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Positional Cloning of a Bombyx Wingless Locus *flügellos* (*fl*) Reveals a Crucial Role for *fringe* That Is Specific for Wing Morphogenesis

Kaoru Sato,* Tomoko Matsuoka Matsunaga,*,[1] Ryo Futahashi,* Tetsuya Kojima,* Kazuei Mita,† Yutaka Banno‡ and Haruhiko Fujiwara*,[2]

*Department of Integrated Biosciences, Graduate School of Frontier Sciences, University of Tokyo, Bioscience Building 501, 5-1-5 Kashiwanoha, Kashiwa, Chiba 277-8562, Japan, †Laboratory of Insect Genome, National Institute of Agrobiological Sciences, Owashi 1-2, Tsukuba, Ibaraki 305-8643, Japan and ‡Laboratory of Insect Genetic Resources, Faculty of Agriculture, Kyushu University, Fukuoka 812-8581, Japan

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ABSTRACT

Mutations at the *flügellos* (*fl*) locus in *Bombyx mori* produce wingless pupae and moths because of the repressed response of wing discs to ecdysteroid. Four recessive *fl* alleles occurred spontaneously and were mapped at 13.0 of the silkworm genetic linkage group 10. By positional cloning, we confirmed that the gene responsible for *fl* is *fringe* (*fng*) encoding Fng glycosyltransferase, which is involved in regulating the Notch signaling pathway. In four different *fl* alleles, we detected a large deletion of the *fng* gene in fl^{k} and nonsense mutations in *fl*, fl^{o}, and fl^{n}. In the wild-type (WT) silkworm, *fng* is expressed actively in the wing discs, brain, and reproductive organs from the fourth to final instars but barely in the other tissues tested. *In situ* hybridization showed that *fng* mRNA is expressed in the dorsal layer of the WT wing discs. The *wingless* (*wg*) mRNA, a downstream marker of Fng-mediated Notch signaling, is localized at the dorsoventral boundary in the WT wing discs but repressed markedly in the *fl* wing discs. Although null mutants of Drosophila *fng* result in postembryonic lethality, loss of *fng* function in Bombyx affects only wing morphogenesis, suggesting different essential roles for *fng* in tissue differentiation among insects.

WING formation is a major morphological change during larval–pupal development in holometabolous insects. Wings develop from the wing imaginal discs, which differentiate in response to insect hormones, juvenile hormone, and pulses of ecdysteroid (Nardi and Willis 1979; Blais and Lafont 1980; Fujiwara and Hojyo 1997; Fujiwara and Ogai 2001; Truman and Riddiford 2007). The wing disc of Drosophila is a well-studied model system for identifying numerous genes involved in pattern formation and understanding the genetic regulation of morphological processes (Williams *et al.* 1993). However, whether the mechanism revealed in Drosophila is actually applicable to other insect species remains to be clarified. Furthermore, although many studies have shown the effects of ecdysteroid on the development of cultured wing discs *in vitro* (Nardi and Willis 1979; Blais and Lafont 1980; Fujiwara and Ogai 2001; Truman and Riddiford 2007), little is known about the molecular mechanisms of hormone-mediated wing morphogenesis and tissue differentiation during metamorphosis.

The silkworm *Bombyx mori* has three wing-deficient mutants: *flügellos* (*fl*), *Vestigial* (*Vg*), and *rudimentary wing* (*rw*) (Fujii *et al.* 1998). The recessive homozygote of the *fl* mutant has the most severe phenotype of the three, with almost undetectable wings in the pupal and adult stages (Figure 1). Four recessive *fl* mutants (*fl*, fl^{k}, fl^{n}, and fl^{o}) occurred spontaneously and independently (Katsuki 1935; Harizuka 1948; Ueda *et al.* 1959; Fujii *et al.* 1998) and were mapped at the same position (13.0) of the silkworm genetic linkage group 10 (LG10) (Figure 2A; Fujii *et al.* 1998). Despite the complete loss of wings, other larval and adult organs appear normal in the *fl* mutants. Histological studies revealed that, although the *fl* wing discs are slightly smaller than those of the wild type (WT), they develop normally until the fourth larval instar (Fujiwara and Hojyo 1997). In the fifth instar, however, developmental events such as wing epithelial invagination and tracheal migration into the lacunal space do not occur (Nagata 1962; Hojyo and Fujiwara 1997). Moreover, *fl* wing discs cultured in medium containing 20-hydroxyecdysone (20E) do not develop, whereas the WT wing discs differentiate normally under the same conditions (Fujiwara and Hojyo 1997). During the prepupal stage, the transcription of two ecdysteroid-induced genes, *BHR3* (early-late gene; Bombyx homolog of *DHR3*) and *Urbain* (wing-specific

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[1]*Present address:* Department of Molecular Virology, Research Institute for Microbial Diseases, Osaka University, 3-1, Yamadaoka, Suita, Osaka 565-0871, Japan.

[2]*Corresponding author:* Department of Integrated Biosciences, Graduate School of Frontier Sciences, University of Tokyo, Bioscience Bldg. 501, 5-1-5 Kashiwanoha, Kashiwa, Chiba 277-8562, Japan.
E-mail: haruh@k.u-tokyo.ac.jp

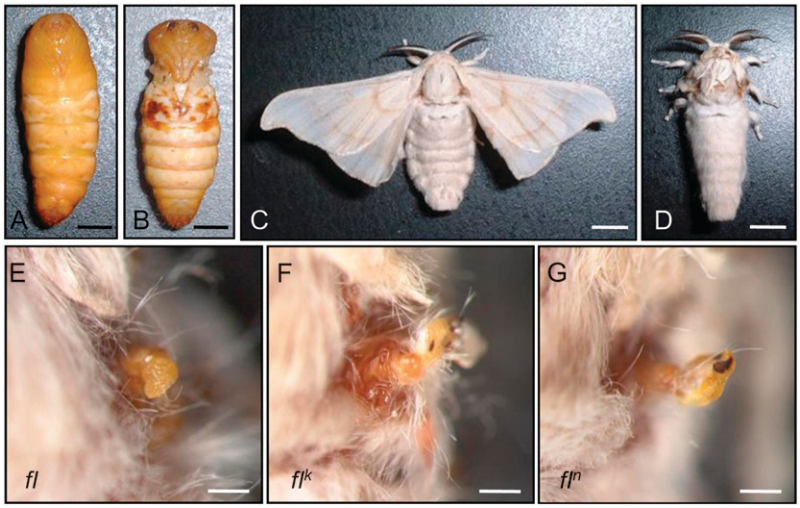

Figure 1.—Bombyx wingless mutant, *fl* lacks four wings in the pupa and adult stages. (Top) The pupa (A and B) and adult (C and D) of wild type (A and C) and *fl* mutant (B and D). Bars, 5 mm. (Bottom) The vestigial wing of *fl* (E), fl^k (F), and fl^n (G). Bars, 0.5 mm.

late gene), is blocked in the fl^k wing disc in a tissue-specific manner (Matsuoka and Fujiwara 2000), and the expression of an ecdysteroid-induced 41-kDa protein is also reduced (Fujiwara and Hojyo 1997). These observations suggest that *fl* wing discs cannot respond to ecdysteroid during metamorphosis in the same way as WT wing discs do.

