed by 25–35 thermal cycles of 30 s at 94°C, 30 s at 50°C and 60 s at 72°C, with a final primer extension step of 7 min at 72°C. *RpL3* was used as an internal standard and amplified for 25 cycles. RT-PCR products were separated on 1.5% (w/v) agarose gels and visualized by ethidium bromide staining. All the data are representative of at least three amplifications.

Preparation of riboprobes and whole-mount RNA in situ hybridization

The primers used for preparation of RNA probes (Supplementary Table S2) were designed by the Primer3 software and used for PCR amplification of the cDNA synthesized from the total RNA of V8 brains. The PCR products were cloned into the pGEM-T Easy plasmid vector (Promega) and confirmed their sequence. Antisense and sense (control) RNA probes with digoxigenin were generated using an RNA-labeling in vitro transcription kit (Roche Diagnostics, West Sussex, UK) with T7 and SP6 RNA polymerase and were collected by 4 M lithium chloride/ethanol precipitation.

Dissected V2 larval brains were hybridized with each of 0.4–1 ng/μl riboprobe, as previously described (Aslam et al., 2011). After hybridization, the brains were stained with 4-nitroblue-tetrazolium chloride (NBT) and 5-bromo-4-chloro-3-indolylphosphate toluidine salt (BCIP) and observed under a microscope (SZ X 100 Olympus, Japan). Hybridization was performed thrice for each probe to authenticate the data.

RESULTS

Identification of 20E-responsive genes in the larval brain of the silkworm by microarray

Our previous observations indicated that EcR transcripts were upregulated by 20E and expressed exclusively in PTPCs in the larval brain, suggesting that PTPCs are under the control of 20E (Hossain et al., 2006, 2008). To explore the downstream genes regulated by 20E, we conducted DNA microarray analysis of RNA samples from the brains of V2 silkworm larvae injected with 20E or insect Ringer's solution. Gene expression levels between the RNA samples

(20E-injected V2 × Ringer-injected V2) were compared by a two-color dye-swap protocol with two replicates. The outcome revealed several hundred 20E-responsive upregulated genes from which forty-one genes were showing greater than a twofold change after the application of 20E. We isolated 26 of these, designated as *UP1* to *UP26*, to be studied further. The upregulation was further investigated by RT-PCR screening of cDNA samples from brains of V2 larvae treated in the same way. Initially, no hits were found using the microarray data in any publically available database (EST database and SilkBase) for four genes (*UP13*, *UP16*, *UP17*, and *UP23*) and therefore they could not be analyzed. The RT-PCR screening of the rest of the genes showed that ten genes (*UP1*, *UP4*, *UP5*, *UP10*, *UP11*, *UP12*, *UP20*, *UP22*, *UP25*, and *UP26*) increased their expressions in response to the exogenous 20E treatment (Fig. 1A). Of these, six genes were already known as 20E response genes (as described in the next section), indicating that our screening functioned properly at least partly. Detailed information about these genes is listed in Table 1. Eight genes (*UP2*, *UP3*, *UP6*, *UP8*, *UP14*, *UP15*, *UP19*, and *UP24*) were downregulated by 20E according to the RT-PCR screening which was different from the microarray results. Although we have identified by microarray analysis that the levels of these transcripts were higher in the +20E samples than in the –20E samples, we found that the levels of these transcripts were reversed on RT-PCR analysis; i.e., the transcription level was higher in the –20E samples than in the +20E samples (Fig. 1B). Information about these genes is listed in Table 2. Homology search for these genes did not describe their direct relationship with ecdysone cascade in any of the cases (Fujii et al., 1989; Mori et al., 1991; Fujiwara and Yamashita, 1992; Tan et al., 2003; Kaneno et al., 2007; Mrinal and Nagaraju, 2008). Expression of the other four genes (*UP7*, *UP9*, *UP18*, and *UP21*) showed no significant difference between the +20E and the –20E samples (Fig. 1C) indicating that these genes may not be influenced by 20E. *RpL3* amplification confirmed that there was no significant difference in the amount of cDNA among these samples. We also confirmed for absence of genomic contamination using –RT samples (Fig. 1D).

