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ORIGINAL ARTICLE

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Expression of ecdysteroid-regulated genes is reduced specifically in the wing discs of the wing-deficient mutant (*fl*) of *Bombyx mori*

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Abstract The wing-deficient mutant, *flügellos* (*fl*), of the silkworm, lacks four wings in the pupa and the adult. Previous studies have suggested that the *fl* wing discs lose responsiveness to ecdysteroid during metamorphosis. To test this hypothesis at the molecular level we compared the expression of 12 genes when the wing discs from the wild-type (WT) and *fl* larvae were cultured in the presence or absence of 20-hydroxyecdysone (20E). Most of the genes tested here, ecdysteroid-inducible *(EcR-A*, *-B1*, and *E75*) and noninducible genes (actin *A3*, β-tubulin, *apterous* (*ap*)*, USP*, and *BHR38)* were normally expressed in the *fl* wing discs. However, the amounts of mRNAs of two ecdysteroid-inducible genes, *BHR3* (early-late gene; *Bombyx* homologue to *DHR3* and *MHR3)* and *Urbain* (wing-specific late gene), were reduced to about 50% and 20% of WT in the cultured *fl* wing discs, respectively. We analyzed developmental profiles of these mRNAs during metamorphosis. They also demonstrated decreased BHR3 and Urbain mRNA 2 days after the onset of wandering. This reduction in transcription of *BHR3* in the *fl* mutant was observed only in the wing disc, not in the testis and fatbody. These results imply that the aberrant expression of the *fl* gene affects the downstream pathway of ecdysteroid signaling specifically in the wing discs and thus leads to a deficiency in wing formation.

Key words Key words: Wing-deficient mutant *fl* · *Bombyx mori* · Wing imaginal discs · Ecdysteroid · Metamorphosis

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Introduction

Holometabolous insects reconstruct their body pattern dynamically during larval-pupal development. Adult appendages develop from imaginal discs, which differentiate in response to pulses of ecdysteroid. Wing imaginal discs, in particular, undergo striking morphological changes. The wing disc of *Drosophila* has been studied as a model system for understanding the genetic regulation of patterning process and for identifying numerous genes controlling spatial patterns (Cohen 1993; Williams et al. 1993). While many studies have also shown the effects of ecdysteroid on development of the cultured wing discs in vitro (Fristrom et al. 1982; Mandaron 1970; Milner 1977; Milner and Muir 1987), little is known about the molecular mechanisms of ecdysteroid-mediated wing differentiation during metamorphosis.

In the silkworm, *Bombyx mori,* there are three wing-deficient mutants: *flügellos* (*fl*; linkage group 10, locus 13.0), *rudimentary wing (rw;* 1–22.8), and *vestigial (Vg;* 1–37.8) (Doira 1983). The recessive homozygote of *fl* mutant has the most severe phenotype of the three, leading to almost undetectable wings in pupal and adult stages. In spite of complete loss of the wings other larval and adult organs appear as normal in the *fl* mutant. Histological studies reveal that *fl* wing discs are slightly small but develop normally until the fourth larval instar. In the fifth instar, however, developmental events such as wing epithelial invagination and tracheal migration into the lacunar space do not occur (Fujiwara and Hojyo 1997; Nagata 1962). Moreover, *fl* wing discs cultured in medium containing 20-hydroxyecdysone (20E) do not develop, although the wild-type (WT) wing discs extend and differentiate under the same conditions (Fujiwara and Hojyo 1997). It has also been shown that a 41-kDa protein is induced by 20E specifically in WT wing discs, not in *fl* discs. These results suggest that the *fl* wing discs cannot respond to ecdysteroid as the same way as WT discs.

Ashburner et al. (1974) proposed a model for an ecdysteroid-activated genetic regulatory hierarchy through

the puffing patterns of *Drosophila* salivary gland chromosomes. In this model, ecdysteroid interacts with a hypothesized receptor and differentially induces the transcription of a few early genes, which then activate many late genes. Recent molecular analyses have revealed many important genes involved in the ecdysteroid-activated pathway predicted by the model of Ashburner (Thummel 1995). The functional ecdysone receptor consists of EcR and USP, both of which belong to the nuclear receptor superfamily (Yao et al. 1993). At present three early genes [*E74*: Burtis et al. 1990; *E75:* Segraves and Hogness 1990; *Broad-Complex (BR-C):* DiBello et al. 1991] and three early-late genes [*DHR3:* Koelle et al. 1992; *DHR39:* Ohno and Petkovich 1992; *E78:* Stone and Thummel 1993] are identified as transcription factors. E75, *Drosophila* hormone receptor (DHR) 3, DHR39, and E78 are orphan nuclear receptors. These transcription factors upstream in the ecdysteroid signaling pathway are expressed in all tissues studied so far (Huet et al. 1993, 1995), except for tissue-specific expression of EcR and BR-C isoform mRNAs (Emery et al. 1994; Kamimura et al. 1997; Talbot et al. 1993). It would be of interest to discover whether and how some of them are involved in tissue differentiation during metamorphosis.