The cause of wing deficiency in *fl* mutants is speculated to involve the functional loss of the "*fl* gene," although the gene has not yet been identified. Previously, we identified genes expressed abnormally in the fl^k wing discs and found that the expression of *Annexin b13* (*Anxb13*) mRNA is repressed completely (Matsunaga and Fujiwara 2002). By comparing the WT and fl^k genomic structures by Southern hybridization using the cDNA of *Anxb13* as the probe, we found that the entire gene is lost from the fl^k genome (Matsunaga and Fujiwara 2002). Moreover, we have found that *Anxb13* is located on the chromosome (LG10) that includes the *fl* locus (Matsunaga and Fujiwara 2002). These findings suggested that the *fl* gene might be located near *Anxb13*. We undertook to identify the *fl* gene by positional cloning, beginning at the *Anxb13* locus, on the basis of bacterial artificial chromosome (BAC) library screening and shotgun sequencing. First, we compared the structures of the surrounding regions in the fl^k mutant with those of the WT. We detected a large chromosomal deletion in fl^k, which includes *Anxb13* and another four genes. We compared the structures of these five genes in other *fl* mutants with the WT and found that all four *fl* mutants have deletion or nonsense mutations in only one gene, the *fringe* (*fng*) ortholog (Irvine and Wieschaus 1994). Fng is an *O*-fucose-specific β-1,3-*N*-acetylglucosaminyltransferase that modifies the receptor Notch (N) and thereby modulates its ligand sensitivities (Panin *et al.* 1997; Haines and Irvine 2003). Fng-mediated N signaling plays a crucial role in the developmental processes of various tissues in *Drosophila melanogaster* (Cho and Choi 1998; Domínguez and De Celis 1998; Papayannopoulos *et al.* 1998; Grammont and Irvine 2001; Rauskolb 2001; Thomas and Van Meyel 2007) and is especially well known for its participation in dorsoventral boundary formation in wing discs (Irvine and Wieschaus 1994; Haines and Irvine 2003). During wing development in Drosophila, *fng* is expressed specifically in the dorsal cells under the control of Apterous (encoded by *ap*), which is a LIM-homeodomain transcription factor that is required for dorsal cell identity (Irvine and Wieschaus 1994; Kim *et al.* 1995). Fng-mediated N signaling is activated along the border between dorsal and ventral cells, leading to the expression of *wingless* (*wg*), which results in the organization of wing growth and patterning (Couso *et al.* 1994; Panin *et al.* 1997; Klein and Martinez Arias 1998; Baonza and Garcia-Bellido 2000; Haines and Irvine 2003). In the *fl* wing discs, the expression of *BmWnt-1*, a Bombyx homolog of *wg*, is completely repressed, suggesting a functional deficiency of Fng in the *fl* mutants. These observations clarify that *fng* is the gene responsible for the *fl* mutant. This is the first report of the positional cloning of an important mutation locus in *B. mori*. It is interesting that null or nonsense mutations in *fng* cause postembryonic lethality in *D. melanogaster* (Irvine and Wieschaus 1994; Correia *et al.* 2003), whereas the functional deficiency of the *fng* mutants in *B. mori* affects only wing morphogenesis. Our findings demonstrate the crucial role of *fng* in Bombyx wing formation and the existence of a *fng*-independent signaling pathway for tissue differentiation in tissues other than wings.

MATERIALS AND METHODS

Silkworm strains: The *fl*, fl^n, and fl^k mutant strains were provided from the silkworm stock center of Kyusyu University supported by the National Bio-Resource Project (NBRP), and the fl^o mutant strain was kindly provided by the National Institute of Agrobiological Sciences (NIAS, Kobuchizawa,

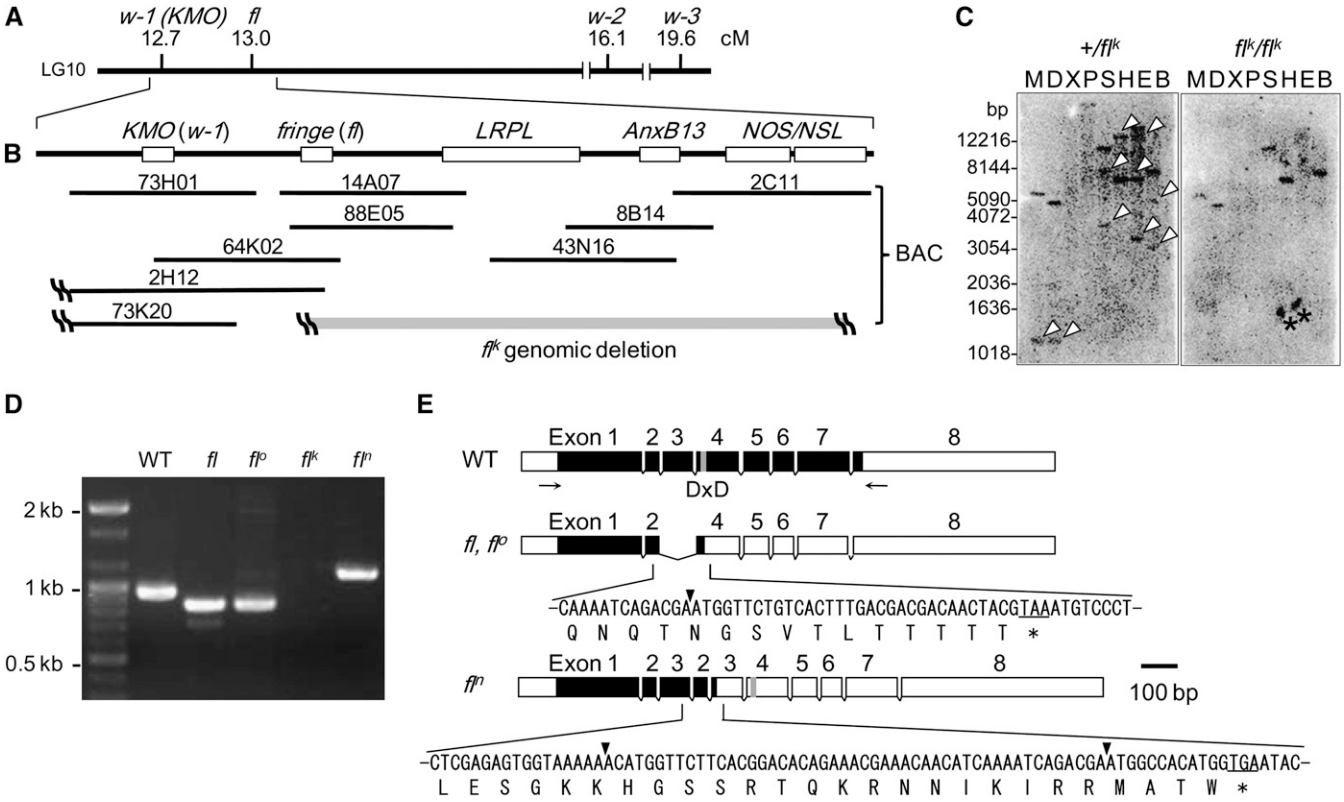

Figure 2.—Map of *fl* locus and mutations in *fng* of each *fl* allele. (A) Genetic map of the silkworm LG10 near the *fl* locus. *fl* is mapped at 13.0 and distant 0.3 cM (~100 kb) from *w-1* (*KMO*). (B) Gene organization of *fng* (*fl*) and other genes across the *fl* locus. Shaded bar indicates the deletion region in the fl^k genome. (C) Genomic Southern analysis for $+/fl^k$ and fl^k/fl^k genome with the *fng* probe. The cDNA of *fng* ORF region is used as the probe (see materials and methods). Arrowheads in $+/fl^k$ indicate the bands that are lost in fl^k/fl^k. The DNA-size markers are shown to the left. Asterisks indicate nonspecific signals. M, *Msp*I; D, *Dra*I; X, *Xho*I; P, *Pst*I; S, *Sac*I; H, *Hin*dIII; E, *Eco*RI; B, *Bgl*II. (D) RT–PCR for *fng* mRNA from wing discs of fifth instar larvae of WT, *fl*, fl^o, fl^k, and fl^n strains, using a primer set indicated by arrows in Figure 2E. (E) Schematic representation of deletion of exon 3 of *fng* in *fl*, fl^o, and duplication of exon 2–3 of *fng* in fl^n. Solid and open boxes indicate ORF and untranslated region (UTR), respectively. The shaded box represents DxD (D, aspartic acid; x, any amino acid) motif. Arrowheads and asterisks indicate exon junction and stop codon, respectively.