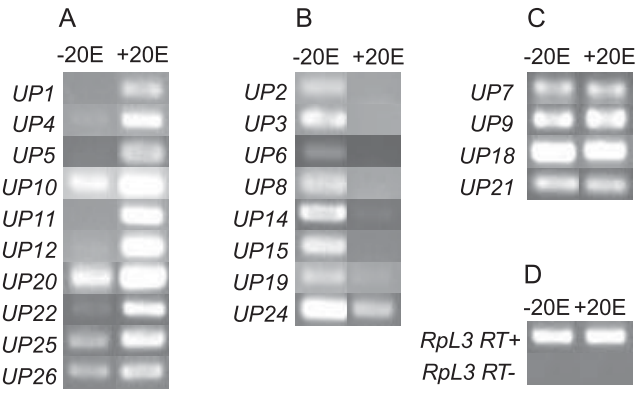

Fig. 1. Expression profiles of 26 20E-responsive candidate genes in the silkworm larval brain. Fifth instar day-2 larvae were injected with either 1 μg 20E or insect Ringer's solution to study the induction by 20E. Expressions of **(A)** 20E-upregulated genes, **(B)** 20E-downregulated genes, and **(C)** 20E–nonresponsive genes are shown here. –20E represents the control treated with insect Ringer's solution, while +20E represents ecdysone-responsive genes following 20E treatment. These genes were analyzed by RT-PCR using *RpL3* as a reference **(D)**. All of the experiments were performed three times to verify reproducibility and a representative was shown.

Table 1. List of 20E-induced genes revealed by RT-PCR.

Gene name	Probe name	FC	Blast result	Hybridization 1		Hybridization 2	
				Cy3* (–20E)	Cy5* (+20E)	Cy3* (+20E)	Cy5* (–20E)
UP1	K03101	100	AB024902.1 Bombyx mori BHR3 mRNA for hormone receptor 3, partial cds, 5e-18	251	28342	24635	309
UP4	K04754	12.4	AY526608.1 Antheraea pernyi vrille (Vri) mRNA, complete cds, 6e-27	2527	31452	27730	2554
UP5	K06460	7.19	EF427620.1 Bombyx mori tal-like protein 1A, tal-like protein 2A, tal-like protein 3A, and tal-like protein 4A mRNA, complete cds, 1e-24	296	2128	1992	219
UP10	K02477	4.09	AY526608.1 Antheraea pernyi vrille (Vri) mRNA, complete cds, 1e-12	987	4032	4122	962
UP11	K03776	3.78	AF332552.2 Bombyx mori orphan nuclear receptor E75C splicing variant precursor RNA, partial cds, 1e-24	521	1973	2142	577
UP12	K12343	3.55	AF073927.1 Bombyx mori nuclear receptor HR3 isoform A (HR3) mRNA, complete cds, 1e-24	382	1355	1339	325
UP20	K03022	2.21	AB219449.1 Bombyx mori BmBR-C gene for Broad-Complex isoform Z2, isoform Z4, isoform Z1, complete cds, alternative splicing, 1e-24	176	390	430	195
UP22	K16637	2.17	No significant homology found	2203	4777	3754	2171
UP25	K11708	2.05	L35266.1 Bombyx mori 20-hydroxy-ecdysone receptor mRNA, complete cds, 1e-24	209	428	408	213
UP26	K02307	2.05	DQ471939.1 Bombyx mori transcription factor E74 isoform A (E74A) mRNA, complete cds, alternatively spliced, e-102	1474	3016	2738	1463

*Cy3 and Cy5 indicate processed signal intensities for Cy3 and Cy5 labeling, respectively.

