lingerer, a Drosophila Gene Involved in Initiation and Termination of Copulation, Encodes a Set of Novel Cytoplasmic Proteins

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ABSTRACT

In an effort to uncover genetic components underlying the courtship behavior of *Drosophila melanogaster*, we have characterized a novel gene, *lingerer* (*lig*), mutations of which result in abnormal copulation. Males carrying a hypomorphic mutation in *lig* fail to withdraw their genitalia upon termination of copulation, but display no overt abnormalities in their genitalia. A severe reduction in the dosage of the *lig* gene causes repeated attempted copulations but no successful copulations. Complete loss of *lig* function results in lethality during early pupal stages. *lig* is localized to polytene segment 44A on the second chromosome and encodes three alternatively spliced transcripts that generate two types of 150-kD proteins, Lig-A and Lig-B, differing only at the C terminus. Lig proteins show no similarity to known proteins. However, a set of homologous proteins in mammals suggest that Drosophila Lig belongs to a family of proteins that share five highly conserved domains. Lig is a cytoplasmic protein expressed in the central nervous system (CNS), imaginal discs, and gonads. Lig-A expression is selectively reduced in *lig* mutants and the ubiquitous supply of this protein at the beginning of metamorphosis restores the copulatory defects of the *lig* mutant. We propose that *lig* may act in the nervous system to mediate the control of copulatory organs during courtship.

THE mating behavior of male Drosophila is characterized by a fixed sequence of actions. The sequence starts with orientation toward a female fly, followed by tracking, tapping of the female's abdomen, generation of courtship songs by alternate vibration of its wings, and licking of the female's genitalia (Hall 1985; Greenspan 1995; Yamamoto *et al.* 1997; Yamamoto and Nakano 1998). As a consequence of such courtship, sexual receptivity in the female increases, inducing a reduction in her locomotion (von Schilcher 1976). This permits the male to mount her back and attempt copulation by curling his abdomen to clasp her genitalia. After ~15 min of copulation, the male withdraws his genitalia and dismounts the female.

This stereotypic pattern of courtship represents an innate behavior that is primarily under genetic control, although many of the genes controlling it remain to be identified (Yamamoto *et al.* 1998). To identify these genes, we employed a forward genetic approach in which mutants of a specific phenotype are isolated to track down the genes responsible for the alteration in courtship behavior. Accordingly, we screened ~2000 *P*-element insertion lines and isolated eight mutants, each of which showed unique anomalies in mating behavior (Yokokura *et al.* 1995; Suzuki *et al.* 1997; Baba *et al.* 1999; Nakano *et al.* 2001). *lingerer* (*lig^P*) is one such mutant. The *lig^P* male flies start copulation normally, but often fail to withdraw their genitalia smoothly at the end of copulation. As a result, the male and female tug at each other, pulling in opposite directions for several seconds to tens of minutes.

Another mutant with a phenotype similar to that of *lig^P* is *stuck* (*sk*). *sk* was isolated by Hall *et al.* (1980), who reported that the mutation was polygenic in nature with a genetic factor on the fourth chromosome contributing significantly to the *sk* phenotype. Although we also found some influence of the genetic background on the *lig* mutant phenotype, mutations in the *lig* locus resulted in an unambiguous *sk*-like phenotype, suggesting a major role for this gene in genital control. Furthermore, when the *lig* gene is severely disrupted, copulation is almost completely blocked although the mutant males continue to repeatedly attempt copulation. The relatively clear-cut behavioral phenotypes found in *lig* mutants enabled us to clone the *lig* gene.

Sequence data from this article have been deposited with the EMBL/GenBank Data Libraries under accession nos. AF276963 (type 1 *lig*), AF276964 (type 2 *lig*), AF276965 (*Mlig-1*), AF276966 (*Mlig-2a*), and AF276967 (*Mlig-2b*).

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Here, we report that three forms of mRNA, type 1, type 2, and type 3, are transcribed from the *lig* locus, and expression of only the type 1 transcript is disrupted in the lig^{P} mutant because of *P*-element insertion. The *lig* gene encodes a set of novel cytoplasmic proteins, which are conserved between flies and mammals. Rescue experiments using the type 1 cDNA driven by a heat-shock promoter revealed that the *lig* function is required only from the late third instar stage to pupation for normal copulation to occur in adults. During the late third instar larval stage, the *lig* gene is expressed in the central nervous system (CNS) as well as in the imaginal discs. These results suggest a role for this gene in the development of the machinery controlling genital movement.

MATERIALS AND METHODS

Fly stocks: lig^{P} is a homozygously viable *P*-element insertion line, obtained from a screen for alterations in mating behavior done in our laboratory. Homozygous lig^{P} males are fertile, whereas lig^{P} females are less fertile. Molecular analyses revealed that lig^{P} is a hypomorphic mutation.

The revertant lig^{R4} and lig^{R11} were isolated from fly lines in which the *P* element at 44A was remobilized by introducing the $P(ry^{+}\Delta 2\text{-}3)$ chromosome into the lig^{P} lines. Precise excision of the *P* element in the revertant lines was confirmed by genomic Southern hybridization and PCR followed by sequencing. lig^{R4} was used for further analyses.

Deficiency lines lacking the *lig* locus [$Df(2R)lig^{X4}$, $Df(2R)lig^{X13}$, and $Df(2R)lig^{X18}$] were obtained by X-ray irradiation of lig^{P}/*SM1* males. All three lines were lethal when homozygous and semilethal when placed *in trans* to lig^{P}. We confirmed that the *lig* transcription unit was deleted in these lines by genomic Southern blot and PCR analyses. In the text, $Df(2R)lig^{X4}$, $Df(2R)lig^{X13}$, and $Df(2R)lig^{X18}$ are referred to as *X4*, *X13*, and *X18*, respectively.

The *lig* alleles with double *P* insertions were generated by the local transposition (Tower *et al.* 1993; Zhang and Spradling 1993). From the progeny of the males bearing both lig^{P} and $P(ry^{+}\Delta 2\text{-}3)$, the flies with darker red eyes than those of the original lig^{P} males were collected and balanced. Among them, five lines (lig^{PP1}, lig^{PP2}, lig^{PP3}, lig^{PP4}, and lig^{PP5}) were semilethal when placed *in trans* to lig^{P}. All of the five alleles showed homozygous pupal lethality forming long puparia and failed to complement pupal lethality of each other. The pupal lethality was observed with equal severity for $lig^{PP1\text{-}5}$ homozygotes and $lig^{PP1\text{-}5}/X4$, indicating that $lig^{PP1\text{-}5}$ are strong loss-of-function or null alleles of this gene. We confirmed that the *lig* product was undetectable in lig^{PP1} larvae by Western blot analysis (Figure 6B). Plasmid rescue followed by sequencing demonstrated that these five lines retained the lig^{P} original insertion plus a new independent insert arising from local hopping (see Figure 4). The second *P* element was inserted in the opposite direction at 878, 456, 503, 507, and 885 bp downstream of the original insertion in lig^{PP1}, lig^{PP2}, lig^{PP3}, lig^{PP4}, and lig^{PP5}, respectively. The lig^{PP1} was used for further analyses.

The *lig* mutant alleles used in this study had been outcrossed to a w^{1118} strain, which had a Canton-Special (CS) wild-type genetic background. The mutant phenotypes were maintained in the CS genetic background.

Behavior assays: To observe mating behavior, virgin males were collected at eclosion and placed individually in food vials for 3–5 days. Each male fly was transferred to a circular mating chamber (0.9-cm diameter, 0.5-cm height) with a wild-type virgin female. The behavior of the fly pairs was recorded using a video recorder. If the flies did not start copulation within the first 25 min, they were discarded. The copulating pairs were carefully observed to check whether the males dismounted normally or not upon termination of copulation. When the male released the female genitalia after, and not before, dismounting, it was considered as a stuck phenotype, irrespective of the time spent to become separated. Mating success was estimated as the percentage of pairs that copulated within the 1-hr or 25-min observation period (the observation period in each experiment is described in the relevant figure legend). The sex appeal parameter index (SAPI) was defined as the proportion of time the male exhibited unilateral wing vibration during a 5-min observation period or during the interval before copulation in the case of males that started to copulate within 5 min.

To examine adult locomotor activity, a male fly was transferred to the mating chamber, and its behavior was recorded by a video recorder for 10 min. The locomotor activity was determined as the number of times the fly crossed a straight line on the floor during a 10-min observation period.

Morphological analysis of male genitalia: For scanning electron microscopy (SEM), the flies were prepared for critical point drying and coated with a 2-nm layer of gold. Images were taken on a low-voltage prototype SEM. For genital cuticle preparation, the male adult terminalia were dissected in PBS, boiled in 10% NaOH for 10 min, and washed in water four times.

Isolation of *lig* genomic DNA and cDNA: Genomic DNA flanking the *P*-element insertion site was isolated by plasmid rescue and used for screening of the λEMBL3 CS genomic library (CLONTECH, Palo Alto, CA). Drosophila adult and pupal cDNA libraries were constructed with oligo(dT)-primed cDNA and λgt10 vector using the TimeSaver cDNA synthesis kit (Amersham Pharmacia Biotech, Buckinghamshire, UK). Using genomic fragments as probes, the cDNA libraries were screened, and several cDNA clones containing large inserts were isolated. For cloning of mouse homologs, PCR primers were designed according to the expressed sequence tag (EST) sequences, and PCR was performed using phage DNA prepared from the lysates of a mouse embryonic cDNA library (CLONTECH) as a template. The amplified fragments were sequenced and used as probes for screening of the mouse embryonic cDNA library. Nucleotide sequences were determined using a 377 DNA sequencer (Perkin-Elmer, Norwalk, CT).

Northern hybridization and RT-PCR: Total RNA was isolated using TRIzol (Life Technologies, Rockville, MD), and poly(A)$^{+}$ RNA was prepared by affinity chromatography using oligo(dT)-cellulose Type 7 (Amersham Pharmacia Biotech). Poly(A)$^{+}$ RNA (0.5 or 2 μg) was separated on a 1% agarose gel containing formaldehyde. Following transfer to BiodynPlus (Pall, Port Washington, NY), the filters were hybridized with digoxigenin (DIG)-labeled probes, and signals were detected using a DIG luminescent detection kit (Boehringer Mannheim, Indianapolis). DIG-labeled antisense single-strand DNA probes, L1 and L2, were prepared by PCR (Lehmann and Tautz 1994) using primers as follows:

L1 sense primer, 5′-ATGCTCGTCAAGGAGCAACAG-3′;
L1 antisense primer, 5′-TGCGGATGTTGCTGTATGTTTC-3′;
L2 sense primer, 5′-TAGGCGGAGGGACGGAAAGT-3′;
L2 antisense primer, 5′-AATCACAGTCAACAAGTGTACAA-3′.

As a control for the amount of RNA loaded in each lane, the blots were rehybridized with a *Drosophila melanogaster ras2* gene probe (Bishop and Corces 1988), which was provided by V. G. Corces.