Japan). The silkworms were reared with leaves of a mulberry or an artificial diet (Nihon Nosan Kogyo, Yokohama, Japan) under a 16-hr light:8-hr dark photoperiod at 25°. The newly molted fourth and fifth instar larvae were segregated immediately after the onset of the photophase and this day was designated as day 0. Under this condition, most larvae began wandering on day 8 and pupated on day 12 of the fifth instar.

BAC library screening and shotgun sequencing: A Bombyx BAC library RPCI-96 was used for screening. We used BAC high-density replica (HDR) filter sets, which are available from BACPAC Resources, Children's Hospital Oakland Research Institute (Oakland, CA). The DNA probe for screening the BAC library containing the genomic sequence of 5′ upstream of *LRPL* was synthesized by PCR with a primer set, 5′-GCCGTCGCCTGTGGTACTGC-3′ and 5′-ACTCTTTAGAC CAGCCTTGGTTGAC-3′. The hybridization of the BAC HDR filter with DNA probes was carried out using the ECL direct nucleic acid labeling and detection systems kit (GE Healthcare, Waukesha, WI), according to the manufacturer's instructions (Koike *et al.* 2003). Isolation and random shotgun sequencing analysis of a BAC DNA were carried out using the large-construction kit (QIAGEN, Valencia, CA) and TOPO shotgun subcloning kit (Invitrogen, Carlsbad, CA). Multiple sequence alignments were carried out using the VectorNTI software (Invitrogen). We predicted the protein-coding regions by the NCBI Blast search program, Silkbase (http://morus.ab.a.u-tokyo.ac.jp/cgi-bin/index.cgi), KAIKOBLAST (http://kaikoblast.dna.affrc.go.jp/) and KAIKOGAAS (http://kaikogaas.dna.affrc.go.jp/) (Mita *et al.* 2004; Xia *et al.* 2004).

Cloning of Bombyx *fng* cDNA: Total RNA was isolated from several tissues by the TRI-reagent kit (Sigma, St. Louis) and reverse transcribed with random primer (N6) by the first-strand cDNA synthesis kit (GE Healthcare). The full-length cDNA was obtained by 5′ and 3′ rapid amplification of cDNA ends (RACE) technique using the Marathon cDNA amplification kit (Clontech, Mountain View, CA). We performed reverse transcription–polymerase chain reaction (RT–PCR) in the open reading frame (ORF) region of Bombyx *fng* of four *fl* alleles using the primer set 5′-ATGGGCGGACGA AGAATGCT-3′ and 5′-CTATCGCTGCACTGTTTCCTCTTCC-3′ in 35–40 cycles of 96° for 30 sec, 55° for 40 sec, and 72° for 3 min. The RT–PCR products were subcloned into pGEM-T easy Vector (Promega, Madison, WI) and sequenced by an ABI3130xl genetic analyzer (Applied Biosystems, Foster City, CA).

Sequence comparison and phylogenetic tree construction: Sequence alignment was constructed using Clustal_X. The neighbor-joining method with the MEGA2 program was used for the construction of a phylogenetic tree for the *fng* sequences. The following *fng* sequences were used to create the

diagram: BmFringe, *Bombyx mori* (this study, AB360592); PcFringe, *Precis coenia* (AAO38754); DmFringe, *Drosophila melanogaster* (AAF51658); SgFringe, *Schistocerca gregaria* (AAF17565); AmFringe, *Apis mellifera* (XP_623898); Bflo-Fringe, *Branchiostoma floridae* (CAD97418); Hsap-LF, -MF, and -RF, *Homo sapiens* (NP_002908, NP_002295, NP_002396); Mmus-LF, -MF, and -RF, *Mus musculus* (NP_033079, BAE31866, NP_032621); Rnor-LF, -MF, and -RF, *Rattus norvegicus* (AAH62031, NP_596884, AAH61801); Ggal-LF, -MF, and -RF, *Gallus gallus* (NP_990278, NP_990279, XP_416278); Xlae-LF and -RF, *Xenopus laevis* (AAB38363, NP_001017051); Nvir, *Notophthalmus viridescens* (AAD10827); Drer-LF, -MF, and -RF, *Danio rerio* (NP_001001830, NP_571046, AAT46070); LF, *Lunatic fringe*; MF, *Manic fringe*; RF, *Radical fringe*.

Semiquantitative RT–PCR for *fng* expression: Total RNA was isolated from several tissues of WT larva at various stages as described above, using the TRI-reagent kit (Sigma) and reverse transcribed with random primer (N6) by a first-strand cDNA synthesis kit (GE Healthcare). We performed semiquantitative RT–PCR analysis for *fng* expression using the cDNAs and following primer sets: 5′-ATGGGCGGACGAAG AATGCT-3′ and 5′-CTATCGCTGCACTGTTTCCTCTTCC-3′ for *fng*, 5′-AGCACCCCGTCATGGGTCTA-3′ and 5′-TGC GTCCAAGCTCATCCTGC-3′ for *ribosomal protein L3* (*rpL3*) as an internal standard. To quantify the transcripts of *fng*, we performed a quantitative RT–PCR of *rpL3* as previously described (Matsuoka and Fujiwara 2000). PCR conditions were as follows: 20 cycles of 96° for 30 sec, 58° for 40 sec, and 72° for 1 min for *rpL3*; 20 cycles of 96° for 30 sec, 55° for 40 sec, and 72° for 2 min for *fng*. The fluorescence intensities with the ethidium bromide staining were determined using the ImageJ free software (National Institutes of Health, http://rsb.info.nih.gov/ij/).

Tissue dissection and *in vitro* tissue culture: The wing discs were dissected from day 2 fifth instar larvae. After rinsing in phosphate-buffered saline (PBS), the discs were cultured in 1 ml of Grace's medium (Sigma). 20E (Sigma) was dissolved in 10% isopropyl alcohol and added to the culture medium to give the desired concentration. Incubation was performed in a 1.5-ml centrifuge tube rotating so that the discs were exposed to the air.

Northern and Southern hybridization: Total RNA (10 μg) was separated on a formaldehyde–agarose (1%) gel and transferred to a Hybond-N nylon membrane (GE Healthcare). DNA probe for *fng* was synthesized by RT–PCR with the primer set, 5′-ATGGGCGGACGAAGAATGCT-3′ and 5′-CTATCGCT GCACTGTTTCCTCTTCC-3′, and labeled with [α-^{32}P]dCTP using a BcaBEST labeling kit (Takara Bio, Japan). Hybridization was performed at 48° for 18 hr in 50% formaldehyde, 5× SSC (0.15 M sodium chloride and 0.15 M sodium citrate, pH 7.4), 10× Denhardt's solution (0.2% each of bovine serum albumin, Ficoll, and polyvinylryrrolidone), 25 μg/ml of sonicated salmon sperm DNA, 50 mM sodium phosphate (pH 7.0), and ^{32}P-labeled DNA. The membranes were washed twice at room temperature for 20 min in 2× SSC containing 0.1% sodium dodecyl sulfate (SDS). Further washes were performed by 30 min at 65° successively in 2× SSC containing 0.1% SDS and in 0.2× SSC containing 0.1% SDS.