Temporal expression analysis

Homology searches for the upregulated genes revealed that six of them (*UP1*, *UP11*, *UP12*, *UP20*, *UP25*, and *UP26*) were previously known to be 20E-response genes (Fletcher et al., 1995; Palli et al., 1995; Eystathioy et al., 2001; Consoulas et al., 2005). The remaining genes (*UP4*, *UP5*, *UP10*, and *UP22*) are of particular interest as they have not been known to be either early or late 20E-response genes in the ecdysone signal pathway. To investigate whether these genes are also responsive to endogenous 20E, we conducted temporal expression profiling by RT-PCR (Fig. 2A). We excluded *UP10* from analysis, as both *UP4* and *UP10* were homologous to *vrille* according to the BLAST search results. We analyzed the temporal expression of these three genes using RNA samples isolated from the brains of larvae from the third instar day-0 through the fifth instar day-8. The expression of two genes, *UP4* and *UP5*, showed a better correlation with the hemolymph ecdysteroid titer (Sakurai et al., 1998; Takaki and Sakurai,

Table 2. List of 20E-downregulated genes revealed by RT-PCR.

Gene name	Probe name	FC	Blast result	Hybridization 1		Hybridization 2	
				Cy3* (–20E)	Cy5* (+20E)	Cy3* (+20E)	Cy5* (–20E)
UP2	K01429	15.2	AP009017.1 Bombyx mori genomic DNA, chromosome 3, BAC clone:503G12, complete sequence, 1e-24	624	9481	11585	559
UP3	K04176	13.6	D12523.1 Bombyx mori hemolymph protein (Slp), 1e-24	231	3146	2886	230
UP6	K07732	7.16	M24370.1 Silkworm (B.mori) storage protein 2 (SP2) gene, exons 2,3,4 and 5, 1e-24	2635	18854	18887	2616
UP8	K13572	6.23	X54736.1 B.mori 19G1 gene for 30K protein, 3e-22	606	3781	3826	491
UP14	K03674	2.99	AB239448.1 Bombyx mori glv2 mRNA for gloverin2, complete cds, 1e-24	193	577	416	259
UP15	K01459	2.73	GU015849.1 Bombyx mori clone Bmhp20 hemolymph protein mRNA, complete cds, e-152	278	760	736	187
UP19	K06785	2.24	AB062102.1 Bombyx mori SCI-SB mRNA for chymotrypsin inhibitor, complete cds, 3e-22	2571	5772	5715	2287
UP24	K01452	2.06	No significant homology found	400	824	716	444

*Cy3 and Cy5 indicate processed signal intensities for Cy3 and Cy5 labeling, respectively.

