logjam Encodes a Predicted EMP24/GP25 Protein That Is Required for Drosophila Oviposition Behavior

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ABSTRACT

A newly characterized Drosophila melanogaster gene, logjam (loj), functions in female reproduction by modulating oviposition behavior. The locus encodes at least six overlapping transcripts with unique 5’ ends. P-element mutants that express very low levels of loj transcripts are unable to oviposit mature eggs. This phenotype can be rescued by the introduction of a transgene expressing the most abundant loj transcript. As for many genes that specify behavioral outputs, loj is present in the adult central nervous system (CNS). Interestingly, it is also observed in vitellogenic egg chambers, suggesting that there may be multiple functions for this gene in egg-laying behavior. loj encodes a predicted protein with homology to the EMP24/GP25 transmembrane components of cytoplasmic vesicles and likely functions in intracellular trafficking.

Using the Drosophila melanogaster model system we have a unique opportunity to clarify our understanding of the genetic and molecular underpinnings of behaviors. D. melanogaster are complex animals with ∼13,500 predicted genes (Adams et al. 2000), yet they are amenable to a genetic dissection of the molecular pathways that regulate biological processes. Drosophila exhibit a wonderfully rich repertoire of interesting behaviors such as courting and responding to potential mates, olfactory learning, and foraging for food. All of these behaviors require integration of multiple sensory inputs and generation of various motor outputs and are recognized to be under genetic control (reviewed in Hall 1994; Dubnau and Tully 1998; Sokolowski 2001).

The sexually dimorphic behaviors associated with Drosophila reproductive success are clearly governed by the actions of multiple genes, some of which function as dedicated components of the somatic sex-determination hierarchy, beginning with the initial setting of Sex-lethal (Sxl) activity (reviewed in Burtis 1993; McKeown 1994; Cline and Meyer 1996). Two genes, doublesex (dss) and fruitless (fru), have been shown to be regulated by the upstream components of this signaling cascade (Baker and Ridge 1980; McKeown et al. 1988; Nagoshi et al. 1988; Ito et al. 1996; Ryner et al. 1996). A third gene, dissatisfaction (dsf), was shown to interact genetically with this pathway but is not regulated directly by known upstream members of the hierarchy (Finley et al. 1997, 1998). All three genes encode DNA-binding proteins (Burtis et al. 1991; Ito et al. 1996; Ryner et al. 1996; Finley et al. 1998), but the identity of genes necessary for reproductive behaviors that function directly downstream of them is not known.

The pathways for regulating female and male sexual behaviors diverge in the requirements for these regulators of downstream functions. dsf and dss, but not fru, control female sexual behaviors (Ito et al. 1996; Ryner et al. 1996; Finley et al. 1997; Waterbury et al. 1999). dsf mutant females have defective uterine muscle innervation, a phenotype that is correlated with an absence of oviposition behavior in these females (Finley et al. 1997). The major function of dss is to control sex-specific morphological differentiation in males and females (Hildreth 1965; Keisman and Baker 2001; Keisman et al. 2001; Ahmad and Baker 2002). Misexpression of DSX in males transforms them to phenotypic females, which allows them to mate with other males (Waterbury et al. 1999).

All three genes affect male behavior. fru is the major regulator of male sexual behavior but has no demonstrable role in female reproduction (Ito et al. 1996; Ryner et al. 1996; Villella et al. 1997). dsf functions in male courtship object choice (Finley et al. 1997, 1998), while DSX is needed for male sine-song production and general robustness of male courtship (Villella and Hall 1996).

The major reproductive behaviors of females are (1) receptivity to courtship followed by copulation and (2) oviposition of eggs, a behavior that is independent of the act of mating and is under voluntary control of the female. Virgin females are refractive to the mating advances of males for the first day of their lives (Manning 1966, 1967). During this time the ovaries mature (reviewed in Mahowald and Kambsellis 1980; Spradling 1993), cuticular pheromonal profiles change to

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make the female more enticing to the male (reviewed in Jallon 1984; Tompkins 1984, 1998), and hormonal fluxes foster the development of female sexual receptivity (Manning 1966). Unmated females retain mature eggs but eventually lay unfertilized eggs beginning at approximately the fifth day after eclosion (Mahowald and Kambsells 1980).

Once the female has mated, her behavior alters as she rebuffs further mating advances. Proteins and other compounds in the male ejaculate affect the female's rate of ovulation, oviposition, and her receptivity to male mating overtures (Chen et al. 1988; Monsma and W olfner 1988; Aigaki et al. 1991; Kubli 1992; Herndon and Wolfner 1995; Wolfner 1997, 2002; Wolfner et al. 1997; Heifetz et al. 2000, 2001; Chapman et al. 2001; Fleischmann et al. 2001; Saudan et al. 2002). Mated females have been observed to retain eggs if they do not find oviposition conditions to be ideal (Grossfield 1978).