We performed genomic Southern hybridization for *fng* in the *fl*^{k} mutant. Genomic DNA isolated from posterior silk glands was digested with several restriction enzymes, separated on an agarose (1%) gel, and transferred to a Hybond-N nylon membrane (GE Healthcare). DNA probe synthesis and hybridization were performed with the same procedures for Northern hybridization, as mentioned above.

Whole-mount *in situ* hybridization: Wing discs were dissected and then fixed immediately in 4% paraformaldehyde in PBS for 30 min at 4°. The peripodial membrane of wing discs was removed during fixation on ice. After washing in PBS containing 0.5% Tween 20 (PBST), the wing discs were treated with proteinase K (20 μg/ml; Merck, St. Louis) in PBS for 20 min at 37° and postfixed in 4% paraformaldehyde in PBS for 30 min. Hybridization was performed at 50° for 24 hr in the hybridization buffer (50% formamide, 5× SSC, 500 μg/ml yeast tRNA, 0.5% Tween 20 and 50 μg/ml heparin sodium) with a digoxigenin-labeled antisense RNA probe. RNA probes for Bombyx *fng* and *BmWnt-1* were prepared using the Roche biochemicals kit and primers for *fng* described above in *Northern and Southern hybridization* and 5′-GTGAAGA CTTGCTGGATGAGGC-3′ and 5′-CTAGGTATCCCCGGCA CGCAC-3′ for *BmWnt-1*. After hybridization, the wing discs were washed at 50° for 10 min in hybridization buffer. The wing discs were then washed for 5 min successively in PBST. After replacing in PBST, the wing discs were incubated with alkaline phosphatase-labeled anti-DIG antibody (Roche, Indianapolis) diluted 1:1,200 in PBST for 2 hr at room temperature. Excess antibody was washed away with at least four changes of PBST. The sample was equilibrated with two changes of the staining buffer (100 mM Tris-HCl, pH 9.5, 100 mM NaCl, 50 mM $MgCl_2$, and 0.1% Tween 20) and then reacted with the staining buffer containing 3.5 μg/ml 5-bromo-4-chloro-3-indolyl-phosphate, 4-toluidine (BCIP; Roche) and 4.5 μg/ml nitroblue tetrazolium chloride (NBT; Roche). After staining, wing discs were embedded in paraffin and sectioned at 7 μm and observed.

RESULTS

Comparison of genomic structures across the *fl* locus in *fl*^{k} and WT: Previous studies have suggested that *Anxb13* is deleted in the *fl*^{k} genome and is located near the *fl* locus (Matsunaga and Fujiwara 2002). We isolated *Anxb13* from *fl* mutants, except the *fl*^{k} mutant, and compared its sequences with that of the WT but found no significant mutation (supplemental Figure 2). Therefore, we inferred that the *Anxb13* gene is not the *fl* gene.

We deduced that there is a large chromosomal deletion in the *fl*^{k} genome that includes *Anxb13* and the true *fl* gene. We undertook to define the deleted region by comparing the genomic structures of *fl*^{k} and WT. The *white egg 1* (*w-1*) locus, which corresponds to the *kynurenine 3-monooxigenase* gene (*KMO*) (Quan *et al.* 2002), has been mapped nearest to the *fl* locus at 12.7 of LG10 (Figure 2A; Fujii *et al.* 1998). The 0.3 cM distance between *fl* and *w-1* (*KMO*) on the classical genetic map is estimated to be ~100–200 kb. The *w-1* mutant is characterized morphologically by its white eggs and white adult eyes. The fact that the eggs and adult eyes are normally colored in the *fl*^{k} mutant indicates that *KMO* is not deleted in *fl*^{k}. Therefore, we presumed that *KMO* is located near the *fl* gene and that one of the ends of the chromosomal deletion lies between *KMO* and *Anxb13* in the *fl*^{k} genome. Using BAC clones containing *Anxb13* as the starting point, chromosomal walking was performed until both ends of the chromosomal deletion were found in the *fl*^{k} genome or the *KMO* (*w-1*) gene was reached (Figure 2, A and B). In the first round of chromosomal walking from *Anxb13*, we screened for BAC clones containing *Anxb13* and analyzed these

clones (43N16, 8B14, and 2C11 in Figure 2B) by random shotgun sequencing. We finally assembled these sequences and obtained contiguous stretches of genomic sequences. Analyzing these with Blast homology searches and gene prediction programs, we predicted that three genes were included in the neighborhood of *Anxb13*, those encoding *low-density lipoprotein receptor-related protein-like protein* (*LRPL*, GenBank accession no. AB360591), *nitric oxide synthase* (*NOS*) (Imamura *et al.* 2002), and *NOS-like protein* (*NSL*, GenBank accession no. AB360590) (Figure 2B). By comparing the WT and fl^k genomic structures by Southern hybridization using each BAC end sequence as the probe, we found that one BAC end of 2C11 (*NOS/NSL* side) is not deleted in the fl^k genome but that the other BAC end is deleted. In addition, both BAC ends of 43N16 and 8B14 are deleted. We inferred that the right end of the chromosomal deletion lies within the 2C11 region of the fl^k genome (shaded bar in Figure 2B). Another round of chromosomal walking from *LRPL* was performed until the left end of the chromosomal deletion in the fl^k genome was detected or *KMO* was reached. We screened the BAC library using the cDNA of *KMO* or the unique genome sequence upstream from *LRPL* (the left end of *LRPL* in Figure 2B; see materials and methods) as the probe and successfully juxtaposed the BAC clones between *KMO* and *LRPL* using each BAC end sequence and contiguous genomic sequences from a Bombyx genome database (Figure 2B). By comparing the genomic structures of the WT and fl^k by PCR analysis using primer sets designed to bind within each BAC end region, we found that one BAC end of 88E05 (*KMO* side) is not deleted in the fl^k genome but that the other end (*LRPL* side) is deleted. Therefore, we inferred that the left end of the chromosomal deletion lies within the 88E05 region of the fl^k genome (shaded bar in Figure 2B). We then analyzed one of these BAC clones (88E05) and obtained contiguous stretches of genomic sequences across the *fl* region. Using Blast homology searches and gene prediction programs to analyze the obtained sequences, we predicted that the *fng* homolog in Bombyx (GenBank accession no. AB360592) is included within this region (Figure 2B).

We further isolated the cDNAs of the genes *fng*, *LRPL*, *NOS*, and *NSL* from the *fl*, fl^o, and fl^n mutants and compared their sequences with those of the WT. The *LRPL*, *NOS*, and *NSL* genes contained no significant nucleotide changes causing severe amino acid mutations in the *fl* mutants (supplemental Figures 3–5). Therefore, we deduced that these genes are not the *fl* gene. However, we detected remarkable mutations in the *fng* genes of all *fl* mutants, as described below (Figure 2, C and D, and supplemental Figure 1B).