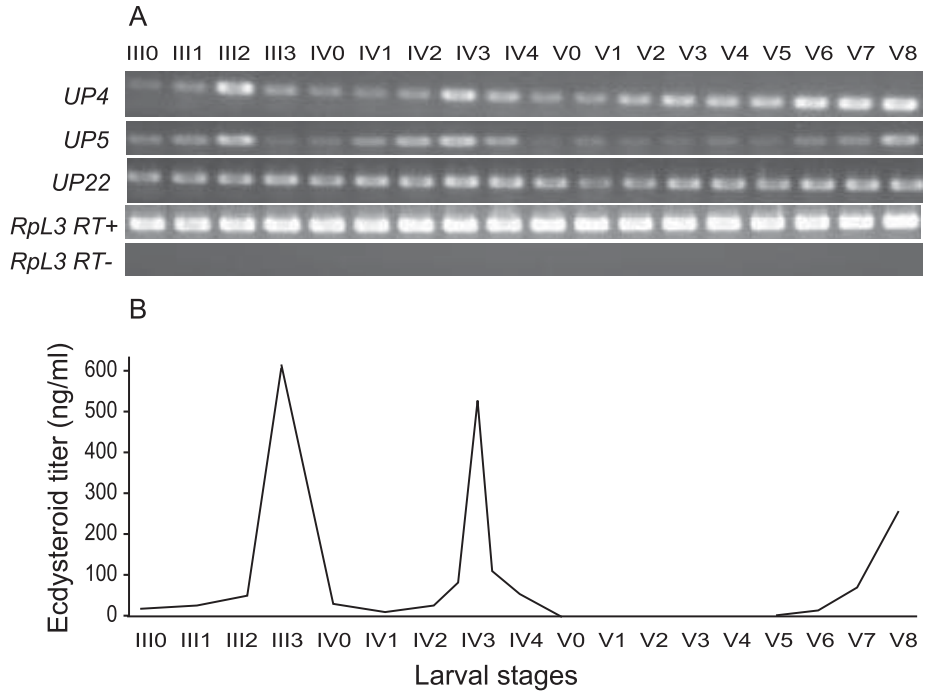

Fig. 2. A temporal expression analysis of 20E-induced genes. **(A)** RT-PCR was performed on brain RNAs from day-0 of third instar to day-8 of fifth instar larvae. *UP4* and *UP5* show an expression pattern that is parallel to the hemolymph ecdysteroid titer, whereas *UP22* have no significant relation pattern in any of the stages. *RpL3* amplification shows similar cDNA amounts and no genomic contamination with or without reverse transcription. All of the experiments were performed three times to verify reproducibility and a representative was shown. **(B)** Developmental changes of ecdysteroid titer in the silkworm hemolymph. The titer from III0 to III3, from IV0 to IV4, and from V0 to V8 was depicted from Takaki and Sakurai (2003), Koyama et al. (2004), and Sakurai et al. (1998), respectively. Concentrations are expressed in 20E-equivalents.

2003; Koyama et al., 2004; Fig. 2B), while no significant relation was found for *UP22* in any stage (Fig. 2A). The temporal expression of *UP4* strongly corresponded to the hemolymph ecdysteroid titer during the fourth and fifth instars, while during the third instar, the expression at III2 preceded the peak ecdysteroid titer at III3. A similar expression profile found in case of *UP5* except for III1. The expression of *UP5* at III1 and III2 clearly preceded the peak hemolymph ecdysteroid titer at III3. The expressions of the fourth and fifth instars showed no inconsistency with the hemolymph ecdysteroid titer. There was no significant difference among the band intensities in *RpL3*, confirming that similar amounts of cDNA were used (Fig. 2A). The developmental profiles therefore suggest that these two genes (*UP4* and *UP5*) are responsive to endogenous as well as exogenous 20E during the last two instars.

Spatial localization of the 20E-induced genes

The action of 20E is coordinately regulated by EcR and USP proteins. Previously, we reported that EcR was upregulated by 20E and expressed in the PTPCs (Hossain et al., 2006) in the brains of V2 larvae of silkworm. Thus, whole-mount RNA in situ hybridization was performed to identify the cells that express these two 20E-induced genes. The experiment was carried out using brains of day-2 fifth instar larvae injected with 1 μg 20E or Ringer's solution. The positive control was performed with specific RNA probes for the *EcR* common region, and the signal was found in two pairs of LNCs (Fig. 3A, arrows). The localization of *UP4* and *UP5* transcripts were also detected exclusively in two pairs of LNCs in the brains of larvae injected with 20E (Fig. 3B, C, arrows). The LNCs are expected to be the PTPCs. No signal was detected in brain samples that were either hybridized with sense probes or were from larvae injected with insect Ringer's solution (Fig. 3D–L). These results suggest that *UP4* and *UP5* are downstream targets of *EcR*.

DISCUSSION

Disruption of the components in PTTH and EcR signaling pathways can cause abnormal development in insects (Gu et al., 2011; Kugler et al., 2011), but it is still unknown how these are correlated with each other at the molecular level. In the present study, we performed a comprehensive screening of genes induced by 20E using DNA microarray