In this study we investigated mRNA expression levels of several classes of gene in wing discs. To compare different responses of each gene to 20E between WT and *fl* we used in vitro culture of wing discs with or without 20E. These molecular approaches confirm the hypothesis that the *fl* wing discs lose the responsiveness to 20E in tissue specific manners during metamorphosis.

Materials and methods

Experimental animals

The *fl*^{k} (*flügellos*) mutant strain was kindly provided from Dr. Y. Banno of Kyusyu University and maintained in the laboratory by sib mating, crossing heterozygotes (*fl*^{k}/+) and homozygotes (*fl*^{k}/*fl*^{k}). The silkworms were reared on an artificial diet (Nihon Nosan Kogyo, Yokohama, Japan) under 16 h light:8 h dark photoperiod at 23±1°C. The newly molted fifth instar larvae were segregated immediately after the onset of the photophase (this day was designated day 0). Under these conditions most larvae began wandering on day 7.5 and pupate on day 11.

Tissue dissection and in vitro culture

The wing discs were dissected from day 2 fifth instar larvae. After rinsed in 1×SSC (0.15 M NaC1, 0.015 M sodium citrate), the discs were cultured in Grace's medium (Gibco). The 20E (Sigma) was dissolved in 10% isopropyl alcohol and added to the medium to give the desired concentration. Each disc was incubated in a 50-µl hanging drop of the medium in a culture well (96-well round bottom plate; Sumilon) at 25°C in an atmosphere of water saturated air.

RNA extraction

Total RNA was isolated by a modification of the guanidinium isothiocyanate-phenol-chloroform method (Chomczynski and Sacchi 1987) and further purified by ethanol precipitation. The RNA concentrations were determined by spectrophotometric measurements.

Cloning of *E75*, *BHR3, RpL3,* and *ap* in *Bombyx mori*

The C (DNA binding) region for *E75* was amplified by reverse-transcription polymerase chain reaction (RT-PCR) with a primer sets designed in the 5'-side of C region (5'-CGGCGCTCCATA CAACAGAA-3'; amino acid residue 56–62 of *Manduca* E75B; Segraves and Woldin 1993) and in the middle of D (hinge) region (5'-AACTCGCAGGTGTCGAGATG-3', amino acid residue 150–156 of MsE75B). Degenerate primers for RT-PCR of *BHR3* were designed in 5'- and 3'- ends of C region (5'-TGCAAA GTTTGCGGNGAYAA-3' and 5'-CATGCCGAGTTTNAGGCAY TT-3') of corresponding sequence of several insects reported so far *(MHR3*: Palli et al. 1992; *GHR3*: Jindra et al. 1994a; *CHR3*: Palli et al. 1996). Based on these PCR products for C region, full-length cDNA of Bm *E75* and the A/B region of *BHR3* were isolated by the rapid amplification of cDNA ends (RACE) technique, respectively. Primers for amplification of *Bombyx ap* were designed in the middle of LIM 2 domain (5'-GTTTTTCACGT CAACTGCTT-3'; amino acid residue 230–236 of *Drosophila ap;* Cohen et al. 1992) and in the middle of homeo domain (5'-GCATTTTGAAACCAGACCTG-3'*;* amino acid residue 412–418 of Dm *ap).* A partial sequence of *Bombyx RpL3* was obtained during construction of an expressed sequence tag database for *Bombyx* wing discs of the fifth instar. The 513-bp cDNA fragment corresponds to amino acid residue 1–171 of *Drosophila* RpL3 (Chan et al. 1998).

Reverse-Transcription Polymerase chain reaction

Total RNA was reverse transcribed into cDNA using the First-Strand cDNA Synthesis Kit (Pharmacia). A 7.5-µl mixture containing 1 µg total RNA and 0.1 µg random hexadeonucleotides was incubated at 37°C for 60 min, heated to 90°C for 5 min, quickly chilled on ice, and diluted with 172.5 µl RNase-free water. The cDNA sample (1 µl) was amplified by PCR in a 10-µl reaction mixture at a final concentration of l×PCR buffer [10 mM Tris-HCl, pH 8.3/50 mM KCl/0.001% (w/v) gelatin], 0.2 mM dNTP, 2 mM $MgC1_2$, 0.5 µM each 5' and 3' primers, 0.25 U *Taq* DNA polymerase (AmpliTaq Gold, Perkin-Elmer), and 37 kBq [α-^{32}P]dCTP (3000 Ci/mmol, ICN). After activating *Taq* DNA polymerase at 95°C for 10 min, PCR was performed for 17–27 cycles of 94°C for 30 s, 54–60°C for 30 s, and 72°C for 1 min. Table 1 summarizes the sequences of primers and the PCR conditions for each target gene. The PCR products were segregated on 5–7% polyacrylamide gel and the radioactivity of target DNA fragments was measured using a bioimaging analyzer (BAS-2500Mac, Fujifilm).