Identification and characterization of the *fng* ortholog in Bombyx: To clarify the structural deficiency of *fng* in *fl* mutants, we determined the complete *fng* cDNA sequence of the WT using RT–PCR and RACE techniques. The complete cDNA sequence (1781 bp) revealed that Bombyx *fng* has eight exons encoding 327 amino acids, constituting a 38-kDa protein (supplemental Figure 1A). On the basis of the shotgun sequence data, the genomic region of *fng* is predicted to be cut into at least four fragments by the *Sac*I restriction enzyme (Figure 3A). Southern hybridization of the *Sac*I-digested genomic DNA of the WT with the full-length *fng* cDNA as the probe showed four bands, which coincided with the predicted *Sac*I sites (Figure 3B). We also performed a tBLASTn search of the Bombyx genome database using Bombyx Fng and Drosophila Fng proteins as the queries, to examine whether there is another *fng*-like gene in Bombyx. However, we did not detect any sequence apart from this *fng* gene (data not shown). Phylogenetic analysis of the amino acid sequences of Fng from Bombyx, Precis, and Drosophila together with other glycosyltransferases from Bombyx indicated that Bombyx Fng clusters with the other insect homologs (supplemental Figure 6). These results suggest that the Bombyx genome contains only one copy of the *fng* gene, although we cannot completely exclude the possibility that there is a gene that compensates for *fng* function in tissues other than the wing discs, despite its low sequence similarity to *fng*. Comparison of Bombyx Fng with that of other insects and vertebrates revealed that Bombyx Fng is most similar to the Fng homolog from another Lepidopteran species, *P. coenia* (Figure 3C and supplemental Figure 7). The amino acid sequence alignment of Bombyx Fng with Drosophila Fng and mouse Manic-fng (Mfng), the crystal structure of which has been revealed (Jinek *et al.* 2006), is shown in Figure 3D. Bombyx Fng is 44.2 and 37.7% identical to the homologous sequences of Drosophila Fng and mouse Mfng, respectively. A DxD (D, aspartic acid; x, any amino acid) motif, which is involved in sugar-transferase activities (Munro and Freeman 2000), is well conserved in all the Fng proteins. Furthermore, the amino acids required for UDP-GlcNAc binding and putative fucose binding are also highly conserved (Figure 3D) (Jinek *et al.* 2006). These observations confirm that the *fng* gene identified here is an authentic and exclusive ortholog of *fng* in *B. mori*.

Mutations of Bombyx *fng* in each *fl* mutant: We determined the complete cDNA sequences of the *fng* genes amplified by RT–PCR from the *fl*, fl^o, and fl^n mutants. In the *fng* ORF of *fl* and fl^o, we found a 103-bp deletion, which corresponds to nearly all of exon 3. This deletion could cause a frameshift within exon 4 and a premature TAA stop codon before the completion of normal translation (Figure 2E, *fl* and fl^o). In the *fng* ORF of fl^n, we found a duplication of exons 2 and 3, which possibly cause a premature TGA stop codon in the second exon 3 (Figure 2E, fl^n). These results demonstrate that the three alleles, *fl*, fl^o, and fl^n, encode nonsense-mutated *fng* genes. The DxD motif, which is important for Fng function (Munro and Freeman

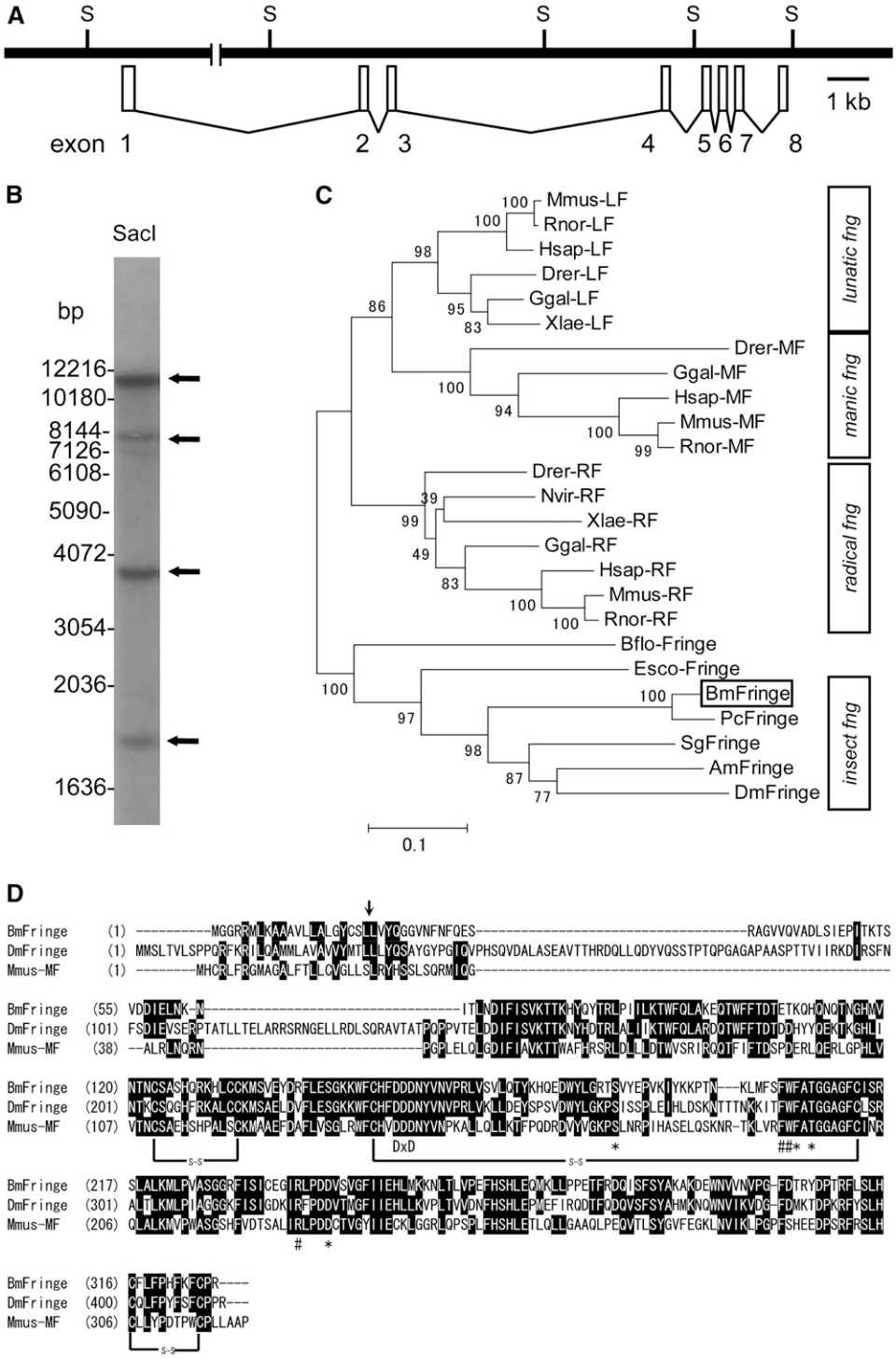

FIGURE 3.—Genomic and phylogenetic characterization of Bombyx *fng*. (A) The genomic structure of Bombyx *fng*, which consists of eight exons. S indicates *Sac*I restriction site. (B) Genomic Southern analysis with the *fng* probe for WT genomic DNA restricted with *Sac*I. The cDNA of *fng* ORF region is used as the probe (see MATERIALS AND METHODS). Arrows indicate four positive bands. The DNA-size markers are shown to the left. (C) Phylogenetic tree of *fng* based on the amino acid sequences. The numbers at the tree edge represent the bootstrap values. The scale bar indicates the evolutionary distance between the groups. Bombyx *fng* (BmFringe) is boxed. Respective *fng* sequences are shown in MATERIALS AND METHODS. (D) Amino acids alignment of Fng from Bombyx (BmFringe), Drosophila (DmFringe), and Manic-fng from mouse (Mmus-MF). Black shading with white letters indicates identical amino acid residues. Arrow shows the putative signal peptide cleavage site. DxD show conserved DxD motif. Asterisks and number signs (#) show the putative amino acid residues involved in UDP-GlcNAc binding and fucose-binding, respectively. s-s indicates disulfide bond.