For RT-PCR experiments, oligo(dT)-primed first-strand cDNA was synthesized from 0.5 μg of poly(A)$^+$ RNA using a RNA PCR kit (AMV), version 2.1 (Takara, Kyoto, Japan). To prepare Lig-A-specific first-strand cDNA, reverse transcription was performed on 0.6 μg of poly(A)$^+$ RNA with a primer complementary to the sequence in the 3′-untranslated region of type 1 cDNA (Lig-A-3′; 5′-GCGAAGGCTATAAAAGTC GTTC-3′) at 65° for 1 hr using *Bca*BEST RNA PCR kit, version 1.1 (Takara). PCR was performed with ExTaq DNA polymerase (Takara) and the following primers:

S-U1, 5′-TGCGTTTCTCGGCGCATTTG-3′;
S-U2, 5′-CTGGTTTTAAGATCGAATCCTTC-3′;
A-P, 5′-CGACGGGACCACCTTATGT-3′;
A-U, 5′-CTGCGGATCCTCTGTGGTGCTATT-3′;
S-D, 5′-TCCAACCAGTCGCAGGCAG-3′;
A-D1, 5′-GTTCTGTCCGGCCCAGTACGA-3′;
A-D2, 5′-GGTATGTAACTTGAGGACCGTT-3′.

Antibody production and Western blot analysis: A *Bam*HI/*Not*I fragment of the *lig* cDNA (amino acids 82–225; Lig-N) was cloned into the pET-32b(+) vector (Novagen, Madison, WI). Using this vector, the Lig-N fragment was expressed into the periplasmic space of *Escherichia coli* as a fusion protein with thioredoxin. The fusion protein was extracted by the osmotic shock method according to the manufacturer's instructions and purified by affinity chromatography using His-Bind Resin (Novagen) immobilizing Ni^{2+}. A synthetic peptide, Lig-A-C, corresponding to the C-terminal 19-amino-acid peptide of Lig-A protein (CQSKSAGKQGYSPSYWAGQN), was conjugated with bovine serum albumin (BSA) using a cross-linking reagent, *N*-hydroxysuccinimidyl 3-(2-pyridyldithio) propionate (Carlsson *et al.* 1978), and the BSA-Lig-A-C conjugate was purified by dialysis. Three female BALB/c mice were immunized for each antigen using standard methods (Harlow and Lane 1988). Antibodies in plasma were prepared as follows. Ten to 14 days after injection, the immunized mice were bled from the tail vein. The blood was collected in heparinized glass capillary tubes and centrifuged to remove cells. The titers of antibodies were checked using the dot-immunobinding assay (Hawkes *et al.* 1982).

For Western blotting, flies or larvae were homogenized in lysis buffer [50 mm Tris-HCl (pH 6.8), 2% SDS]. After 10 min boiling in the presence of 0.1 m dithiothreitol, the lysate was applied to 7.5% polyacrylamide gels and then transferred to an Immobilon polyvinylidene difluoride membrane (Millipore, Bedford, MA). The blots were incubated with anti-Lig-A-C (1:10000) and visualized using an alkaline phosphatase-conjugated secondary antibody (Bio-Rad, Hercules, CA).

***P*-element transformation and rescue experiment:** The following transgenic vectors were made and injected into w^{1118} embryos together with a phsπ helper plasmid (Steller and Pirrotta 1986) to establish transgenic lines (Rubin and Spradling 1982):

1. pCaSpeR4-*Glig*: The 12-kb *lig* genomic fragment (*Glig*), which included the whole transcribed region of all types of *lig* transcripts as well as 0.3 and 1 kb of flanking sequences at the 5′ and 3′ ends, respectively (see Figure 4), was isolated using PCR technique and ligated into the *Eco*RI/*Xho*I sites of the pCaSpeR4 transformation vector.
2. pCaSpeR-*hs-lig1*: The *hs-lig1* fusion gene, in which the expression of type 1 *lig* cDNA was controlled by the *hsp70* heat-shock promoter, was constructed by subcloning the full-length type 1 *lig* cDNA into the *Eco*RI site of the pCaSpeR-hs vector in the same direction to the *hsp70* promoter.

For rescue experiments of stuck phenotype with *hs-lig1*, two independently generated transgenic lines, *hs-lig1(#4)* and *hs-lig1(#5)*, were used. The *hs-lig1(#4)* line showed leaky expression of the Lig-A protein at 25° (Figure 6B). The *hs-lig1(#5)* line showed little leaky expression under similar conditions (Figure 6B) and therefore was used as a negative control. Fly lines carrying both the lig^p mutation and the *hs-lig1* transgene were reared at 25° and subjected to behavioral assays.

For rescue experiments of noncopulating phenotype using *hs-lig1,* fly lines carrying both the lig^p mutation and the *hs-lig1* transgene were crossed with the *X4/SM1* line. The progeny from the third instar larval stage to the pupal stage were exposed to a single heat-shock treatment at 37° for 1 hr. After eclosion, mutant hemizygous male flies carrying the *hs-lig1* transgene were collected and subjected to behavioral assays.

***In situ* hybridization:** *In situ* hybridization in whole-mount tissues and embryos of Drosophila was performed according to the protocol laid out by Lehmann and Tautz (1994) with some modifications (Juni *et al.* 1996). The larvae at the late third instar larval stage were cut into anterior and posterior halves and turned inside out in Drosophila Ringer's solution [183 mm KCl, 46 mm NaCl, 3 mm $CaCl_2$, 10 mm Tris-HCl (pH 7.2), 1% BSA] using a fine metal needle. The dissected larvae were fixed with 4% paraformaldehyde in PBS for 15–30 min on ice followed by incubation with 4% paraformaldehyde and 0.5% Triton X-100 in PBS for 15 min at room temperature. All incubations and washes were performed at room temperature. Embryos were dechorionated, fixed, and devitellinized according to the method described by Lehmann and Tautz (1994). After three 5-min washes in PBS/0.1% Tween 20, the fixed larvae and embryos were digested using proteinase K (12.5 μg/ml in PBS/0.1% Tween 20) for 5–15 min. The digestion was stopped by incubation in PBS/0.1% Tween 20 and 2 mg/ml of glycine for 10 min. After three 5-min washes in PBS/0.1% Tween 20, the larvae and embryos were refixed for 20 min with 4% formaldehyde and 0.1% glutaraldehyde in PBS. Thereafter, following five 5-min washes in PBS/0.1% Tween 20, the larvae and embryos were hybridized with DIG-labeled single-strand DNA probes at 48°, and signals were detected using an alkaline-phosphatase-conjugated anti-DIG antibody (Boehringer Mannheim) using the method described by Lehmann and Tautz (1994). Sense or antisense DIG-labeled single-strand DNA probes were prepared by PCR (Lehmann and Tautz 1994) using the following primers that hybridize to the region common to all types of the *lig* transcripts:

sense primer, 5′-ACAAAGTGCTTACCAGTCAAG-3′;
antisense primer, 5′-CCGCTGTTGCTGCTCACTC-3′.

In situ hybridization to polytene chromosomes was performed using DIG-labeled probes and the DIG DNA detection kit (Boehringer Mannheim), following the method of Engels *et al.* (1986).

Immunohistochemistry: For immunohistochemistry, the larval brains and discs were dissected in PBS and fixed with 4% formaldehyde in PBS for 1 hr at room temperature. After blocking with 2% normal goat serum in PBS containing 0.3% Triton X-100 for 1 hr at room temperature, samples were incubated with the mouse anti-Lig-N antibody (1:500) overnight at 4°. The signals were visualized using a biotinylated secondary antibody and horseradish peroxidase-conjugated ABC reagent (Vector, Burlingame, CA) with diaminobenzidine as the chromogen according to the manufacturer's protocol.

RESULTS

Phenotypes of *lig* mutant: lig^p is a hypomophic mutant induced by a *P* insertion at cytological location 44A on the second chromosome. Homozygous lig^p males were

completely viable and fertile and did not show any generalized behavioral abnormality, such as inactivity (see Figure 2B), sluggishness, or uncoordinated walking.

To compare mating behavior between mutant and wild-type males, a single male was placed with a single wild-type female in a small mating chamber. In the case of wild-type male and female pairs, 77% of the tested pairs started copulation within 25 min. Just before the termination of copulation, the female fly often kicked the legs of the mounting male with her hindlegs. The male fly then released the female's genitalia and dismounted from the female (Figure 1A). In contrast, although the *lig*^{P} homozygous male flies initiated copulation with wild-type females just as often as wild-type males did (mating success within 25 min = 68%), they were frequently unable to release the female's genitalia while mounted on the female's back. The mutant males tended to dismount the females without withdrawing their genitalia. As a result, the male and female often tugged at each other, pulling in opposite directions (Figure 1B). In many cases, the *lig*^{P} male and wild-type female remained stuck for several seconds to tens of minutes after copulation, until finally managing to separate from each other. We call this the "stuck phenotype." As shown in Figure 1C, 75% of the copulating *lig*^{P} males exhibited the stuck phenotype, while only a small percentage of the mated wild-type males became stuck. The frequency of the stuck phenotype in the *lig*^{R4} line, which was obtained by precise excision of the *P* element in *lig*^{P}, was close to that in the wild-type strain (Figure 1C), demonstrating that the stuck phenotype found in *lig*^{P} resulted from the *P*-element insertion.

Interestingly, *lig*^{P} females did not show any abnormality in courtship behavior. *lig*^{P} females mated normally with wild-type males (mating success within 25 min = 65%, n = 63) although they showed a reduction in fertility (~25% of the fertility of CS wild-type or *lig*^{R4} females).

The *lig*^{P} mutation was semilethal in both sexes when placed *in trans* to chromosomes lacking the *lig* activity, such as null *lig* alleles (*lig*^{PP1}, *lig*^{PP2}, *lig*^{PP3}, *lig*^{PP4}, and *lig*^{PP5})