Quantitative analysis of relative amount of mRNA by RT-PCR

A wing disc of an early fifth instar larva used for culture is so small that only a few amount of total RNA (0.05–0.1 µg) can be obtained. To analyze expressions of various genes in cultured wing discs the RT-PCR is a more practical method than northern hybridization analysis. First, to overcome "plateau effects" of PCR (Mocharla et al. 1990) we determined the number of PCR cycles for each target gene to ensure the exponential phase (Table 1). Second, to avoid tube-to-tube variation (Wang et al. 1989), PCR reactions for each target gene were always performed simultaneously in three separate tubes in various cycles (e.g., 18, 19, and 20 cycles for RpL3; Table 1). From three sets of data in the exponential phase the mean value of each reaction set was calculated based on the method of Nakayama et al. (1992). The relative amount of mRNA for a given tested gene was defined as "the mean value of the gene, divided by the mean value of RpL3," when using the same cDNA as template. This method enables nearly 200 times as many PCR reactions when starting from 1 µg total RNA.

Table 1 Oligonucleotide primers and number of amplification cycles used for PCR (*RpL3* ribosomal protein L3, *EF-1α* elongation factor 1α)

Gene	PCR primer		Annealing temperature (°C)	Number of cycles in exponential phase	Number of cycles for quantification	References
	5' primers	3' primers				
EcR-A	TCGCCCTCCCCAGGCGCGCC	CTCTTGCTGTCGAGGTGCAG	57	23–28	25–27	Kamimura et al. (1997)
EcR-B1	ATGAGACGCCGCTGGTCTGA	CTCTTGCTGTCGAGGTGCAG	57	24–28	25–27	Kamimura et al. (1996)
USP	TAACCATCCCTTGAGCGGCT	GGACCGACACGTAGGATCTG	57	22–28	24–26	Tzertzinis et al. (1994)
E75	GATACAATACCGGCCCTGCA	AACCGAGGAGCGTCATCAAG	56	22–26	22–24	This study
BHR3	ATCGTCGGGGGTGCACTATG	CTGTAGTCTGCAGTACTGGC	55	18–23	20–22	This study
BHR38	CTGCCCTCAAAGCCTAAATG	GAAGCCAGTCTCCGAACGAT	54	22–29	26–28	Sutherland et al. (1995)
Urbain	CTGGTGCTCAGTCTACTGAG	TCCGCTTTCAACCCGTCACT	57	16–20	17–19	Besson et al. (1996)
RpL3	AGCACCCCGTCATGGGTCTA	TGCGTCCAAGCTCATCCTGC	58	16–21	18–20	This study
EF-1α	GGCAGAGTTGAAACTGGTGTG	ATGTGGGCAGTGTGGCAATC	56	16–21	18–20	Kamiie et al. (1993)
Actin A3	TCGATCGTCGGAAGGCCCC	GTAGTCTGTGAGGTCACGACCGG	58	16–20	18–20	Mounier and Prudhomme (1986)
β-Tubulin	GGTCCTCGATGTAGTCCGCA	GTCTGCTTCCACGGGATGTG	58	16–21	18–20	Quan et al. (1998)
Apterous	CACCAGCTACGGACCATG	TAAGACGGTTTTCGAGAGTCC	55	22–28	25–27	This study

All the primers are 100% match for the nucleotide sequences of *Bombyx mori.* The primers for *EcR-A* and *EcR-B1* were designed within A/B (isoform-specific) region. The primers for *E75* and *BHR3* were designed within C (DNA-binding) and D (hinge) region. Numbers of amplification cycles were determined as explained in the text.

Northern analysis

Total RNA (10 μg) was separated on a formaldehyde-agarose (1%) gel and transferred to a Hybond-N nylon membrane (Amersham). Northern hybridizations were performed at 42°C for 18 h in 50% formamide, 5×SSC, 10×Denhardt's solution (0.2% each of bovine serum albumin, Ficoll, and polyvinylpyrrolidone), 25 μg/ml sonicated salmon sperm DNA, 50 mM sodium phosphate, pH 7.0, and ^{32}P-labeled DNA. The DNA probe was labeled with [α-^{32}P] dCTP using BcaBEST Labeling Kit (TaKaRa). The membranes were washed twice at room temperature for 20 min in 2×SSC with 0.1% sodium dodecyl sulfate (SDS). The further washes were followed by 30 min at 65°C successively in 2×SSC with 0.1% SDS, in 1×SSC with 0.1% SDS, and in 0.1×SSC with 0. 1% SDS.