2000), is encoded in exon 4 of Bombyx *fng* (Figure 2E, WT). It is noteworthy that premature stop codons in the three nonsense mutants, *fl*, fl^{o}, and fl^{n}, appear before the DxD motif, which would cause the expression of incomplete Fng proteins without essential enzymatic activity (Figure 2E, *fl*, fl^{o}, and fl^{n}). We further characterized the deletion of the *fng* gene in the fl^{k} mutant by Southern hybridization in the whole genomes of the WT ($+/fl^{k}$ heterozygote) and fl^{k} (fl^{k}/fl^{k} homozygote) using the full-length *fng* cDNA as the probe (Figure 2C). One to three bands appeared in each lane of the $+/fl^{k}$ genome, and some of these were also present in the fl^{k}/fl^{k} genome, suggesting that the *fng* gene is not completely deleted from the fl^{k} genome. We designed primer sets to the genomic region of Bombyx *fng* and found that the genomic region containing exon 8 of *fng* was amplified in the fl^{k} genome by PCR but exons 1–3 of *fng* were not amplified (supplemental Figure 8). We

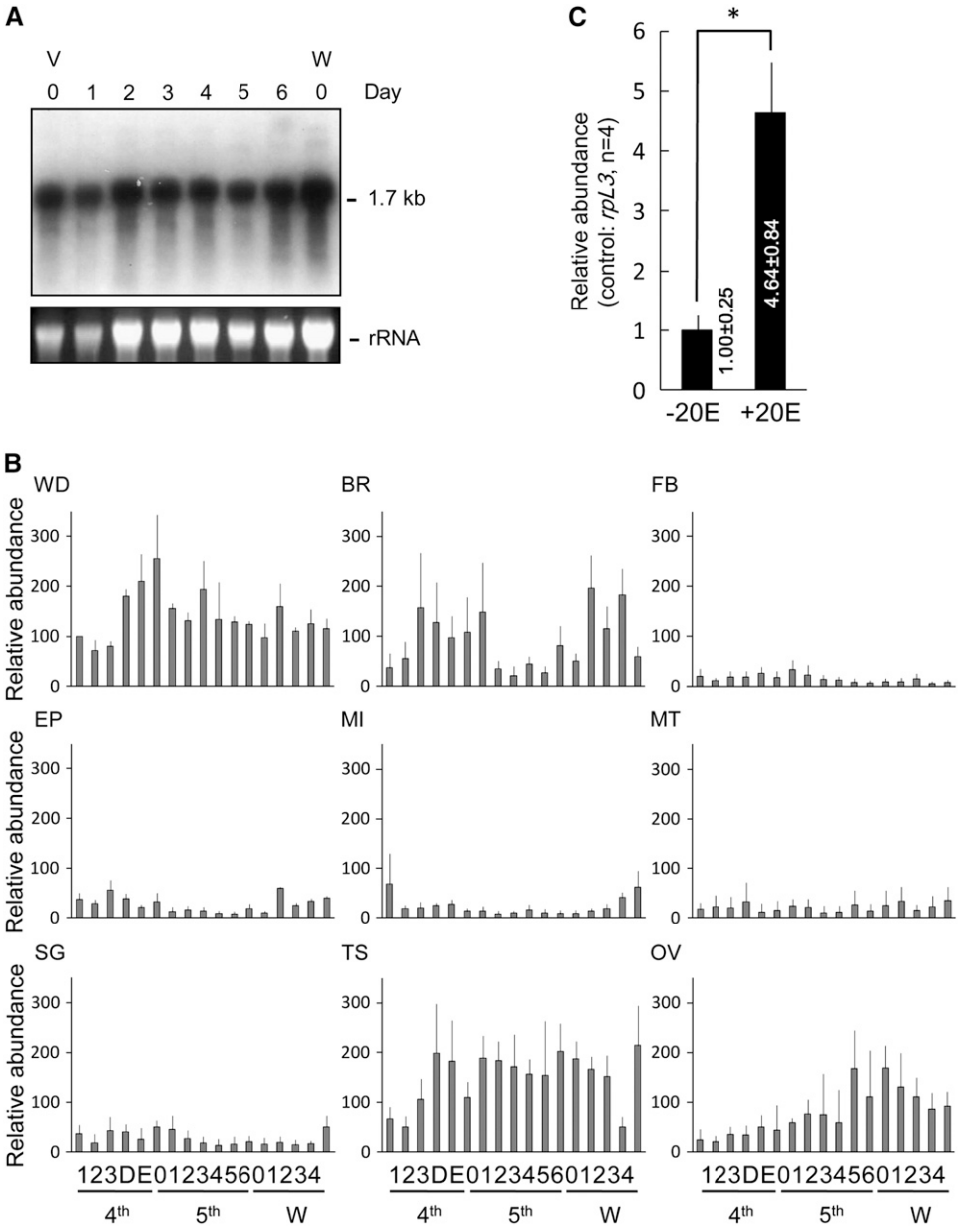

FIGURE 4.—Expression patterns of Bombyx *fng* in WT larva. (A) Northern analysis of *fng* in wing discs during fifth larval instar. Ethidium bromide staining of rRNA is shown as a loading control. V0 to V6, days 0–6 of fifth instar larva; W0, day 0 of wandering stage. (B) Developmental profiles of *fng* expression in wing discs (WD), brain (BR), fat body (FB), epidermis (EP), midgut (MI), Malpighian tubule (MT), silk gland (SG), testis (TS), and ovary (OV), by semiquantitative RT–PCR. Relative abundance of *fng* mRNA during days 1–3 of fourth larval instar (4th), D and E of 4th molting stage (4th), days 0–6 of fifth larval instar (5th) and days 0–4 of prepupal (W, wandering stages) is shown by shaded columns. Vertical thin line indicates standard deviation ($n = 3$) (see supplemental Table S1). The *rpL3* expression was used as an internal control. (C) Induction of mRNA of the *fng* gene by 20E in the wing discs of Bombyx. The discs were cultured for 12 hr with Grace's medium with 2 μg/ml 20E (+20E) or without (−20E), and the relative amount of mRNA was estimated by semiquantitative RT–PCR using *rpL3* as an internal control. $*P < 0.001$.

inferred that the 5′ end of the *fng* gene has been removed from the fl^k genome. We also found that the complete *Anxb13* and *LRPL* and most of *NOS/NSL* have been deleted from the fl^k genome (Figure 2B). From the average size of the BAC clones, we estimated that a region of >300 kb, from *fng* to *NOS/NSL*, is deleted (Figure 2B). This structural analysis of the genomes of the four *fl* mutant strains clearly shows that the functional Fng protein is not expressed from any of the *fl* alleles.

Expression profiles of Bombyx *fng* in wing discs and other larval tissues: To identify the functional role of Bombyx Fng in wing morphogenesis, we first analyzed the expression of *fng* mRNA in the wing discs of WT fifth instar larva by Northern hybridization (Figure 4A). Bombyx *fng* is expressed continually as a single 1.7-kb transcript during the last larval instar. To compare *fng* expression semiquantitatively among larval organs, we used RT–PCR to amplify transcripts from various larval organs from the fourth instar to the prepupal stages (Figure 4B and supplemental Table S1). We observed significant expression of Bombyx *fng* in the wing discs (WD), brain (BR), testis (TS), and ovary (OV), but only a little in the other tissues, including the epidermis (EP), posterior silk gland (SG), midgut (MI), fat body (FB), and Malpighian tubule (MT). In Drosophila, the expression of *fng* transcripts has also been observed in the wing, eye, leg imaginal discs, ovarian follicle cells, and longitudinal glial cells of the central nervous system and is necessary for the correct development of those tissues (IRVINE and WIESCHAUS 1994; CHO and CHOI 1998; DOMÍNGUEZ and DE CELIS 1998; PAPAYANNOPOULOS *et al.* 1998; GRAMMONT and IRVINE 2001; RAUSKOLB 2001; THOMAS and VAN MEYEL 2007). It is intriguing