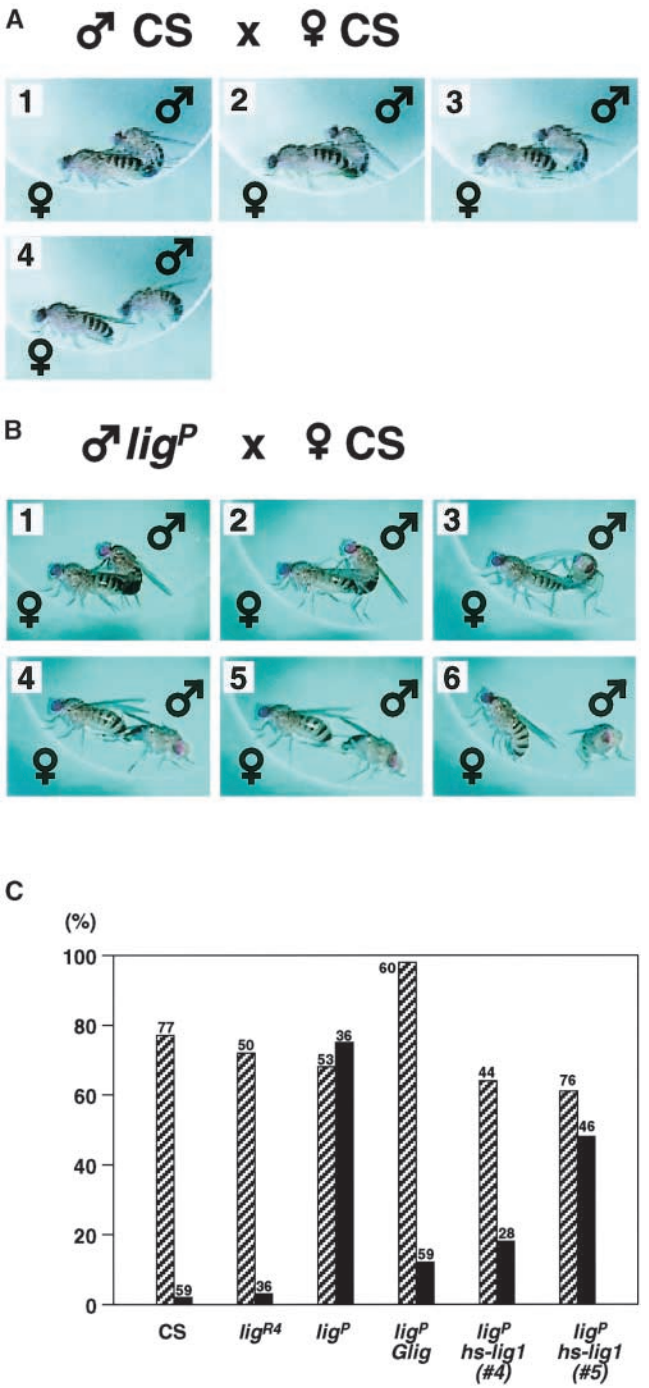


FIGURE 1.—The stuck phenotype of a *lig*^{P} homozygote. (A and B) Dismounting behavior of a CS wild-type male (A) and a *lig*^{P} mutant male (B). The images were selected from continuous videotape recordings. The females were all CS wild type. (A, 1) A wild-type male mounts a wild-type female. (A, 2) The female kicks the legs of the male with her legs just before the end of copulation. The male fly then releases the female's genitalia (A, 3) and dismounts the female (A, 4). The female copulating with the *lig*^{P} male (B, 1) also kicks the male's legs (B, 2). The *lig*^{P} male dismounts the female while clasping the female's genitalia with his genitalia (B, 3). The male and the female tug at each other, pulling in opposite directions for a while (B, 4) and finally succeed in separating (B, 5 and 6). (C) The mating success rate and the frequency of stuck copulation among pairs involving wild-type (CS), *lig*^{R4}, *lig*^{P}, *lig*^{P}; *Glig*, *lig*^{P}; *hs-lig1(#4)*, and *lig*^{P}; *hs-lig1(#5)* males. High levels of mating success in 25 min (hatched column) were attained in the six genotypes. The stuck phenotype (solid column) is represented as the percentage of stuck pairs among the copulating pairs. The majority of *lig*^{P} males exhibited the stuck phenotype. The incidence of the stuck phenotype was very low in CS wild-type or revertant line (*lig*^{R4}). The stuck phenotype in *lig*^{P} was rescued in the presence of *Glig*. Leaky expression of the type 1 *lig* transcript from the *hs-lig1(#4)* transgene was sufficient to suppress the stuck phenotype, whereas the *hs-lig1(#5)* transgene with little leaky expression did not rescue the phenotype. The number of flies observed to estimate the mating success rate and the frequency of the stuck phenotype is shown above each column.

or deficiency lines (*X4*, *X13*, and *X18*). Male escaper flies in lig^{P}/lig^{PP1} or $lig^{P}/X4$ were tested for mating behavior with the expectation that a lower dosage of the *lig* gene would result in more severe behavioral phenotypes. As shown in Figure 2A, only 26% of lig^{P}/lig^{PP1} males started copulation, whereas lig^{R4}/lig^{PP1} or lig^{P} homozygous males mated at normal wild-type rates. Also, mating success in $lig^{P}/X4$ was significantly decreased in comparison with that in $lig^{R4}/X4$ flies (Figure 2A). Similarly, $lig^{P}/X13$ and $lig^{P}/X18$ males showed lower mating success rates (0%, $n = 22$ and 6%, $n = 35$, respectively) than those of $lig^{R4}/X13$ (77%, $n = 36$) or $lig^{R4}/X18$ (87%, $n = 31$) males. Thus, a further reduction in *lig* activity resulted in an additional defect in the ability of the male to initiate copulation, hereafter referred to as the "noncopulating phenotype."

Although in the absence of females, lig^{P}/lig^{PP1} or $lig^{P}/X4$ males showed low locomotor activity (~35% of the activity of wild-type males; Figure 2B), they courted females as vigorously as wild-type males did; the SAPI (see MATERIALS AND METHODS) estimated for the $lig^{P}/X4$ males (0.27 ± 0.04, $n = 10$) was comparable to that for wild-type males (0.30 ± 0.03, $n = 10$; Mann-Whitney *U*-test, $P = 0.8$). Thus, the noncopulating phenotype was unlikely to be a result of a reduced level of courtship activity.

We hypothesized that the *lig* mutants disrupted one step in the stereotyped sequence of courtship and mating behavior and that this disruption caused the noncopulating phenotype. To address this, we compared the proportion of wild-type and lig^{P}/lig^{PP1} males who displayed respective steps of mating behavior (Figure 2C). Once they had started chasing (ch) a female, 90% of wild-type males reached the final step, copulation (co), after engaging in all the elementary behavioral steps, including tapping (t), singing (s), licking (l), and attempted copulation (a). The lig^{P}/lig^{PP1} males were as active as the wild-type males in performing the early steps of mating behavior; practically all the flies performed chasing, tapping, singing, and licking. After these steps, 73% of lig^{P}/lig^{PP1} males performed attempted copulation, which is defined as a contact of male genitalia with female genitalia. Nevertheless, only 18% of lig^{P}/lig^{PP1} males succeeded in copulation. This means that, among mutant males that attempted copulation, only 24% succeeded in copulation, while 100% of males could copulate after attempted copulation in the case of wild type. These results indicate that *lig* male flies with more severe mutant alleles are impaired in their ability to clasp female genitalia with their own genitalia.

The stuck and noncopulating phenotypes could be due to morphological abnormalities in the mutant male genitalia. To investigate this possibility, we compared the external genitalia of lig^{P} and $lig^{P}/X4$ males with those of wild-type and revertant (lig^{R4}) males by SEM (Figure 3, A–D). We found that the morphology of the genitalia of mutant males was indistinguishable from that of wild-type or lig^{R4} males. We also examined the internal genitalia in cuticle preparations and found no obvious abnormalities in mutant males (Figure 3, E–G). These results suggest that both phenotypes do not appear to result from morphological defects in the male genitalia.

The most severe phenotypes were observed in the null lig^{PP1} allele. lig^{PP1} homozygotes die during the early pupal stage. Because the mutant larvae form puparia without contraction, they remain long and slender even after pupariation, unlike the barrel-shaped wild-type pupae (Figure 3, H and I). This phenotype is similar to that found in certain mutants of the ecdysone receptor (BENDER *et al.* 1997) and ecdysone-inducible genes such as *E74* (FLETCHER *et al.* 1995). Histological analyses of late third instar larvae in the lig^{PP1} revealed that wing discs are flimsy and morphologically aberrant, as can be seen in Figure 3K (*cf.* Figure 3J for the wild type). On the other hand, no discernible abnormalities in cellular composition or structure were found in the lig^{PP1} larval CNS when stained with anti-Elav and anti-Repo antibodies (data not shown).

Cloning and molecular analyses of *lig* gene: The 34-kb genomic region adjacent to the *P*-element insertion site was cloned by plasmid rescue and subsequent chromosomal walking. The genomic fragments in this region were used as probes for screening adult and pupal cDNA libraries. A group of cDNA clones, which hybridized to each other, were isolated and analyzed in detail. The longest cDNA clone (4.6 kb) was used in Northern blot analysis. It recognized a 4.6-kb transcript whose abundance was decreased in the lig^{P} mutant compared with wild-type flies (Figure 5A), suggesting that these cDNAs correspond to the *lig* gene. Sequence analysis of these cDNA clones revealed two types of alternatively spliced *lig* transcripts of a similar size, designated type 1 and type 2. By comparing the cDNA sequences with the genomic sequence, we determined the exon/intron boundaries and the genomic organization of the *lig* gene (Figure 4). Type 1 and type 2 mRNAs have different 5′ untranslated regions since they are transcribed from different transcription initiation sites. These transcripts also differ from each other in their 3′ regions including their open reading frames. Type 1 cDNA potentially encodes a novel protein composed of 1332 amino acids (designated Lig-A), and type 2 cDNA encodes a 1375-amino-acid protein (designated Lig-B) similar to the type 1 product, but having a unique C terminus (Figure 8A).

To examine the expression of Lig-A- or Lig-B-encoding transcripts separately, we performed Northern blot analysis using probes hybridized to the Lig-A- or Lig-B-specific exon (Figure 5, B and C). The Lig-A-encoding transcript was expressed abundantly in both sexes and decreased in amount in lig^{P} homozygotes (Figure 5B). To confirm that the amount of the Lig-A protein is reduced in the mutant, a Lig-A-specific antibody (anti-Lig-A-C) that recognizes the C terminus of Lig-A was raised and used for Western blot analysis. As shown in