Results

Identification of several genes *in Bombyx mori*

To obtain more information about nucleotide sequences of ecdysone-inducible genes and marker genes we newly identified *E75, BHR3, ribosomal protein L3 (RpL3)* and *ap* genes of the silkworm in this report (Table 2). The identified sequences of C (DNA binding) regions of *E75* and *BHR3* revealed 100% amino acid identity with *Manduca E75* and *MHR3,* respectively. We further determined complete cDNA sequences for *Bombyx E75A* and *B* (Ogai and Fujiwara, in preparation) and more than 1 kb in the A/B region of *BHR3* using the RACE technique. The deduced amino acid sequences from these cDNAs show a high degree of similarity with homologues of other Lepidoptera in corresponding regions (Table 2). The sequence identity between overall regions of BmE75 and DmE75 is much lower (43%), however, because only 127 amino acids of BmE75 have high identity (83%) corresponding to C and D region, but low similarity in other regions of DmE75. Bombyx hormone receptor 3 (BHR3) has an A/B region different in length than that of DHR3 and *Caenorhabditis elegans* hormone receptor 3 (CHR3), although the amino acid identity in C region is high (97% and 89%, respectively). Overall in the sequenced region the putative *Bombyx ap* shows only 52% and 49% amino acid identity to *Artemia* and *Drosophila* homologues (Table 2) because the region between LIM 2 and the homeo domain is not conserved. However, the homeo domain of *Bombyx ap* exhibits high degree of identity with Af *ap* (97%) and Dm *ap (*91%*),* respectively, suggesting that the identified sequence here is the *Bombyx* homologue of *ap.*

Gene expressions in cultured WT and *fl* wing discs

To compare the response of each gene to ecdysteroid precisely we cultured wing discs explanted from WT and *fl* larvae in Grace's medium with or without 20E. The peak ecdysteroid titer during the fifth instar of *Bombyx* is approximately 1.5 μg/ml (Kiguchi et al. 1985) and thus a higher concentration of 20E that can activate early-late and late genes was fixed at 2 μg/ml. A lower concentra-

Table 2 Newly identified *Bombyx* genes and sequence comparison with homologues of other species (*Ms Manduca sexta, Cf Choristoneura fumiferana, Gm Galleria mellonella, Dm Drosophila melanogaster, Ce Caenorhabditis elegans, Af Artemia franciscana*)

Bombyx genes	DDBJ accession no.	Identified region	Amino acid identity	Reference
E75A	AB024904	Complete cds	MsE75A (91%) CfF75A (89%) GmE75A (88%) DmE75A (43%)	Zhou et al. (1998) Palli et al. (1997) Jindra et al. (1994b) Segraves and Hogness (1990)
E75B	AB024905	Complete cds	MsE75B (92%) GmE75B (88%) DmE75B (37%)	Segraves and Woldin (1993); Jindra et al. (1994b) Segraves and Hogness (1990)
BHR3	AB024902	5'UTR ~ DNA binding region	GHR3 (94%) MHR3 (93%) *Choristoneura fumiferana* HR (82%) DHR3 (54%) *Caenorhabditis elegans* HR3 (25%)	Jindra et al. (1994a) Palli et al. (1992) Palli et al. (1996) Koelle et al. (1992) Kostrouchova et al. (1998)
apterous	AB024903	LIM 2 domain ~ homeo domain	Af *ap* (52%) Dm *ap* (49%)	Averof and Cohen (1997) Cohen et al. (1992)
RpL3	AB024901	Amino acid residue 1~173	DmRpL3 (81%) CeRpL3 (74%)	Chan et al. (1998) Zhu et al. (1996)

tion of 20E (0.2 μg/ml) was used for activation of *EcR* (Fujiwara et al. 1995; Kamimura et al. 1996) and early genes.

To determine the internal standard gene we first compared expression levels of four housekeeping genes, actin *A3,* β-tubulin, *EF-1α*, and *RpL3*. Of the four genes, *RpL3* showed the most ubiquitous expression. The amount of mRNA of RpL3 was not changed between WT and *fl* wing discs and with or without 20E (data not shown). Since the *RpL3* genes of *Drosophila* and human (Chan et al. 1998; Van Raay et al. 1996) are also expressed in non-stage- and non-tissue-specific manners, we chose *RpL3* as an internal standard.

Ecdysteroid-regulated genes

Ecdysone receptor genes, EcR-A, -B1*, and USP*. Figure 1A and B shows the expression levels of two EcR isoform mRNAs (Kamimura et al. 1996, 1997; Swevers et al. 1995) when WT and *fl* wing discs were cultured for 6–24 h with 0–2 μg/ml 20E. Both *EcR-A* and *B1* isoforms seemed to be activated normally in *fl* wing discs because there was no significant difference of expression levels between WT and *fl* wing discs following 6–12 h culture. Exposure to 20E increased the amount of EcR mRNA three- to fivefold compared with the basal level without 20E. Northern hybridization also showed that the *EcR* isoforms were normally transcribed in *fl* wing discs from day 0 to day 6 fifth instar (data not shown). The level of EcR-B1 mRNA decreased 50–60% in WT, but not in *fl* wing discs, when cultured with 2 μg/ml 20E for more than 18–24 h. This reduction was not observed in *EcR-A*. When the relative amount of USP mRNA (BmCF1; Tzertzinis et al. 1994) was analyzed (Fig. 1C), there was no difference in expression levels between WT and *fl*. There was constitutive transcription regardless of concentration of 20E.