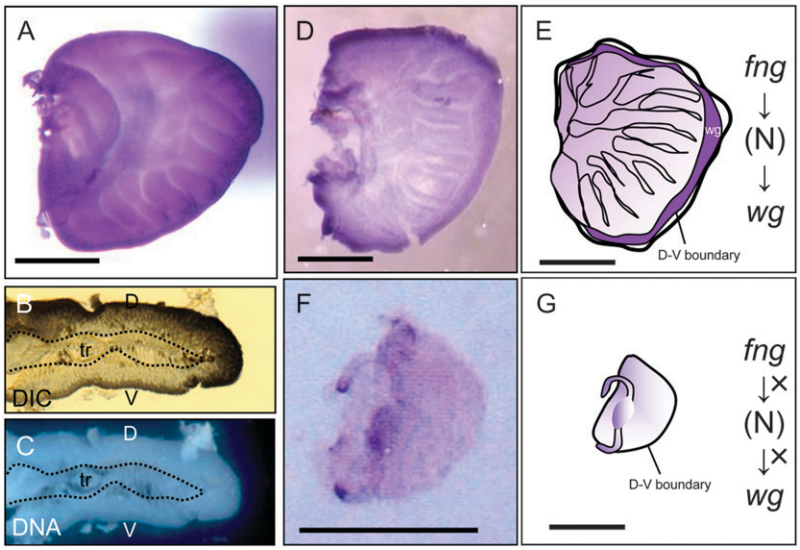

Figure 5.—Whole mount *in situ* hybridization of Bombyx *fng* and *BmWnt-1*, Bombyx homolog of *wg* in wing discs. (A and B) Expression of Bombyx *fng* in wing discs (A, dorsal view; B, cross-section). The *fng* cDNA antisense probe was hybridized to the wing discs of last instar larvae. (C) DAPI staining in cross-section. D, dorsal; V, ventral; tr, trachea. (D–G) Expression of *BmWnt-1* in wing discs. *BmWnt-1* cDNA antisense probe was hybridized to the wing discs of the last instar larvae. D and F show the expression patterns in wild-type and *fl* wing discs, respectively. E and G show the schematic representation of the *BmWnt-1* expression in wild-type and *fl* wing discs, respectively. Bars, 0.5 mm.

that the major defect in the *fl* mutants, which have lost the Fng function, appears only in wing morphogenesis, even though Bombyx *fng* is expressed in several tissues other than the wing discs.

The increase of the *fng* expression at the fourth molting stage (4th D and E) and after the wandering stage in several tissues, such as the WD, seems to be concurrent with the rising titer of ecdysteroid in those stages. To examine whether the expression of Bombyx *fng* is ecdysteroid inducible, we cultured the wing discs from the WT fifth instar larvae in Grace's medium with or without 20E and compared their responses to ecdysteroid by semiquantitative RT–PCR (Figure 4C). We observed an upregulation of *fng* mRNA expression in the wing discs cultured in the presence of 20E (Figure 4C). The general responses to ecdysteroid and the ecdysteroid-activated pathway have been studied extensively in Drosophila and *Manduca sexta* (Thummel 1995; Riddiford *et al.* 2003). Ecdysteroid exerts its effects by the transcriptional stimulation of its response genes, which define the functional and morphological properties of the tissue. This result suggests that Bombyx *fng* expression is induced by ecdysteroid and is involved in the ecdysteroid-activated pathway.

Expression patterns of Bombyx *fng* and *BmWnt-1* mRNAs in wing discs: In Drosophila wing discs, *fng* mRNA is especially detected in the dorsal region from the second to mid-third instars (Irvine and Wieschaus 1994). The expression pattern of Schistocerca *fng* has been investigated in segment morphogenesis and in the compound eye, legs, and ovary, but not in the wing (Dearden and Akam 2000). Among the *fng* orthologs, Precis *fng* (Correia *et al.* 2003) is most closely related to Bombyx *fng* (Figure 3C), but its expression pattern has not been established. To examine whether Bombyx *fng* localizes in the wing discs like Drosophila *fng*, whole-mount *in situ* hybridization was performed on the wing imaginal discs using the full-length ORF of Bombyx *fng* as the probe. In the wing imaginal discs of Bombyx, the *fng* transcripts were expressed in the dorsal apical region (Figure 5, A and B). This expression pattern of Bombyx *fng* is consistent with that of Drosophila *fng*. To our knowledge, this is the first report of the *fng* expression pattern in the wing discs of a Lepidopteran insect or of an insect other than Drosophila.

Shutting down the Fng function in *fl* wing discs was expected to severely affect the expression of genes in its downstream signaling pathway. The expression of *wg* is induced in the dorsoventral boundary region by the activation of Fng-mediated N signaling (Panin *et al.* 1997; Haines and Irvine 2003), and *wg* expression along the wing margin is important for wing morphogenesis in Drosophila (Couso *et al.* 1994; Klein and Martinez Arias 1998). Therefore, we first analyzed the expression pattern of *BmWnt-1* mRNA in the wing discs of fifth instar WT larvae with whole-mount *in situ* hybridization using the full-length *BmWnt-1* cDNA as the probe (Figure 5D). *BmWnt-1* expression was observed along the entire dorsoventral boundary of the WT wing disc, which occupies the margin of the wing disc in the Lepidoptera (Figure 5, D and E). Next, we examined *BmWnt-1* expression in the *fl* wing disc of fifth instar larvae, which is smaller than the WT disc and does not show any morphological changes, such as tracheal invasion. In the *fl* wing disc, we detected no distinct signals for *BmWnt-1* mRNA at the wing margin or in any other wing area (Figure 5F). These results suggest that the Fng-mediated N signaling pathway and *wg* expression are conserved and are essential for the process of wing morphogenesis in Bombyx, as in Drosophila and other organisms. These data also suggest that the loss of Fng function inhibits the activation of the N signaling pathway, which results in the wing-deficient phenotype observed in the *fl* mutants of the silkworm (Figure 5G).

DISCUSSION

In this study, we elucidated the genome structure across the *fl* locus and identified that the responsible gene for *fl* is *fng*. To our knowledge, this is the first report to use positional cloning of the responsible gene for an intriguing allele, such as the wing-deficient mutant in the silkworm, *B. mori*. We initially thought that the wing-deficient mutant of the silkworm, *fl*, may be caused by the functional loss of some known genes identified in Drosophila, such as *ap* or *vestigial*, mutations of which cause a wingless phenotype similar to that of *fl*. On this point, the finding that *fng* is the *fl* gene is an unexpected result, because the severe loss-of-function mutants of *fng* are the postembryonic lethal in Drosophila (Irvine and Wieschaus 1994; Correia *et al.* 2003).

***fng* is the gene responsible for *fl*:** In the fl^k mutant, we identified a large chromosomal deletion that includes five putative genes: *fng*, *Anxb13*, *LRPL*, *NOS*, and *NSL* (Figure 2, B and C). In the other three *fl* alleles, we detected nonsense mutations in the *fng* gene (Figure 2, D and E), but only synonymous or insignificant amino acid changes in the other four genes (supplemental Figures 2–5), strongly suggesting that *fng* is the gene responsible for *fl*. In the fl^k homozygote, all five genes are deleted from the chromosomes, but the loss of *Anxb13*, *LRPL*, *NOS*, and *NSL* seems to have no vital effect on fl^k development, because there is no remarkable difference in the phenotypes of fl^k and the other *fl* mutants. We also estimated the distance between *fng* and the nearest known gene, *KMO*, to be <200 kb by calculating the average size of the BAC clones. This value coincides well with the 0.3-cM distance between them on the classical genetic map (Figure 2A).