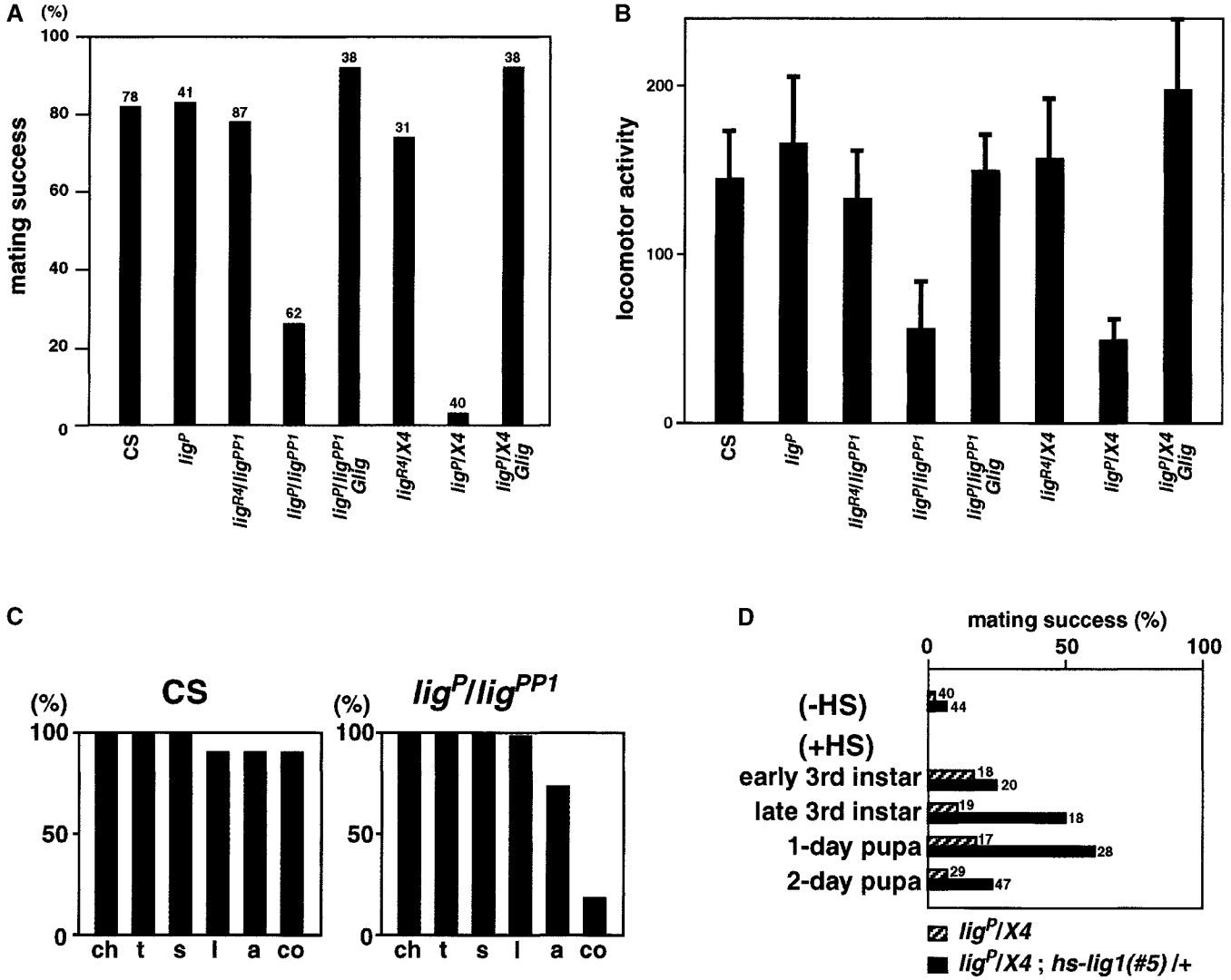


FIGURE 2.—The noncopulating phenotype and locomotor activity observed in *lig* mutant and transgenic flies. (A) The mating success rate in 60 min estimated for males of the various genotypes. The *trans*-heterozygous males of lig^{P} in combination with a null allele (lig^{PP1}) or a deficiency chromosome (*X4*) exhibited low mating success rates as compared with wild-type (CS), lig^{P} homozygote, lig^{R4}/lig^{PP1}, and $lig^{R4}/X4$ flies. The low mating success observed in lig^{P}/lig^{PP1} or $lig^{P}/X4$ was completely rescued by *Glig*. The number of flies observed is shown above each column. (B) The locomotor activity of male flies of the various genotypes. The values shown as locomotor activity represent the actual number of crossings of a line by the experimental fly (see MATERIALS AND METHODS). Error bars indicate plus one standard error of mean. The locomotor activity in lig^{P}/lig^{PP1} and $lig^{P}/X4$ was significantly reduced when compared with CS wild-type control males (Mann-Whitney *U*-test, $P < 0.01$). No significant difference can be found between CS wild type and lig^{P} homozygote ($P = 1.0$), lig^{R4}/lig^{PP1} ($P = 0.7$), lig^{P}/lig^{PP1}; *Glig*/+ ($P = 0.9$), $lig^{R4}/X4$ ($P = 0.8$), or $lig^{P}/X4$; *Glig*/+ ($P = 0.3$). Ten flies were tested for each genotype. (C) The percentage of male flies displaying different actions of mating behavior, *i.e.*, chasing (ch), tapping (t), singing (s), licking (l), attempted copulation (a), and copulation (co). When the tested male exhibited the respective action at least once in a 25-min observation period, the fly was considered as having performed that action. In preliminary observations, we noted that lig^{P}/lig^{PP1} males walked more slowly than wild-type female courtship partners. To examine mating behavior without the effect of reduced walking speed in the mutant, the males were paired with slow-walking females who had their midlegs removed. In this condition, lig^{P}/lig^{PP1} males showed low mating success rate (18% within 25 min, $n = 45$) comparable to that of lig^{P}/lig^{PP1} males paired with normal virgin females (13% within 25 min, $n = 62$); no significant difference can be found (χ^2 test, $0.4 < P < 0.5$). The majority of lig^{P}/lig^{PP1} males showed attempted copulation but failed to copulate. The number of flies tested was 10 (CS wild type) and 45 (lig^{P}/lig^{PP1}). (D) Rescuing effect of the *hs-lig1* transgene on mating success. The mating success rate in 60 min was estimated for $lig^{P}/X4$ (hatched column) and $lig^{P}/X4$; *hs-lig1(#5)*/+ (solid column) males. The flies were raised with (+HS) or without heat-shock treatment (−HS). The former flies were exposed to a single heat-shock treatment at the developmental stages indicated. Male $lig^{P}/X4$; *hs-lig1(#5)*/+ flies without heat-shock treatment showed a low mating success comparable to that of $lig^{P}/X4$. When the heat-shock treatment was given to $lig^{P}/X4$; *hs-lig1(#5)*/+ at the late third instar larval or 1-day pupal stage, more than one-half of the tested flies succeeded in copulation. Heat-shock treatments in the early third instar larval or 2-day pupal stage did not result in rescue. The same heat-shock treatment given to $lig^{P}/X4$ flies did not result in rescue. The number of flies observed is shown on the right of each column.

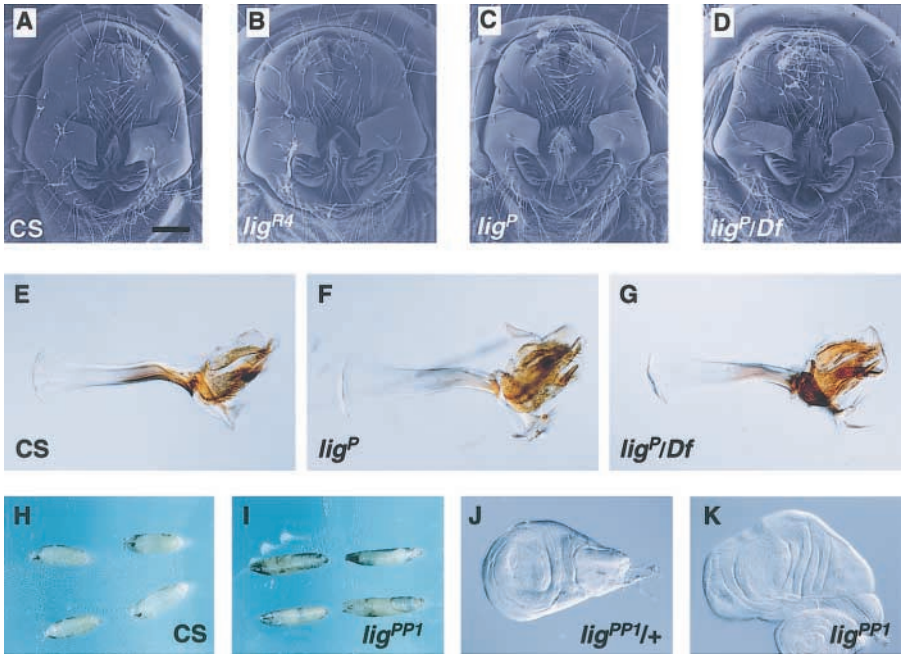


FIGURE 3.—The structural characteristics of *lig* mutants. (A–D) Scanning electron microscopic observations of the external genitalia of CS wild-type (A), lig^{R4} (B), lig^{P} (C), and lig^{P}/Df (D) male flies. No significant difference was found among the four genotypes. (Dorsal is at the top.) Bar, 60 μm. (E–G) Male genital cuticles of CS wild-type (E), lig^{P} (F), and lig^{P}/Df (G) flies. The structure of internal genitalia seems normal for all cases (lateral views: anterior to the left, dorsal to the top.) (H and I) Comparison of the shape of the pupae from CS wild-type (H) and a null allele lig^{PP1} (I) strains. lig^{PP1} larvae form elongated puparia. (J and K) The wing discs dissected from a wandering-stage larva of wild type (lig^{PP1}/+) (J) or lig^{PP1} (K). The lig^{PP1} mutant wing disc is distorted in shape. In K, the smaller disc attached to the wing disc is a haltere disc.

Figure 6A, the amount of the Lig-A protein (150 kD) was significantly reduced in lig^{P} homozygotes. In contrast, the amount of the Lig-B-encoding transcript, which was rare and specific to the male, was unchanged in the mutant compared with the wild type (Figure 5C). These results suggest that the decrease in the amount of the Lig-A, rather than in that of the Lig-B, protein was likely responsible for the lig^{P} phenotypes. We therefore focused our analyses on the Lig-A protein.

Analysis of the genomic structure of the lig^{P} mutant revealed that the *P* element was inserted into the first intron of the type 1 transcript, 1.5 kb upstream of the transcription initiation site of the type 2 transcript (Figure 4). It is conceivable that type 1 transcription is interrupted by the *P*-element insertion while type 2 transcription is unaffected. To examine if this was the case, we performed RT-PCR experiments in which the 5′ region of each transcript type was specifically amplified, using the primers shown in Figure 4. In RT-PCR using primers S-U1 and A-U, the product corresponding to the 5′ region of the type 1 transcript was found in the wild type, but not in the lig^{P} mutant (Figure 7B, lanes 1–4). RT-PCR with S-U2 and A-U amplified the DNA fragment corresponding to the 5′ region of the type 2 transcript in both the wild type and the mutant (Figure 7C, lanes 1–4). In addition, when the S-U1 primer was used in combination with the antisense primer specific to the *P*-element sequence (A-P), a 0.3-kb product was amplified from lig^{P} but not from the wild type (Figure 7A, lanes 1–4). The size of this PCR product coincided with that of the genomic region between the sequences complementary to the S-U1 and A-P primers in the lig^{P} chromosome (Figure 7A, lane 6). It is likely that aberrant transcripts containing the first exon of the type 1 transcript and *P*-element sequence are produced from the transcription initiation site of the type 1 transcript. This hypothesis is supported by the results of Northern blot analysis using a probe specific to the pUC9 sequence contained in the *P* element (*Bm*-Δ*w*); in this experiment, two aberrant transcripts, 7.5 and 4.2 kb in length, were detected in lig^{P} homozygotes but not in the wild type (Figure 5D). These results collectively suggested that the *P* insertion between the first and second exons led to the generation of the aberrant fusion transcripts in place of the functional type 1 transcript.

In spite of the complete loss of the type 1 transcript, the Lig-A protein was detected in the lig^{P} homozygote (Figure 6A). This suggests the existence of another type of Lig-A-encoding transcript, which should be transcribed from a different transcription initiation site. One candidate for such a transcription initiation site is found in the type 2 transcript. To investigate the 5′ region of Lig-A-encoding transcripts, Lig-A-specific first-strand cDNA was synthesized with a primer, Lig-A-3′, which anneals to the 3′ untranslated region of the Lig-A-encoding transcript (Figure 4), and was used for RT-PCR with the primers shown in Figure 4. Using primers S-D and A-D2, the 3′ region of the male-specific Lig-B-encoding transcript was amplified from oligo(dT)-primed cDNA (Figure 7E, lanes 1–4), but not from Lig-A-3′-primed cDNA (Figure 7E, lanes 7–10). On the other hand, the 3′ region of the Lig-A-encoding transcript was amplified from Lig-A-3′-primed cDNA (Figure 7D, lanes 7–10) as well as from oligo(dT)-primed cDNA (Figure 7D, lane 1–4). These results indicate that the Lig-A-3′-primed first-strand cDNA contains the Lig-A-encoding cDNA but not the Lig-B-encoding cDNA. The fragment corresponding to the 5′ region of the