Early gene, E75. Six hours' exposure to 0.2 μg/ml 20E was sufficient to induce a maximum level of E75 mRNA (Fig. 1D). The *E75* primers used for quantitative RT-PCR were designed within the DNA binding and hinge region, and thus the data represent the sum of mRNAs for E75A and E75B. There was no significant difference in 20E induction levels between WT and *fl*.

Early-late gene, BHR3. The level of transcription of early-late gene *BHR3* in the *fl* wing discs was reduced to about 50–60% of WT discs (Fig. 1E) under all conditions of 20E induction. We also analyzed the effect of the protein synthesis inhibitor cycloheximide on the *BHR3* expression. When cultured with 10 μg/ml cycloheximide and 2 μg/ml 20E, the amount of BHR3 mRNA in the *fl* wing discs was also reduced to about 60% of WT discs cultured under the same conditions (data not shown). This suggests that the reduction in BHR3 mRNA in *fl* wing discs is related to the pathway (that is) independent of protein synthesis.

Late gene, Urbain. Reduction in mRNA level in the *fl* wing discs was more significant for the *Urbain,* a wing-specific late gene (Besson et al. 1996; Fig. 1F). Accumulation of its mRNA in *fl* wing discs was less than 20% of WT in the most inducible conditions, with 2 μg/ml 20E in 12-h culture. While the function of *Urbain* is still unclear, its stage- and tissue-specific expression suggests some roles in morphogenesis of wing epithelium (Besson et al. 1996). Thus, lower induction of *Urbain* in *fl* wing discs may be related directly to its aberrant morphogenesis.

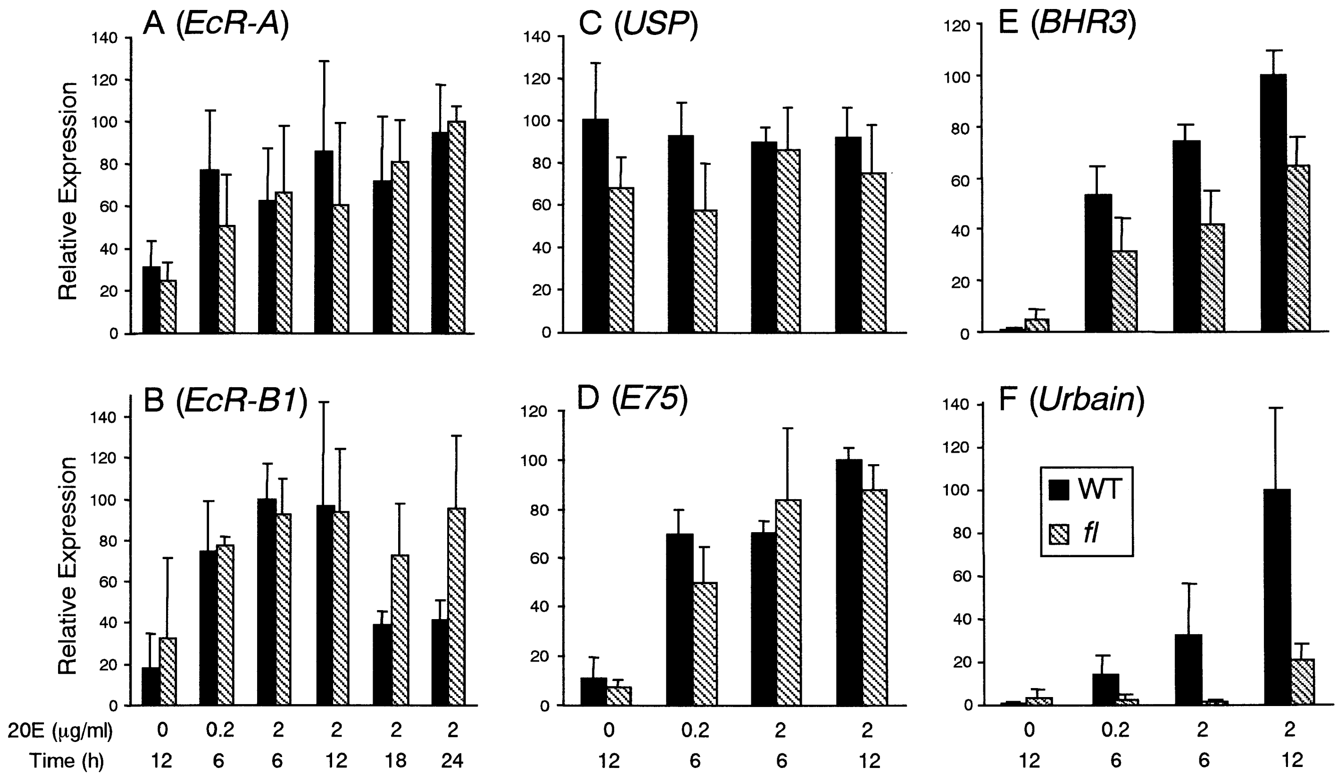

Fig. 1A–F Induction of mRNA of ecdysteroid-regulated genes by 20E in the wing discs of WT and *fl*. The discs were cultured for 6–24 h with 0–2 μg/ml 20E, and the relative amount of mRNA was estimated by quantitative RT-PCR, using *RpL3* as an internal standard. **A** *EcR-A*. **B** *EcR-B1*. **C** *USP*. **D** *E75*. **E** *BHR3*. **F** *Urbain*. For each sample three to ten silkworms were used to collect wing discs. *Bars* means ±SD (n=4–5). Relative expression is based on the maximum data as 100