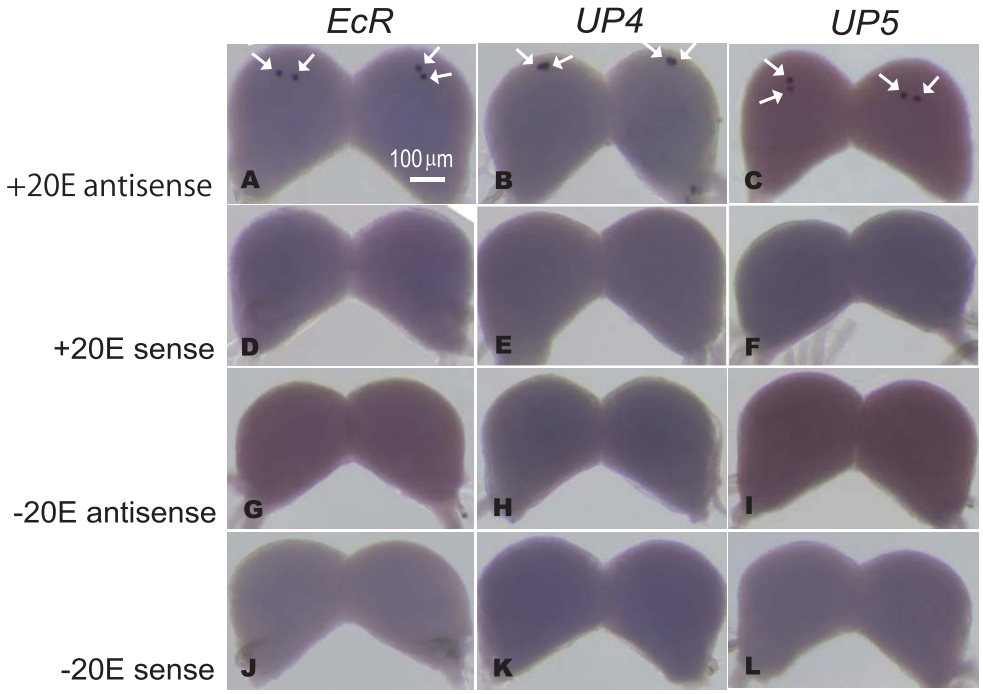

Fig. 3. Spatial localization of the mRNA transcripts of 20E-induced genes in the silkworm larval brain revealed by whole-mount RNA in situ hybridization. An EcR signal was detected in two pairs of LNCs in the brains of fifth instar day-2 larvae **(A)** (arrows) that were injected with 1 μg 20E. *UP4* and *UP5* mRNAs were also detected at the same stage and condition **(B–C)**(respectively, arrows). Signals could not be detected in brains injected with insect Ringer's solution **(G–L)**. No signal was found in brain samples that were hybridized with sense probes, irrespective of the application of 20E or insect Ringer's solution **(D–F)** [+20E] and **(J–L)** ([–20E]). Hybridization was performed thrice to authenticate the data. Scale bar = 100 μm.

in brains of fifth instar day-2 silkworm larvae in the presence or absence of 20E, and identified 41 genes that showed greater than twofold changes on artificial 20E application. Twenty-six of these genes have been studied so far. After semiquantitative screening and developmental profiling, we determined that two genes, *UP4* and *UP5*, which were not previously identified as 20E-response genes were expressed coordinately with the endogenous ecdysteroid titer of the late (fourth and fifth) larval stages. The *UP4* and *UP5* genes are *Bombyx* homologs of *vrille* and *tarsal-less*, respectively as revealed by the BLAST search. According to Ashburner's model for control of sequential gene activation by ecdysone (Ashburner et al., 1974), the early genes are activated first, whose products repress the early genes themselves and activate downstream late genes. We regard that *UP4* and *UP5* could be categorized as early genes because transcripts for each of those genes appeared within 2 h of incubation with 20E. The stage-specific expression patterns of these genes (Fig. 2A) are correlated with the fluctuation of hemolymph ecdysteroid titer in the late larval stages of silkworm (Fig. 2B) (Sakurai et al., 1998; Takaki and Sakurai, 2003; Koyama et al., 2004), indicating that these genes are influenced by endogenous 20E titer during those stages and may have specific function in distinct physiological and developmental context. The temporal expression patterns also showed high levels of transcripts in the fifth instar larvae (Fig. 2A), which suggests their involvement in the regulation of cell death, cell proliferation, and differentiation in a temporally and spatially controlled manner during insect development. In situ hybridization showed that the spatial localization of these genes is restricted to two pairs of LNCs, suggesting that these cells are the EcR-expressing PTPCs (Hossain et al., 2006) in larval brain (Fig. 3B–C, arrows). PTTH, the critical peptide hormone in insect development, stimulates prothoracic gland to synthesize and release ecdysone (Kawakami et al., 1990) and is considered to be a growth factor (Noguti et al., 1995) and a decision maker for larval diapause in many insect species (Denlinger, 2002). Localization of these gene transcripts in EcR-expressing cells, therefore, suggests that they may play roles in 20E regulation of PTPCs.