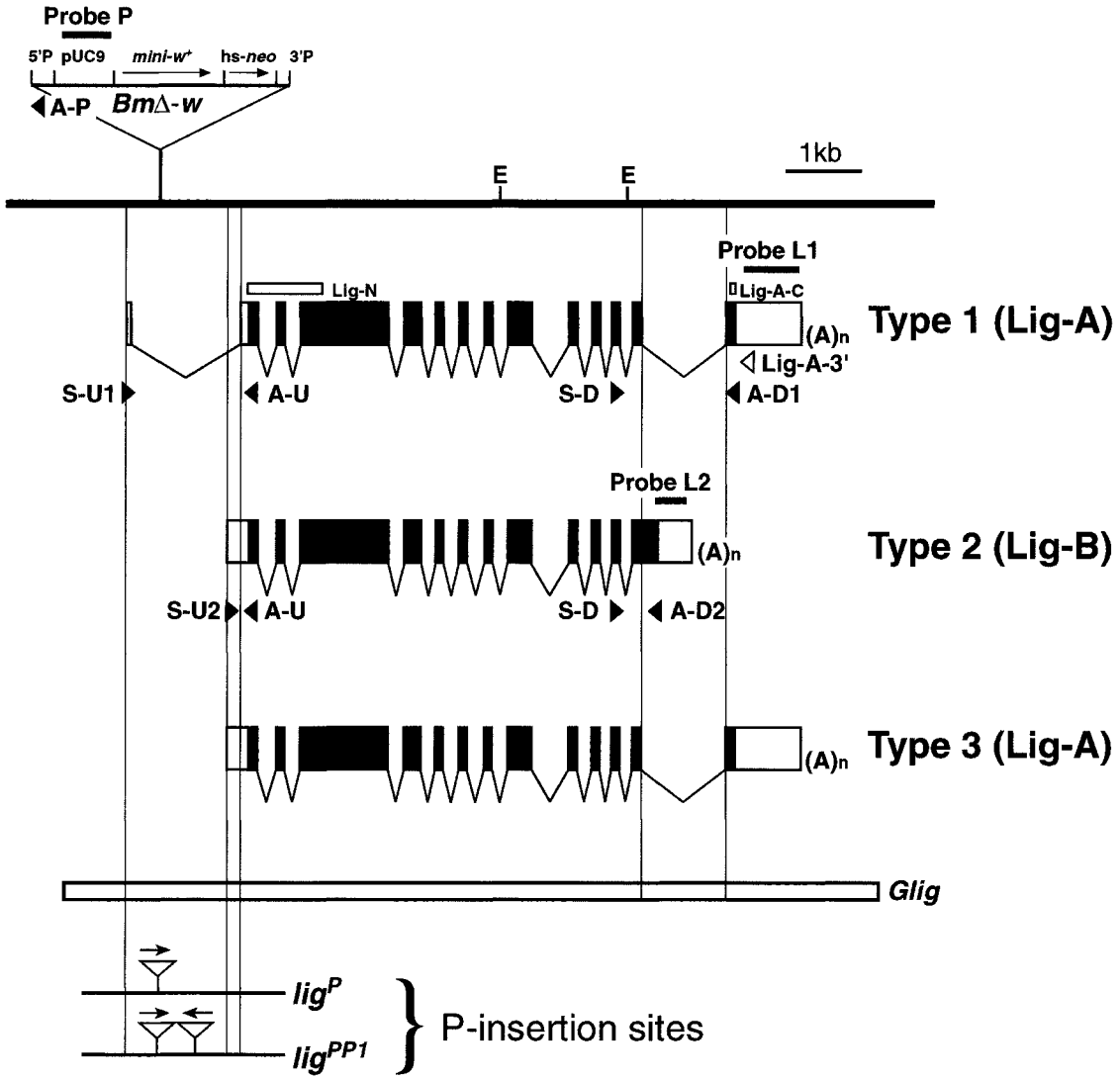


FIGURE 4.—The exon-intron organization of the *lig* gene. Solid and open boxes represent the open reading frames and untranslated regions, respectively. Solid and open bars indicate the regions used as probes for Northern blot analysis and as antigens for raising antibodies, respectively. An open triangle indicates the location of sequences used as a primer for synthesizing the Lig-A-specific first-strand cDNA. Solid triangles indicate the location of sequences used as primers in RT-PCR experiments. E refers to the *Eco*RI restriction site. The thick open bar below the exon-intron structure indicates the genomic fragment (*Glig*) used for rescue experiments. The *P*-insertion sites in lig^{P} and lig^{PP1} are shown at the bottom. Open triangles above the lines indicate the positions of *P*-element insertions, and the arrows above them indicate the direction of the *P* elements. The *P*-element insertion site in the lig^{P} mutant is located in the first intron of the type 1 transcript, 1.5 kb upstream of the transcription initiation site of the type 2 and type 3 transcripts. The null allele, lig^{PP1}, carries an additional *P* insertion in the opposite direction at 878 bp downstream of the original insertion.

type 1 transcript was amplified from Lig-A-3′-primed cDNA in the wild type but not in the *lig* mutant (Figure 7B, lanes 7–10). Using primers S-U1 and A-P, the PCR product corresponding to the aberrant transcript in lig^{P} was obtained from oligo(dT)-primed cDNA (Figure 7A, lanes 1–4) but not from Lig-A-3′-primed cDNA (Figure 7A, lanes 7–10). This result suggests that the aberrant transcript in the lig^{P} mutant does not encode the Lig-A protein. Importantly, the 5′ region of the type 2 transcript was amplified with S-U2 and A-U from Lig-A-3′-primed cDNA in both the wild type and the mutant (Figure 7C, lanes 7–10). Therefore, the Lig-A-encoding mRNA must be transcribed from the transcription initiation site of the type 2 transcript. This type of transcript detected in the aforementioned RT-PCR experiment is designated as the type 3 transcript (Figure 4). Thus, the Lig-A protein can be produced by both type 1 and type 3 transcripts in the wild type. The *P*-element insertion in the lig^{P} mutant inhibits expression of type 1 mRNA, leaving type 3 mRNA intact and reducing the level of the Lig-A protein.

The deduced amino acid sequences of Lig proteins do not contain any known protein motifs, nor any hydrophobic stretches such as a signalpeptide or a trans-

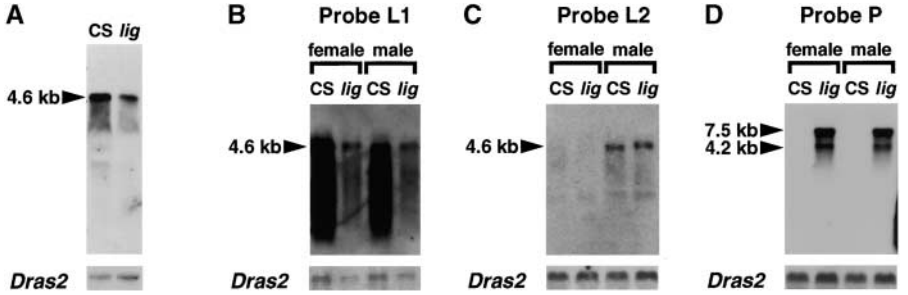


FIGURE 5.—Northern blot analysis. (A) Northern hybridization using the full-length type 1 cDNA as a probe. The probe detected a 4.6-kb signal, which is decreased in amount in the lig^P (*lig*) homozygous flies compared with wild-type flies (CS). The blot was reprobed with the *Dras2* gene (BISHOP and CORCES 1988) to normalize variations in mRNA loading. (B–D) Northern blot analysis using probes from the Lig-A-specific exon (Probe L1; B), the Lig-B-specific exon (Probe L2; C), and the pUC9 sequence in the *P* element (Probe P; D). See also Figure 4. The amount of the Lig-A-encoding transcript decreased in lig^P in both sexes compared with wild type (B), while the amount of the male-specific transcript encoding Lig-B was not changed (C). The mutant has two aberrant transcripts with sizes of 7.5 and 4.2 kb, which contained the *P*-element sequence (D). The blots were reprobed with the *Dras2* gene (BISHOP and CORCES 1988) to normalize variations in mRNA loading.

membrane domain. Our immunohistochemical analysis revealed cytoplasmic localization of the Lig proteins (see Figure 10, A–C). A homology search of the databases indicated that the Lig proteins exhibited no significant homology to any protein with known functions. However, human genes with unknown function, KIAA0144 and KIAA1491 (NAGASE *et al.* 1995, 2000), and several partial sequences from the mouse, human, and rat EST databases were homologous to the *lig* gene. To determine the entire sequences of the mouse *lig* homologs (*Mlig*) found in EST databases, we cloned *Mlig* cDNAs from the mouse embryonic cDNA library. Sequence analysis revealed two kinds of *Mlig* genes, *Mlig-1* and *Mlig-2*, and that the *Mlig-2* gene produces two types of transcripts, *Mlig-2a* and *Mlig-2b*, by alternative splicing (Figure 8B).

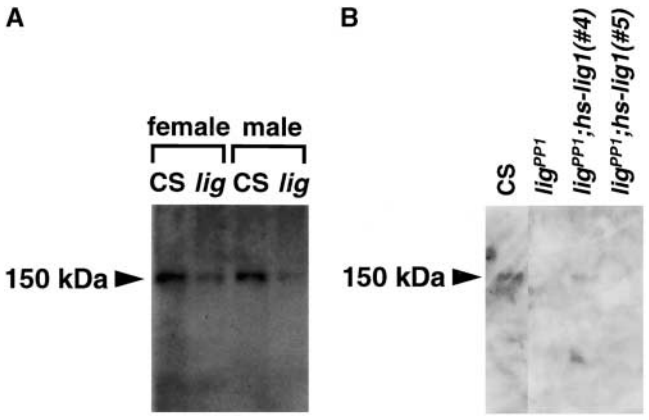


FIGURE 6.—Western blot analysis using an antibody against the Lig-A-specific C-terminal portion, Lig-A-C. (A) Lysate equivalent to one-tenth of male or female fly was loaded onto each lane. The antibody recognized a 150-kD band corresponding to the Lig-A protein. As compared with wild type (CS), the amount of the Lig-A protein was decreased in lig^P (*lig*) in both sexes. (B) Lysate equivalent to one-fourth of male larvae was loaded onto each lane. As compared with wild type (CS), the amount of the Lig-A protein was below the detectable level in lig^{PP1}. In lig^{PP1}; *hs-lig1(#4)*, leaky expression of Lig-A from the *hs-lig1(#4)* transgene was detected, whereas lig^{PP1}; *hs-lig1(#5)* showed little leaky expression.

The proteins encoded by the *Mlig-2a* and *Mlig-2b* transcripts, designated MLig-2A and MLig-2B, respectively, differed from each other in the C-terminal portion. The human *lig* homologs KIAA1491 and KIAA0144 encode proteins homologous to MLig-1 (the protein encoded by *Mlig-1*) and MLig-2B, respectively. As was the case with Drosophila Lig (DLig), none of these proteins contained known protein motifs or significant hydrophobic stretches.