Non-ecdysteroid-regulated genes

While the expression of *BHR3* and *Urbain* was reduced in *fl* wing discs, it is not evident whether the ecdysteroid cascade is specifically blocked, or whether other gene expressions are also disturbed. To test this, the expressions of non-ecdysteroid-regulated genes were analyzed by RT-PCR when WT and *fl* wing discs were cultured with or without 2 μg/ml 20E for 6 h. Table 3 summarizes the results for both ecdysteroid-regulated and non-ecdysteroid-regulated genes.

Housekeeping genes, actin A3, *β-tubulin, and* EF-1*α*. The expressions of actin *A3*, encoding a cytoplasmic actin (Mounier and Prudhomme 1986), and of β-tubulin (Quan et al. 1998) were unaffected by 20E addition and showed the similar patterns in both WT and *fl* wing discs (Table 3). It is known that expression of β-tubulin is af-

Table 3 Relative expression of various genes in cultured wing discs

Gene	Class	WT		*fl*	
		–20E	+20E	–20E	+20E
EcR-A	Ecdysone receptor	50.4 ±19.3	100±39.3	39.6±14.0	106.9±50.1
EcR-B1	Ecdysone receptor	18.2 ±16.2	100±16.9	32.5±38.5	92.7±17.0
USP	Ecdysone receptor	112 ±30.1	100±7.7	75.5±17.1	96.5±21.9
E75	Early	15.7 ±12.3	100±6.9	10.9±4.2	119 ±41.5
BHR3	Early-late	0.70±0.93	100±9.1	6.9±4.7	56.6±16.9**
Urbain[a]	Late	1.2 ±0.5	100±38.5	3.5±4.0	21.1±6.9*
BHR38	Nuclear receptor	51.3 ±21.6	100±42.7	71.8±23.6	98.6±35.7
Actin *A3*	Housekeeping	109 ±32.2	100±7.0	111 ±27.1	105 ±4.6
β-Tubulin	Housekeeping	60.2 ±21.6	100±43.5	61.1±9.83	68.5±15.4
EF-1*α*	Housekeeping	115 ±12.0	100±18.0	69.2±11.3	61.8±3.6**
Apterous	Homeotic	92.6 ±23.6	100±30.3	93.1±2.4	96.4±26.9

*P<0.05, **P<0.005, WT+20E vs. *fl*+20E (Student's t test)

Total RNA of cultured wing discs of WT and *fl* larva was analyzed by RT-PCR (see Fig. 1). Wing discs were cultured with 2 μg/ml 20E (+20E) or Grace's medium only (–20E) for 6 h. Data are presented as means ±SD (n=3–6). Relative expression is based on the means of WT (+20E) as 100.

[a]For +20E, discs were cultured with 2 μg/ml 20E for 12 h.

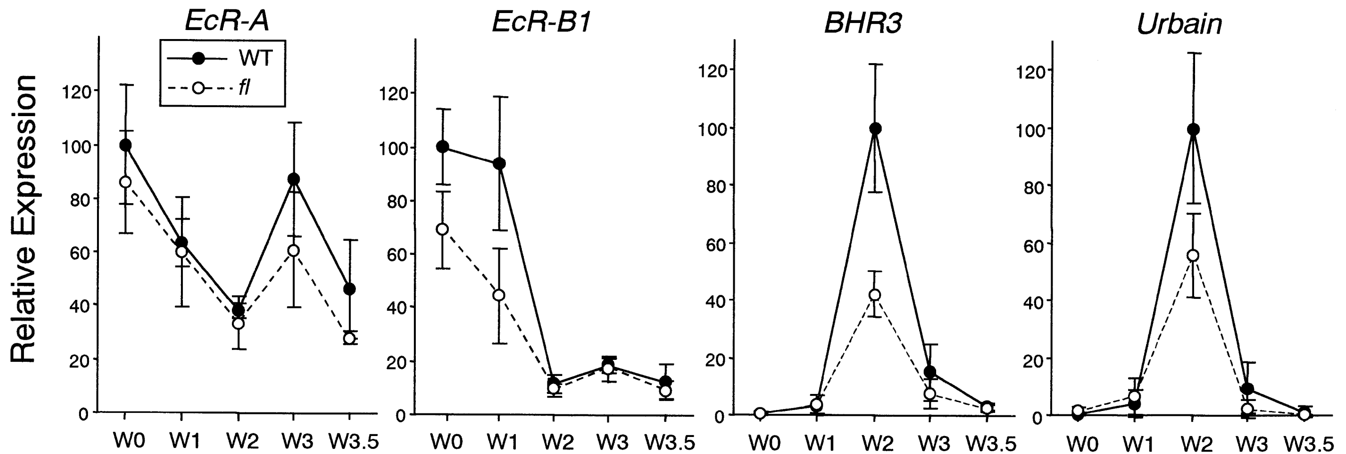

Fig. 2 Developmental profiles for ecdysteroid-regulated genes in the wing discs of WT and *fl* at pharate pupal stages. *W0–3.5* 0–3.5 days after the onset of wandering. The larvae pupate at *W3.5–W4*. The relative expression of each gene was estimated by quantitative RT-PCR, and is shown based on the maximum data as 100. *Bars* means ±SD (*n*=3)