We found no *fng* homolog other than Bombyx *fng* in the silkworm genome or expressed sequence tag (EST) databases (data not shown and supplemental Figure 6). Southern hybridization also demonstrated that the silkworm genome contains a single copy of the *fng* homolog. These results suggest that *fng* at the *fl* locus is the only functional *fng* ortholog in *B. mori*, although we cannot completely exclude the possibility that there is a gene that compensates for *fng* function in the tissues other than the wing discs, despite its low sequence similarity to *fng*. Bombyx Fng has the amino acids that are required for UDP-GlcNAc binding and putative fucose binding, as in mouse Mfng and Drosophila Fng. On the basis of a phylogenetic tree constructed from the amino acid sequences of Fng from a wide variety of organisms, Bombyx Fng (BmFringe) on silkworm LG10 is most closely related to butterfly Fng (PcFringe) (Figure 3C). Furthermore, Lepidopteran Fng proteins (BmFringe and PcFringe) are more distantly related to Drosophila or Apis Fng than to Schistocerca Fng. Several unique amino acid residues are observed, which are either Lepidopteran type (conserved in Lepidopteran Fng) or non-Lepidopteran type (conserved in Fng of other insects) (supplemental Figure 7). Although the biological significance of these differences is unknown, we presume that these amino acid residues are responsible for the phylogenetic relationships of the Lepidopteran Fng proteins and other insect Fng proteins.

Tissue- and temporal-specific *fng* expression: Males and females of the recessive *fl* homozygote, which lack wings, can be crossed with each other to produce an F_1 generation that also develops normally, except for wing morphogenesis, suggesting that these homozygotes are fertile and their sexual behavior is normal. As shown in Figure 4B, RT–PCR analysis of various tissues from the fourth instar to the prepupal stages showed that *fng* mRNA is expressed abundantly in wing discs, brain, testis, and ovary but only to a small extent in other tissues. Bombyx Fng in the wing discs must be involved in wing morphogenesis because the expression of a downstream marker of Fng-mediated N signaling pathway, *wg*, is repressed in the *fl* wing discs (Figure 5, F and G). However, we cannot explain the exact functional role of Bombyx *fng* in the brain or reproductive organs because it seems that the reproductive ability and behavior of the *fl* mutants are not affected. As a trivial effect, we usually observed that the third thoracic legs of the *fl* mutants move more slowly than the corresponding WT legs, suggesting that the neurogenesis of the *fl* mutants is affected through the loss of *fng* function in the brain and nervous systems.

The *fng* expression in the wing discs and brain increased in the fourth molting stage and after the wandering stage (Figure 4B). Previous morphological studies have shown that several defects in *fl* wing development become evident immediately after the fourth larval ecdysis, suggesting that the *fl* gene product is required around the fourth molting stage. The titer of ecdysteroid, which regulates various morphological events, peaks at each molting stage and at the prepupal stages. The correlation between the *fng* expression pattern and the ecdysteroid titer implies that *fng* mRNA is induced by ecdysteroid. We demonstrated that *fng* is an ecdysteroid-inducible gene in Bombyx (Figure 4C). These results suggest that Bombyx *fng* is part of the ecdysteroid-activated pathway and plays a wing-specific role.

We have shown previously that *fl* wing discs lose their responsiveness to ecdysteroid in a wing-disc specific manner (Fujiwara and Hojyo 1997; Hojyo and Fujiwara 1997; Matsuoka and Fujiwara 2000). The expressions of a 20E-inducible 41-kDa protein, *BHR3*, and *Urbain* were also reduced in *fl* wing discs. These results imply that the loss of *fng* function specifically affects the downstream pathway of ecdysteroid signaling and leads to a deficiency in wing formation. Although we do not have direct evidence at present, the *fl* wing disc is a good model in which to study the unknown crosstalk between the ecdysteroid and Fng-mediated N signaling pathways in insect development.

Crucial role of *fng* in Bombyx wing morphogenesis: Here, we detected *fng* expression in the dorsal-specific region of Bombyx wing discs, which is similar to that in Drosophila (Figure 5, A and B). Previously, the analysis of Precis homologs of Drosophila appendage patterning genes revealed that butterfly *ap* and *wg* are expressed exclusively in dorsal cells and in cells along the future wing margin of the Precis wing discs, respectively (Carroll *et al.* 1994; Weatherbee *et al.* 1999). This fact implies that Butterfly *fng* is also expressed in the dorsal region of the wing discs, under the control of *ap*, as in Drosophila. Furthermore, from our results, *wg* is expressed along the border between the dorsal and ventral cells in the WT but not in *fng* null mutants of *B. mori* (Figure 5, D–G). These results suggest that the wing fields of Drosophila and Lepidopteran insects are organized and regulated in a similar manner and that the function of *fng* is conserved in Bombyx and Drosophila, although the mode of action of Bombyx *fng* remains speculative at present.

The Lepidopteran wing margin contains a special structure called the "bordering lacuna" (BL) (Fujiwara and Ogai 2001). In the early pupal stage of wing development, ecdysteroid induces cell proliferation and differentiation proximal to the BL and programmed cell death distal to the BL (Fujiwara and Ogai 2001). The outline of the adult wings of moths and butterflies emerges as a result of the disappearance of the peripheral region during pupal stages. These phenomena are specific to the Lepidoptera and are widely observed in most butterflies and moths. We recently found that two ecdysteroid receptor isoforms, *EcR-A* and *EcR-B1*, are expressed precisely in the cell death and cell proliferation areas in the wings, respectively (Lobbia *et al.* 2007). It is noteworthy that the *EcR-A* expression region corresponds to the region of the *wg* expression region during wing morphogenesis. Fng-mediated N signaling induces *wg* expression, which is involved in the formation of the wing margin in Drosophila and probably also in Bombyx. Therefore, it is possible that Bombyx *fng* regulates the expression of *wg*, *EcR-A*, or both, to produce the BL structure in Lepidoptera, although further studies are required to support this hypothesis.

Different developmental effects of the *fng* defect on Bombyx and Drosophila: In Drosophila, *fng* null mutants or *fng* mutants with an abnormal DxD motif are lethal during early larval stages (Irvine and Wieschaus 1994; Correia *et al.* 2003). Drosophila Fng plays essential roles in the morphogenesis of the leg and compound eye imaginal discs and in several other tissues, as well as in the wing imaginal discs (Papayannopoulos *et al.* 1998; Grammont and Irvine 2001; Rauskolb 2001; Thomas and Van Meyel 2007). In contrast, null mutants of Bombyx *fng* are viable and fertile, and Bombyx *fng* seems to be required for wing development in Bombyx, unlike in Drosophila. We are interested in understanding the difference in *fng* functions between Bombyx and Drosophila. It has been suggested that the developmental processes of organogenesis differ between the Lepidoptera and Drosophila (Kim 1959; Tanaka and Truman 2005; Allee *et al.* 2006; Franco *et al.* 2007). Drosophila imaginal organs develop from each imaginal disc in a concentric region. However, in Bombyx and other Lepidoptera, most adult organs are derived from the immature epithelial region called the "primordium," except for the wings, which develop from the wing imaginal discs. For example, Lepidoptera legs do not develop from imaginal discs but from the cylindrical epidermis of the caterpillar's legs (Kim 1959; Tanaka and Truman 2005). In the developing leg imaginal discs of Drosophila, Fng-mediated N signaling functions to establish segmentation and the promotion of leg growth (Rauskolb 2001). Our results suggest that Bombyx legs and adult organs, other than the wings, develop and grow independently of *fng* function. Thus, the critical difference in the developmental systems of Drosophila and the Lepidoptera may relate to the different functional roles of *fng*. It will be interesting to investigate in more detail the mechanism underlying *fng* function in the development of the adult organs of Lepidoptera and to determine the developmental systems in the Lepidoptera.

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