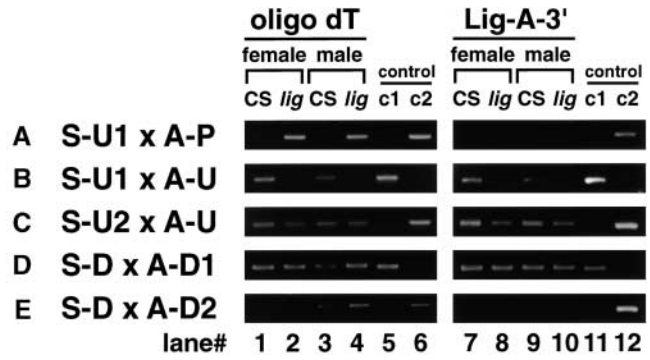
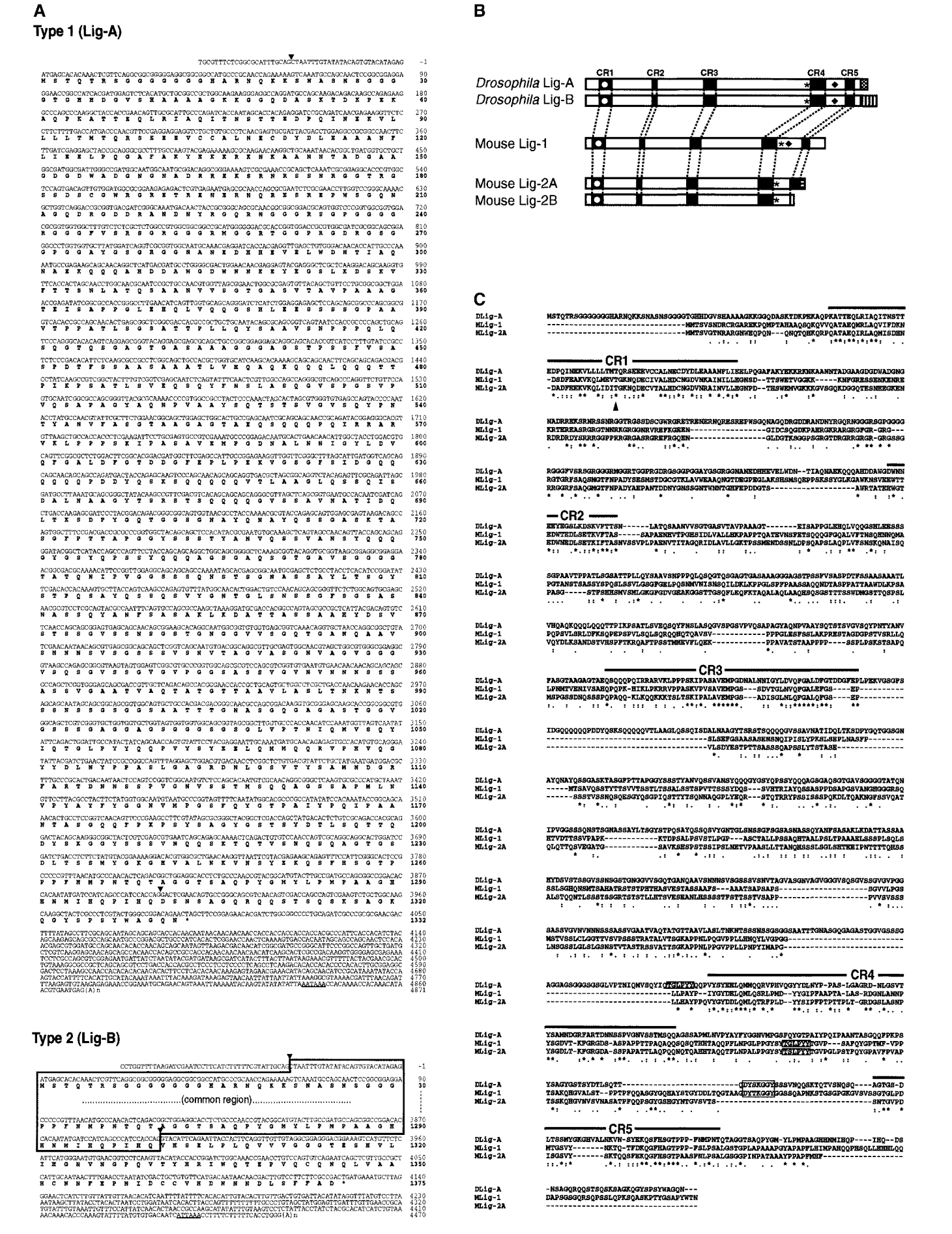


FIGURE 7.—RT-PCR using the primers shown in Figure 4. The first-strand cDNAs primed with oligo(dT) (lanes 1–4) or Lig-A-3′ (lanes 7–10) primers were synthesized from wild type (CS) or lig^P (*lig*) mRNA. (A) In RT-PCR with primers S-U1 and A-P, a 0.3-kb product was generated from the aberrant transcripts (shown in Figure 5D) in lig^P when the oligo(dT)-primed cDNA was used as a template (lanes 1–4). Genomic DNA of CS wild type and lig^P were used as controls c1 (lanes 5 and 11) and c2 (lanes 6 and 12), respectively. In the other four RT-PCR experiments (B–E), type 1 cDNA and type 2 cDNA were used as controls c1 (lanes 5 and 11) and c2 (lanes 6 and 12), respectively. (B) When primers S-U1 and A-U were used, the 0.4-kb product corresponding to the 5′ region of the type 1 transcript was obtained in the CS wild type, but not in the lig^P flies. (C) RT-PCR with S-U2 and A-U amplified the 0.4-kb product corresponding to the 5′ region of the type 2 and type 3 transcripts in both CS and lig^P. (D and E) When the sense primer S-D was used in combination with antisense primers A-D1 (D) or A-D2 (E), PCR fragments containing Lig-A- or Lig-B-specific sequences, respectively, were amplified in both CS and lig^P. In the RT-PCR using the cDNA primed with Lig-A-3′ as a template, the Lig-B-specific product was not amplified (E, lanes 7–10).


A
Type 1 (Lig-A)
Type 2 (Lig-B)
(common region)
B
Drosophila Lig-A
Drosophila Lig-B
Mouse Lig-1
Mouse Lig-2A
Mouse Lig-2B
CR1
CR2
CR3
CR4
CR5
C
DLig-A
MLig-1
MLig-2A

Comparisons of the amino acid sequences between mouse and Drosophila Lig proteins revealed five segments that are highly conserved (50–80% similarity) among them, *i.e.*, conserved regions 1–5 (CR1–5; Figure 8, B and C). Interestingly, a putative protein kinase C (PKC) phosphorylation site (S/T-X-R/K) exists in CR1 (Figure 8C, arrowhead). Because of alternative splicing, MLig-2B lacked CR5, whereas MLig-2A possessed this region. Furthermore, two conserved sequences, T(G/S)LPYY and DY(S/T) KGGY, were found in the Lig proteins (Figure 8, B and C). The first sequence was flanked by CR4 at the N-terminal side in the Drosophila Lig proteins and at the C-terminal side in the mouse Lig proteins. The second sequence was located between CR4 and CR5 in DLig and Mlig-1, but was absent in MLig-2A and MLig-2B.

Rescue of *lig* phenotypes: To establish the causal relationship between the mutant phenotypes and the putative *lig* gene, we performed rescue experiments using a 12-kb *Glig*, which encompassed the entire putative *lig* transcription unit but not any other genes (Figure 4). The *Glig* transgene successfully rescued all the *lig* phenotypes described above: stuck phenotype in lig^{P} male flies (Figure 1C), noncopulating phenotype in lig^{P}/lig^{PP1} and $lig^{P}/X4$ male flies (Figure 2A), low locomotor activity in lig^{P}/lig^{PP1} and $lig^{P}/X4$ adults (Figure 2B), and pupal lethality in lig^{PP1}. Hence, we conclude that the transcription unit included in the *Glig* is the *lig* gene.

Both the Lig-A and the Lig-B proteins could be generated from the *Glig* fragment. Molecular analyses suggested that the Lig-A, rather than the Lig-B, was likely to be responsible for the *lig* phenotypes. To investigate the specific effect of rescue with Lig-A, we constructed the fusion gene (*hs-lig1*), in which the expression of type 1 *lig* cDNA that encodes the Lig-A protein was controlled by the *hsp70* heat-shock promoter. Among six independent transgenic lines generated, two lines, *hs-lig1(#4)* and *hs-lig1(#5)*, were characterized in detail and used for rescue experiments. In Western blot analysis with the anti-Lig-A-C antibody (Figure 6B), a significant amount of Lig-A protein was detected in the lig^{PP1}; *hs-lig1(#4)* larvae raised at 25°. Since lig^{PP1} larvae expressed no detectable level of the protein, the Lig-A expression observed in lig^{PP1}; *hs-lig1(#4)* was likely due to the leaky expression from the *hs-lig1(#4)* transgene at 25°. On the other hand, the Lig-A protein was undetectable in the lig^{PP1}; *hs-lig1(#5)* larvae raised under similar conditions, suggesting that the *hs-lig1(#5)* transgene had little leaky expression of Lig-A at 25°. In an attempt at rescuing the stuck phenotype, the lig^{P} males carrying the *hs-lig1(#4)* or *hs-lig1(#5)* transgene were reared at 25° and then tested for their performance in mating behavior. As shown in Figure 1C, the stuck phenotype was suppressed in lig^{P}; *hs-lig1(#4)* males, in which the Lig-A protein was supplied from the *hs-lig1(#4)* transgene, while the nonleaky transgene, *hs-lig1(#5)*, did not suppress the phenotype. Thus, the stuck phenotype in the lig^{P} males was rescued by the expression of Lig-A.

To confirm that Lig-A could rescue the noncopulating phenotype as well, and to determine when the Lig-A protein is required to rescue this phenotype, expression of type 1 *lig* cDNA was induced in the $lig^{P}/X4$ males at different stages of development using the *hs-lig1(#5)* transgene. As in the case of $lig^{P}/X4$ males without the *hs-lig1(#5)*, $lig^{P}/X4$; *hs-lig1(#5)*/+ males reared at 25° rarely copulated (Figure 2D). When $lig^{P}/X4$; *hs-lig1(#5)*/+ males were administered a single heat-shock treatment at 37° for 1 hr during the late third instar larval or 1-day pupal stage, they exhibited a higher mating success score (50 and 61%, respectively) than that of $lig^{P}/X4$ males, while the same heat-shock treatment at earlier or later stages gave little rescue (Figure 2D). This rescue of the noncopulating phenotype is due to the *hs-lig1(#5)* transgene, because $lig^{P}/X4$ males given the same heat-shock treatment exhibited no such increase in mating success. Similar rescue was observed when the *hs-lig1(#4)* line was used instead of *hs-lig1(#5)* (data not shown). Thus, the supply of type 1 *lig* mRNA in the late third instar larvae or early pupae is able to rescue the noncopulating phenotype, suggesting that the Lig-A protein is required during this developmental period for normal copulation at the adult stage.

Expression pattern of *lig* product: Developmental expression of the *lig* transcripts in wild-type animals was analyzed by Northern blotting using the full-length type 1 cDNA as a probe, which can detect all types of *lig* mRNA. As shown in Figure 9, a high level of *lig* was found throughout development.

To investigate spatial and temporal distribution of *lig* mRNA during embryonic development, we performed whole-mount *in situ* hybridization with a probe hybridizing to the sequence common to all types of transcripts. In early cleavage embryos, maternal *lig* mRNA was distributed uniformly (Figure 9A). Zygotic expression of

FIGURE 8.—Primary structures of the Lig proteins and their mouse homologs. (A) The DNA sequence and conceptual open reading frame of type 1 and type 2 *lig* cDNA clones. Solid triangles indicate the beginning and the end of the region common to both type 1 and type 2. The common region is boxed in the type 2 sequence. The putative polyadenylation signals are underlined. (B) Schematic alignment of Drosophila Lig proteins with their mouse homologs. Five conserved regions (CR1–5) are shown as solid boxes. The putative PKC phosphorylation sites in CR1 are indicated by open circles. The conserved sequences, T(G/S)LPYY and DY(S/T)KGGY, are marked with asterisks and solid diamonds, respectively. Mouse Lig-2B lacks CR5. (C) Alignment of the amino acid sequences of Drosophila and mouse Lig proteins. Identical residues are marked with asterisks. Conservative substitutions are marked with dots. The putative PKC phosphorylation sites in CR1 are indicated by an arrowhead. The conserved sequences, T(G/S)LPYY and DY(S/T)KGGY, are boxed.