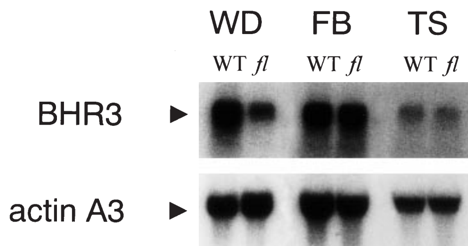

Fig. 3 Northern blot analysis of *BHR3* in various tissues of WT and *fl* at W2 stage. Total RNA (10 μg) was hybridized with a 1-kb cDNA probe for *BHR3* and 468-bp cDNA probe for actin *A3*. *WD* wing disc; *FB* fat body; *TS* testis. Actin *A3* is used as a quantitative control

fected by ecdysteroid titer in wing discs during spinning stage (Quan et al. 1998), although we could not detect the increase in β-tubulin mRNA by 20E in cultured wing discs. On the other hand, the mRNA of elongation factor-lα (EF-lα; Kamiie et al. 1993) was less abundant in *fl* wing discs than in WT discs.

LIM homeo-box gene, apterous. The *ap* is a member of the LIM family of developmental regulatory genes (Cohen et al. 1992). A homozygous mutant of *ap* of *Drosophila* lacks virtually all wings. We examined whether a gene involved in pattern formation of wing discs (such as *ap*) is transcribed normally in the *fl* wing discs. The relative expression levels of *ap* with or without 20E were almost the same between WT and *fl* wing discs (Table 3). In the wing discs from day 0 to day 6 during fifth instar the expression of *ap* was not changed and at the same level in WT and *fl* (data not shown).

Nuclear receptor gene, BHR38. BHR38 is a *Bombyx* homologue of rat nerve growth factor-induced protein B (NGFI-B) and a presumed partner of USP (Sutherland et al. 1995). Fisk and Thummel (1995) reported that the levels of 1.9-kb main transcript of *Drosophila* homologue DHR38 are not affected by 20E in cultured larval organs. The mRNA of BHR38 was expressed in *fl* wing discs in a similar manner to those in WT discs (Table 3).

Developmental profiles of gene expression during metamorphosis

As shown above, induction of BHR3 and Urbain mRNAs with 20E was repressed in vitro culture, indicating aberrant regulation of ecdysteroid signaling pathway in the *fl* wing discs. Our previous studies suggested that the titer of humoral factors and hormones in hemolymph of the *fl* larva is maintained normally as in WT (Hojyo and Fujiwara 1997). We next examined how ecdysteroid-regulated genes are expressed during the pharate pupal stages in wing discs of *fl* larva by RT-PCR (Fig. 2). In wing discs of WT, amounts of mRNA of EcR-A and Bl decreased gradually after wandering, while expression of *BHR3* and *Urbain* reached a maximum 2 days after the onset of wandering (W2). Temporal expression patterns of *EcR* isoforms were similar between *fl* and WT wing discs. However, the amounts of mRNA of BHR3 and Urbain in *fl* wing discs were repressed about half in WT discs. These results support the idea that the ecdysteroid signaling is aberrant in *fl* wing discs in vivo.

Normal expression of *BHR3* in fat body and testis of *fl* larva

The larval and pupal organs, other than wings, seem to develop normally in *fl* mutants (Fujiwara and Hojyo 1997). It would be of interest to know whether the signal of ecdysteroid transmits normally in tissues other than wing discs in *fl* individuals. We therefore dissected fat bodies and testes from the same individuals that were used for isolation of wing discs and compared expression of *BHR3* between WT and *fl* larva by northern hybridization. A 1-kb cDNA probe that covers 5'-UTR to the end of DNA binding domain of *BHR3* was hybridized with

10 μg total RNA from each tissue at W2 stage (Fig. 3). In every tissue wing disc (WD), fat body (FB), and testis (TS), a single 4.6-kb band was detected. The intensities of the band in FB and TS were similar between WT and *fl* mutant, although the band intensity was reduced in *fl* wing discs as shown by RT-PCR. This finding demonstrates that the transcription of *BHR3* is repressed specifically in wing discs but not in other tissues.