A database analysis of *UP4* reveals its homology to the *vrille* (*vri*) of *Antheraea pernyi*. This gene has not been extensively studied in *Antheraea pernyi* but in *Drosophila* and is known to be a transcription factor of the bZIP family. The *vri* gene was initially described as a maternal enhancer of *decapentaplegic* (*dpp*) mutations, producing impaired zygotic development and resulting in ventralization (George and Terracol, 1997). Later it was found in a screening of circadian clock genes of adult flies (Blau and Young, 1999; Cyran, 2003), but there has been no previous report that it responds to 20E. On the other hand, the signaling protein of *dpp*, which is the downstream target of *vri*, cooperates in the subdivision of legs in *Drosophila* in a cell signal triggered by *tarsal-less* (*tal*) (Pueyo et al., 2008), the homolog of *UP5*. The *tal* gene is a new type of putatively polycistronic and noncanonical gene that links patterning and morphogenesis in the *Drosophila* leg (Galindo et al., 2007) and triggers a cell signal of very short peptides (Pueyo et al., 2008). According to some authors, 20E and 20E-responsive gene products regulate the morphogenesis of adult organs derived from imaginal discs (Natzle, 1993; Oberlander, 1985). The induction of *UP4*/*UP5* by 20E (Fig. 1A) and localization of their transcripts in PTPCs (Fig. 3B–C, arrows), together with their developmental profile (Fig. 2A) during the last two instars, therefore reveal an integrated pathway to be analyzed by further biochemical and cellular studies.

Other 20E-upregulated genes identified in this study show similarities to hormone receptor 3 (*BHR3*), orphan nuclear receptor *E75C*, broad-complex gene (*BmBR-C*), 20E receptor (*EcR*), and transcription factor *E74A*, which have been reported to be under direct regulation of 20E

(Fletcher et al., 1995; Palli et al., 1995; Eystathioy et al., 2001; Consoulas et al., 2005). *BHR3* and *E75C* are involved in ovarian development in a 20E-dependent manner (Eystathioy et al., 2001; Swevers et al., 2002) and *BR-C* and *E74* are induced directly by 20E in overlapping temporal and spatial patterns during development (Fletcher et al., 1995). These genes thus occupy important places in ecdysone signal cascade as in *UP4* and *UP5*: especially, *EcR* is expressed in PTPCs in the *Bombyx* larval brain and suggested to play critical role on PTPCs function (Hossain et al., 2006). Therefore, these genes might be involved in ecdysone signal hierarchies underlying pupal–adult development and/or the 20E regulation of PTPCs.

By contrast to these 20E-upregulated genes, eight 20E-downregulated genes were also identified. The BLAST search for these downregulated genes revealed that the homologs include genes (or mRNAs) encoding hemolymph protein (slp), storage protein, 19G1 (30K) protein, gloverin, Bmhp20 hemolymph protein, and chymotrypsin inhibitor (Table 2). These genes are not yet been reported in direct relation with ecdysone signal pathway except for the storage protein (Manohar et al., 2010), which shows a stimulatory effect of 20E during synthesis. Although these genes were identified as 20E-upregulated genes by the microarray analysis, we regard them as false-positive outputs of this experiment (Fig. 1B). We compared the transcripts by a two-color dye-swap protocol with two replicates, but the number of repetition was small, which may cause such false-positive results.

It is hypothesized that various tissue responses induced by ecdysone are due to the differential roles of genes involved in the ecdysone signal cascade through combinatorial action of different receptor and transcription factors isoforms. During the larval and pupal development, tremendous morphogenetic changes are induced by 20E-induced genes through transcription factor complexes of EcR in the transition from the larval brain to the adult brain. Genetic analysis suggests that these 20E-induced genes may have some modulating function, not only in the ecdysone regulatory pathway, but also in the morphogenetic pathway (Beckstead et al., 2005). Further molecular experiments with gene-knockdown or other functional analysis may advance our understanding of the function of these genes during the postembryonic development.

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