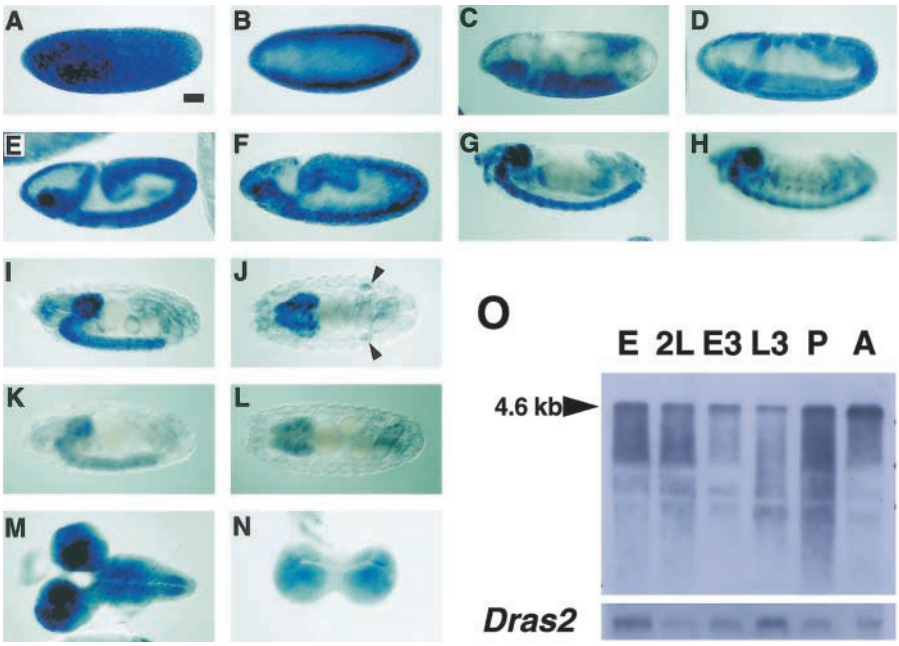


FIGURE 9.—The spatial and temporal expression of *lig* mRNA. (A–L) Expression pattern in CS wild-type (A–J) and lig^{P} (K and L) embryos revealed by *in situ* hybridization with a probe hybridizing to the sequence common to all types of transcripts. (A) Stage 4; (B) stage 5; (C) stage 6; (D) stage 8; (E) stage 9; and (F) stage 11. At stage 13, the *lig* transcript is concentrated in the CNS (G) and the PNS (H, the same embryo as illustrated in G in a different focal plane). At stage 16 (I and J), *lig* expression was detected in the gonads (arrowheads) in addition to the CNS. (K and L) mRNA expression was decreased in the lig^{P} embryo (stage 16). Lateral views in A–I and K show anterior to the left and dorsal to the top; dorsal views are shown in J and L. Bar, 80 μm. (M and N) *lig* expression in the CNS (M) and the male genital disc (N) in wild type during the late third instar larval stage. The *lig* gene is expressed in the other imaginal discs (eye-antenna, wing, halter, leg, and labial discs) as well as in the testes and ovaries (data not shown). In these tissues, *lig* is expressed ubiquitously. Bar: (M) 100 μm; (N) 50 μm. No signal was detected when the sense probe was used in *in situ* hybridization of embryos and larvae (not shown). (O) Developmental Northern blot analysis using a probe that detects all types of *lig* mRNA. High-level expression of the *lig* transcript was detected during various stages: embryos (E), the second instar larvae (2L), the early and late third instar larvae (E3 and L3, respectively), pupae (P), and adults (A). The blot was also probed with the *Dras2* gene (BISHOP and CORCES 1988) to assess the amount of RNA loaded in each lane.

lig started at the cellular blastoderm stage (Figure 9B). Shortly after gastrulation, *lig* mRNA was present in the germ band at relatively low levels (Figure 9, C and D); expression increased markedly from stage 9, at which neuroblasts delaminate from the ectoderm (Figure 9E). At stage 11, *lig* expression became restricted mainly to neuroblasts (Figure 9F). After germ-band shortening, expression was predominantly observed in the CNS, including the brain and the ventral nerve cord (Figure 9G), as well as in the peripheral nervous system (PNS; Figure 9H). During later stages of development, *lig* expression was detected not only in the nervous system (Figure 9I) but also in the gonads (Figure 9J). In the lig^{P} homozygous embryos, *lig* expression was uniformly diminished with negligible changes in spatial and temporal patterns (Figure 9, K and L).

The results of the rescue experiment suggest that *lig* gene function is required in the late third instar larval to early pupal stage for normal copulation behavior of the adult male (Figure 2D). For a survey of tissues expressing the *lig* gene at this stage, whole-mount *in situ* hybridization was performed on "inside-out" preparations of late third instar larvae with the same probe as that used for embryos. The *lig* gene was expressed in the CNS (Figure 9M), gonads (ovaries and testes; data not shown), and imaginal discs, including genital (Figure 9N), eye-antennal, leg, wing, and haltere discs (data not shown). *lig* mRNA is expressed ubiquitously in these tissues.

The Lig protein localization was visualized by immunostaining of the late third instar larval CNS (Figure 10) with an anti-Lig antibody that recognizes the N terminus common to both Lig-A and Lig-B (anti-Lig-N; Figure 4). Many neuronal and glial cells in brain and ventral ganglion were immunoreactive to the anti-Lig-N antibody. In these cells, the staining was found predominantly in the cytoplasm, but not in the nuclei (Figure 10, A–C). The axons and dendrites appeared unstained. Also in the imaginal discs, the Lig protein was localized in the cytoplasm (data not shown). No staining was observed in the lig^{PP1} homozygous larvae (Figure 10, D–F), demonstrating that the antibody specifically detected the *lig* gene products. Similar results were obtained when the Lig-A-specific antibody (anti-Lig-A-C) was used (data not shown).

DISCUSSION

In the present work, we demonstrated that the reduction in gene activity at a single locus results in defects in adult sexual behavior; mutations in the *lig* locus interfere with initiation and termination of copulation.

Mutations that affect initiation of copulation have previously been reported. A classic example is $fruitless^{1}$ (fru^{1}), males of which court females vigorously and attempt to copulate without success. HALL (1994) considered that insufficient bending of the abdomen in fru^{1} males is a possible cause for the lack of success in copulation (see also GAILEY and HALL 1989; GAILEY *et al.* 1991; TAYLOR *et al.* 1994). Since the *fru* gene encodes a putative tran-

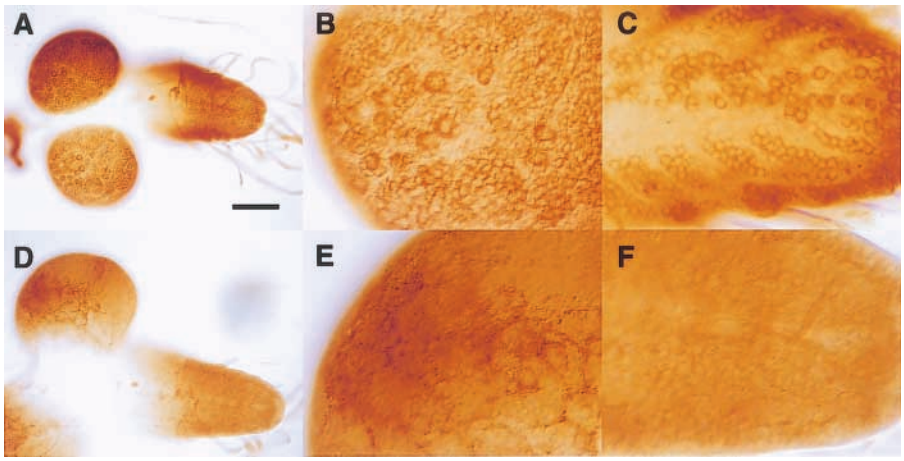


FIGURE 10.—Expression of the Lig protein in the late third instar larval CNS. Immunohistochemical detection of the Lig proteins in CS wild type (A–C) and lig^{PP1} CNS (D–F) with the anti-Lig-N antibody, which recognizes the N terminus common to both Lig-A and Lig-B. Higher magnification views of the brain (B and E) and the ventral ganglion (C and F) are shown. Many neurons and glial cells are stained in the brain and the ventral ganglion of CS wild-type larvae, and the immunopositive material is concentrated in the cytoplasm of cell bodies (A–C). No staining was observed in the lig^{PP1} CNS (D–F), demonstrating that the antibody was specific to the Lig proteins. Bar: (A and D) 100 μm; (B, C, E, and F) 25 μm.

scription factor believed to play a role in the sex determination of certain neurons (ITO *et al.* 1996; RYNER *et al.* 1996; HEINRICHS *et al.* 1998), the inability of fru^1 males to copulate is likely due to incorrect sex determination of these cells (YAMAMOTO *et al.* 1996; VILLELA *et al.* 1997; *cf.* FERVEUR *et al.* 1995). Males with more severe *fru* mutations do not exhibit attempted copulation with females (YAMAMOTO *et al.* 1998).

Another example of mutants with a noncopulating phenotype is *dissatisfaction* (*dsf*). The *dsf* mutant prevents males from bending their abdomens sufficiently to copulate (FINLEY *et al.* 1997). *dsf* has been postulated to play a role in the neuronal sex determination mechanism, although *dsf* and *fru* appear to function in different branches of the cascade (FINLEY *et al.* 1998; YAMAMOTO *et al.* 1998). The *dsf* mutant also affects female-specific behavior, *i.e.*, oviposition. Both the oviposition defect in females and the noncopulating phenotype in males have been shown to result from aberrant innervation to muscles involved in the respective behavior (FINLEY *et al.* 1997, 1998).

Unlike fru^1 and *dsf* mutants, the lig^P/lig^{PP1} males bend their abdomen as much as wild-type males do and can make genital-genital contact with a female (Figure 2C). We suggest that the lig^P/lig^{PP1} blocks a behavioral step subsequent to abdominal bending, *e.g.*, clasping of female genitalia. This notion is consistent with the fact that males with a weaker allele (lig^P) are able to copulate but show the stuck phenotype failing to release the female's genitalia.

This latter phenotype has been reported in the *sk* mutant (HALL *et al.* 1980). To characterize the *sk* phenotype, HALL *et al.* (1980) measured changes in the strength of "genital coupling" over time after the initiation of copulation by counting the number of copulating pairs that released genital connection in response to vortex stimuli applied at various intervals. They found that, in the case of wild-type flies, a larger proportion of pairs tended to separate in response to the stimuli over a period of time after copulation initiation. In other words, the strength of genital coupling declined with time after the start of copulation in wild-type males. In contrast, a larger number of *sk* pairs tolerate vortex stimulation, maintaining genital connection following the initiation of copulation. Thus, in the case of the *sk* mutant, the strength of genital coupling increases toward the end of copulation. As a result, the *sk* males remain stuck at the end of copulation (HALL *et al.* 1980). This observation, together with the finding that the genital structure is normal, led HALL *et al.* (1980) to suggest that the *sk* mutation affects motor control of genital movements rather than the genital structure *per se*.