Discussion

We show here that mRNA of BHR3 and Urbain were reduced in amount in *fl* wing discs, both in in vitro culture and in vivo. In contrast, *EcR* and *E75,* which have higher sensitivity to ecdysteroid, and most non-ecdysteroid-regulated genes, were not repressed in the *fl* mutant. These results indicate that the ecdysteroid-inducible gene activation cascade is blocked in downstream points in the *fl* wing discs.

The BHR3 is a counterpart of DHR3 and an orthologue of the vertebrate RORα, which is an orphan nuclear receptor required for postnatal brain development (Giguère et al. 1994; Sashihara et al. 1995). DHR3 is expressed widely in various tissues during the onset of metamorphosis (Lam et al. 1997). Mutant analyses reveal that DHR3 is also essential for embryogenesis, especially for peripheral neurogenesis (Carney et al. 1997). CHR3 protein distributes in epidermal cells during embryogenesis in *C. elegans,* and inhibition of function by injection of double-stranded CHR3 RNA causes several defects related to abnormal epidermal cell function during postembryonic development (Kostrouchova et al. 1998). These data suggest that the complete loss of BHR3 function could have vital effects on development. In the *fl* mutant, however, *BHR3* was repressed only in wing discs at about 50% of normal level and expressed normally in other tissues. Therefore the reduction in BHR3 may cause a restricted defect, that is, loss of wings.

Cycloheximide and anisomycin reduce the level of *Manduca* hormone receptor 3 (MHR3) mRNA in cultured *Manduca* epidermis, but not completely (Palli et al. 1992). This suggests that half of MHR3 mRNA is induced directly by 20E (primary response), while the rest of mRNA transcription depends on some proteins activated by 20E (secondary response). In our experiments the level of BHR3 mRNA was also about half of WT wing discs cultured with cycloheximide and ecdysteroid. This indicates that the transcriptional regulation mediated by both primary and secondary responses to 20E is involved in the reduction in the BHR3 mRNA in the *fl* wing discs.

A possible explanation for the BHR3 mRNA reduction is through the selective repression of *BHR3* isoforms. The *MHR3* and *CHR3* genes may have isoforms of different length (Jindra et al. 1994a; Palli et al. 1992, 1996). At present, however, we cannot detect any isoforms in the A/B region of *BHR3* by 5'-RACE analysis. In this study we used the primer set designed in the DNA binding domain of *BHR3* for RT-PCR. We also used another primer set designed in A/B region of the gene for RT-PCR, which resulted in the same as shown in Fig. 1C (data not shown). In addition, northern analysis using the A/B region probe showed a single band for BHR3 mRNA (Fig. 3). These results suggest that the selective repression of isoforms, at least in A/B region, is not related to reduction in the BHR mRNA.

The BHR3 mRNA was reduced in wing discs but expressed normally in fat body and testis of *fl* larva (Fig. 3). This suggests that the ecdysteroid activation pathway may be blocked only in wing discs, not in other tissues, because of the aberrant expression of the *fl* gene. The reduction in a wing disc-specific gene *Urbain* in the *fl* wing discs supports this idea. To test the above hypothesis it will be necessary to define and detect more tissue-specific marker genes and to compare their expressions.

The repression of EcR-B1 in WT wing discs cultured over a longer period (Fig. 1B) can be explained in relation to BHR3 regulation. White et al. (1997) reported that DHR3 protein inactivates early gene expression through interaction with EcR protein. Since the *EcR* gene itself is activated by EcR/USP, BHR3 protein induced by ecdysteroid may decrease the amount of EcR mRNA. In this case the reduced level of BHR3 in the *fl* wing discs may cause insufficient repression of EcR-B1. However, it is unclear why only EcR-B1 and not EcR-A is reduced in the level of mRNA. Recently Lan et al. (1999) showed that EcR-B1-USP-1 heterodimer is critical for induction of MHR3. This result suggests a "EcR-B1-BHR3 feedback loop," which may account for B1-specific repression in WT wing discs.

Many *Drosophila* mutants for ecdysteroid-inducible regulatory gene have been isolated so far. Mutants of early genes, *E74* and *BR-C,* cause malformed appendages in some cases, but finally die in various developmental stages from embryo to pupa (*E74:* Fletcher et al. 1995; *BR-C:* Emery et al. 1994). Mutants that have lost the complete function of EcR die in embryogenesis, while *EcR-B1* mutants survive until prepupa (Bender et al. 1997). The mutants of *crol* gene, which is required for ecdysteroid-regulated gene expression, die during prepupal stages with defects in adult head eversion and leg structure (D'Avino and Thummel 1998). These observations suggest that the regulatory genes upstream in the cascade are involved in various and general processes of development of whole body. On this criterion, the *fl* mutant may be categorized into a different class from the mutants above because its phenotype is normal except for wing morphogenesis and only downstream genes in the ecdysteroid signaling are repressed. All four *fl* mutants that have been isolated independently so far show a similar wingless phenotype and no lethality (Fujiwara and Hojyo 1997). The *fl* mutant is therefore a good material for understanding the roles of the downstream genes induced by ecdysteroid on wing differentiation. The identification and characterization of the *fl* genes will help to clarify the gene activation pathway in wing development.

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