In our screening for mating behavior mutants, we isolated a few mutants whose behavioral anomalies seemed to be ascribed to aberrant male genital structures. One such example is the *fickle* mutant, males of which show shortened copulatory duration (BABA *et al.* 1999). This behavioral phenotype appeared to be associated with duplication of the apodeme, a structure that supports the penis. Unlike the case of *fickle*, we were unable to find any obvious deficits in the genital structure of the *lig* mutant males (Figure 3, A–G). In addition, *lig* gene products are expressed in neuronal and glial cells in the CNS (Figure 10, A–C), but not found in muscle cells and/or myoblasts, including those associated with genitalia. Taking these observations into account, we consider it likely that the *lig* mutations affected the neural mechanism controlling genital movements. However, the *lig* gene is expressed in imaginal discs, including the male genital disc (Figure 9N), and the lig^{PP1} mutation interferes with development of wing discs (Figure 3K). Therefore, we cannot exclude the possibility that the lack of *lig* activity generates a very subtle defect in the male genital structure, leading to the observed deficits in mating behavior.

The late third instar larval to the early pupal stage was the critical period for rescue by the expression of *lig* cDNA of the noncopulating phenotype in the *lig*

hemizygotes (Figure 2D). This observation indicates that the failure of the hypomorphic *lig* mutants to copulate resulted from developmental defects during the late larval to early pupal period. This developmental stage coincided with the lethal phase of the null allele, lig^{PP1} (Figure 3I). Thus, the *lig* gene products are likely essential for normal development at this stage. *lig* expression at this stage is confined to the imaginal discs, the CNS, and the gonads, which undergo dynamic growth and/or reconstruction during the larval to pupal molt. The *lig* gene products may be required for processes associated with metamorphosis. In fact, the elongated shape of lig^{PP1} dead pupae was reminiscent of that reported for mutants in ecdysone-related genes (Fletcher *et al.* 1995; Bender *et al.* 1997). The distortion of the wing-disc structure observed in lig^{PP1} mature larvae also suggests a role for *lig* in metamorphosis. However, the gross structure of the brain-ventral ganglion complex in the late third instar larvae appeared normal (Figure 10D) although the lig^{PP1} CNS did not contain Lig proteins at a detectable level. No obvious cellular changes such as degeneration or ectopic proliferation were observed in the lig^{PP1} CNS. Fine anatomical analysis with single cell resolution is required to determine whether the *lig* mutations affect structural and/or functional aspects of the nervous system.

Our molecular analyses revealed that the *lig* locus generates two types of 150-kD proteins, Lig-A and Lig-B, each of which is encoded by type 1 and type 3 or type 2 transcripts, respectively. We further demonstrated that only the type 1 transcript is disrupted by the *P*-element insertion in lig^{P}. The finding that ubiquitous expression of type 1 cDNA in lig^{P} (Figure 1C) or $lig^{P}/X4$ males (Figure 2D) rescued the copulatory abnormalities supports the assumption that a reduced level of the Lig-A protein in mutants is responsible for the behavioral phenotypes. However, we have not determined whether overexpression of the type 2 cDNA, which encodes the Lig-B protein, can compensate for the reduction in the amount of Lig-A protein in the *lig* mutants so as to restore normal copulatory behavior. If this happens to be the case, the Lig-A and Lig-B proteins would be considered to play similar roles.

By comparing the primary structures of the Lig and related proteins, five regions conserved across phyla were identified. None of these corresponded to protein domains with known functions. However, we found an amino acid sequence that matched the consensus sequence for phosphorylation by PKC in one conserved region. It would thus be of interest to examine whether the *lig* behavioral phenotypes can be modulated by mutations that change the activity of PKC.

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LITERATURE CITED

Baba, K., A. Takeshita, K. Majima, R. Ueda, S. Kondo *et al.*, 1999 The Drosophila Bruton's tyrosine kinase (Btk) homolog is required for adult survival and male genital formation. Mol. Cell. Biol. **19:** 4405–4413.

Bender, M., F. B. Imam, W. S. Talbot, B. Ganetzky and D. S. Hogness, 1997 Drosophila ecdysone receptor mutations reveal functional differences among receptor isoforms. Cell **91:** 777–788.

Bishop, J. G., III, and V. G. Corces, 1988 Expression of an activated *ras* gene causes developmental abnormalities in transgenic *Drosophila melanogaster*. Genes Dev. **5:** 567–577.

Carlsson, J., W. Drevin and R. Axen, 1978 Protein thiolation and reversible protein-protein conjugation *N*-succinimidyl 3-(2-pyridyldithio)propionate: a new heterobifunctional reagent. Biochem. J. **173:** 723–737.

Engels, W. R., C. R. Preston, P. Thompson and W. B. Eggleston, 1986 *In situ* hybridization to Drosophila salivary chromosomes with biotinylated DNA probes and alkaline phosphatase. Focus **8:** 6–8.

Ferveur, J.-F., K. F. Stortkuhl, R. F. Stocker and R. J. Greenspan, 1995 Genetic feminization of brain structures and changed sexual orientation in male Drosophila. Science **267:** 902–905.

Finley, K. D., B. J. Taylor, M. Milstein and M. McKeown, 1997 *dissatisfaction*, a gene involved in sex-specific behavior and neural development of *Drosophila melanogaster*. Proc. Natl. Acad. Sci. USA **94:** 913–918.

Finley, K. D., P. T. Edeen, M. Foss, E. Gross, N. Ghbeish *et al.*, 1998 *dissatisfaction* encodes a Tailless-like nuclear receptor expressed in a subset of CNS neurons controlling Drosophila sexual behavior. Neuron **21:** 1363–1374.

Fletcher, J. C., K. C. Burtis, D. S. Hogness and C. S. Thummel, 1995 The Drosophila *E74* gene is required for metamorphosis and plays a role in the polytene chromosome puffing response to ecdysone. Development **121:** 1455–1465.

Gailey, D. A., and J. C. Hall, 1989 Behavior and cytogenetics of *fruitless* in *Drosophila melanogaster*: different courtship defects caused by separate, closely linked lesions. Genetics **121:** 773–785.

Gailey, D. A., B. J. Taylor and J. C. Hall, 1991 Elements of the *fruitless* locus regulate development of the muscle of Lawrence, a male-specific structure in the abdomen of *Drosophila melanogaster* adults. Development **113:** 879–890.

Greenspan, R. J., 1995 Understanding the genetic construction of behavior. Sci. Am. **272:** 72–78.

Hall, J. C., 1985 Genetic analysis of behavior in insects, pp. 287–373 in *Comprehensive Insect Physiology, Biochemistry and Pharmacology*, edited by G. A. Kerkut and L. I. Gilbert. Pergamon Press, Oxford.

Hall, J. C., 1994 The mating of a fly. Science **264:** 1702–1714.

Hall, J. C., R. W. Siegel, L. Tomkins and C. P. Kyriacou, 1980 Neurogenetics of courtship in Drosophila. Stadler Genet. Symp. **12:** 43–82.

Harlow, E., and D. Lane, 1988 *Antibodies: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Hawkes, R., E. Niday and J. Gordon, 1982 A dot-immunobinding assay for monoclonal and other anitbodies. Anal. Biochem. **119:** 142–147.

Heinrichs, V., L. C. Ryner and B. S. Baker, 1998 Regulation of sex-specific selection of *fruitless* 5′ splice sites by *transformer* and *transformer-2*. Mol. Cell. Biol. **18:** 450–458.

Ito, H., K. Fujitani, K. Usui, K. Shimizu-Nishikawa, S. Tanaka *et al.*, 1996 Sexual orientation in Drosophila is altered by the *satori* mutation in the sex-determination gene *fruitless* that encodes a zinc finger protein with a BTB domain. Proc. Natl. Acad. Sci. USA **93:** 9687–9692.

Juni, N., T. Awasaki, K. Yoshida and S. H. Hori, 1996 The *Om(1E)* mutation in *Drosophila ananassae* causes compound eye overgrowth due to *tom* retrotransposon-driven overexpression of a novel gene. Genetics **143:** 1257–1270.

Lehmann, R., and D. Tautz, 1994 *In situ* hybridization to RNA, pp. 575–598 in *Drosophila melanogaster: Practical Uses in Cell and*

Molecular Biology, edited by L. S. B. Goldstein and E. A. Fryberg. Academic Press, San Diego.

Nagase, T., N. Seki, A. Tanaka, K. Ishikawa and N. Nomura, 1995 Prediction of the coding sequences of unidentified human genes. IV. The coding sequences of 40 new genes (KIAA0121-KIAA0160) deduced by analysis of cDNA clones from human cell line KG-1. DNA Res. **2:** 167–174.

Nagase, T., R. Kikuno, K. Ishikawa, M. Hirosawa and O. O'Hara, 2000 Prediction of the coding sequences of unidentified human genes. XVII. The complete sequences of 100 new cDNA clones from brain which code for large proteins *in vitro*. DNA Res. **7:** 143–150.

Nakano, Y., K. Fujitani, J. Kurihara, J. Ragan, K. Usui-Aoki *et al.*, 2001 Mutations in the novel membrane protein Spinster interfere with programmed cell death and cause neural degeneration in *Drosophila melanogaster*. Mol. Cell. Biol. **21:** 3775–3788.

Rubin, G. M., and A. C. Spradling, 1982 Genetic transformation of Drosophila with transposable element vectors. Science **218:** 348–353.

Ryner, L. C., S. F. Goodwin, D. H. Castrillon, A. Anand, A. Villella *et al.*, 1996 Control of male sexual behavior and sexual orientation in Drosophila by the *fruitless* gene. Cell **87:** 1079–1089.

Steller, H., and V. Pirrotta, 1986 *P* transposons controlled by the heat shock promoter. Mol. Cell. Biol. **6:** 1640–1649.

Suzuki, K., N. Juni and D. Yamamoto, 1997 Enhanced mate refusal in female Drosophila induced by a mutation in the *spinster* locus. Appl. Entomol. Zool. **32:** 235–243.

Taylor, B. J., A. Villella, L. C. Ryner, B. S. Baker and J. C. Hall, 1994 Behavioral and neurobiological implications of sex-determining factors in Drosophila. Dev. Genet. **15:** 275–296.

Tower, J., G. H. Karpen, N. Craig and A. C. Spradling, 1993 Preferential transposition of Drosophila *P* elements to nearby chromosomal sites. Genetics **133:** 347–359.

Villella, A., D. A. Gailey, B. Berwald, S. Oshima, P. T. Barnes *et al.*, 1997 Extended reproductive roles of the *fruitless* gene in *Drosophila melanogaster* revealed by behavioral analysis of new *fru* mutants. Genetics **147:** 1107–1130.

von Schilcher, F., 1976 The role of auditory stimuli in the courtship of *Drosophila melanogaster*. Anim. Behav. **24:** 18–26.

Yamamoto, D., and Y. Nakano, 1998 Genes for sexual behavior. Biochem. Biophys. Res. Commun. **246:** 1–6.

Yamamoto, D., H. Ito and K. Fujitani, 1996 Genetic dissection of sexual orientation: behavioral, cellular, and molecular approaches in *Drosophila melanogaster*. Neurosci. Res. **26:** 95–107.

Yamamoto, D., J.-M. Jallon and A. Komatsu, 1997 Genetic dissection of sexual behavior in *Drosophila melanogaster*. Annu. Rev. Entomol. **42:** 551–585.

Yamamoto, D., K. Fujitani, K. Usui, H. Ito and Y. Nakano, 1998 From behavior to development: genes for sexual behavior define the neuronal sexual switch in Drosophila. Mech. Dev. **73:** 135–146.

Yokokura, T., R. Ueda and D. Yamamoto, 1995 Phenotypic and molecular characterization of *croaker*, a new mating behavior mutant of *Drosophila melanogaster*. Jpn. J. Genet. **70:** 103–117.

Zhang, P., and A. C. Spradling, 1993 Efficient and dispersed local *P* element transposition from Drosophila females. Genetics **133:** 361–373.

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