Many identified genes function in one or both of these behaviors, but their relationship to the sex-determination hierarchy is unclear. Some of these genes may function downstream in the sex-determination regulatory cascade, while others may function in convergent pathways that interact with sex-determination genes.

dsf regulates both receptivity and oviposition (Finley et al. 1997). Other loci involved in female reproductive behaviors may function in one pathway but not the other. Mutations in the ec dysone receptor (Ecd; Carney and Bender 2000) or Tyramine-B-hydroxylase (TBH; Montastriotti et al. 1996) affect oviposition behavior. chaste females are unresponsive to wild-type male courtship (Yamamoto et al. 1997), while spinster (spin) mutant females are unresponsive and fail to make normal eggs (Nakano et al. 2001), making an unambiguous determination of spin's role in oviposition difficult. Expression and function of these genes is required in the female for manifestation of sexual behaviors, distinguishing them from the gene products present in the male ejaculate that affect female postmating behaviors when they are transferred to females during copulation (Chen et al. 1988; Monsma and Wolfner 1988; Aigaki et al. 1991; Kubli 1992; Herndon and Wolfner 1995; Wolfner 1997, 2002; Wolfner et al. 1997; Heifetz et al. 2000, 2001; Chapman et al. 2001; Fleischmann et al. 2001; Saudan et al. 2002).

Clearly, the genes described to date compose an incomplete list of those that are required for female reproductive behaviors in Drosophila. From genetic screens designed to isolate additional loci needed for these sex-specific behaviors, we identified a new gene, logjam (loj), whose expression is required for oviposition. The predicted protein from this locus shares identity with the EMP24/GP25 family of cytoplasmic vesicle membrane proteins. The unexpected finding that loj encodes a protein family highlights the importance of intracellular trafficking for proper behavioral outputs.

**MATERIALS AND METHODS**

**Strains:** The loj00898/TM3, ry Sb + P element strain (originally designated fs(3)00898; Karpen and Spradling 1992) was provided by C. Berg. loj00898 fails to complement the third chromosomal deficiency Df(3L)ZN47 (64C-65C). This deficiency stock and the l(2)04026/TM3, ry Sb Ser P element insertion line were obtained from the Drosophila stock center in Bloomington, Indiana. l(2)04026 was originally identified as a lethal allele. However, when the stock is grown at 25°C under noncrowded conditions, homozygous adults eclose, albeit at low frequency. Here we refer to l(2)04026 as log0082, log0082 and log0082/ Df(3L)ZN47 animals eclose in the expected Mendelian ratio, suggesting that a second site on the log0082 chromosome reduces viability of homozygotes.

Both P element insertions were mobilized by standard methods and a total of 59 ry stocks were generated by introduction of the Δ2-3 transposase source (Robertson et al. 1988). Eight of these lines fail to complement a loj P insertion for fertility. By PCR analysis, at least two of these lines appear to have large deletions in the loj region and one is limited to the region upstream of the loj00898 insertion. The remaining alleles, when heterozygous with a loj P insert, produce females that are fertile. Many of these alleles have been shown to be partial deletions in the log0082 P element sequence, indicating that complete or partial loss of the P insertion is sufficient to rescue the loj phenotype.

**Mating assays:** Virgin females were collected and aged individually in food vials for 4–6 days at 25°C. Each female was placed into a courtship chamber with a wetted filter paper and an aged Canton-S male and observed for 30 min or until successful mating occurred (Finley et al. 1997).

**RNA hybridization:** For Northern blotting, total RNA was prepared from Canton-S, log0082, log0082/TM3, log0082, and log0082/ TM3 adult flies using TRIzol (Invitrogen, San Diego), and 6 μg of RNA was electrophoresed in each lane of a 1% agarose gel with MOPS/formaldehyde and then blotted to nylon membranes. For in situ analyses, 30-μm cryostat sections of Canton-S and log0082 adult females and males were hybridized for detection of RNA expression as described previously (Goodwin et al. 2000). Riboprobes for Northern and in situ analyses were derived from the LD30746 cDNA [Berkeley Drosophila Genome Project (BDGP)] that corresponds to the class I transcript (see Figure 3) and labeled with digoxigenin (DIG) using the DIG RNA-labeling system (Roche, Indianapolis). The DIG luminescent detection kit (Roche) was used for Northern signal detection.

These probes are expected to hybridize to all six log tran- script classes. However, the smaller class IV and class V messages are not detected on the blots, suggesting that they are present at low levels or that there is temporal specificity to their expression. Both hypotheses are supported by the fact that few rapid amplification of cDNA end (RACE) clones that correspond to class IV and V transcripts were isolated from adults (see below).

**5’ and 3’ RACE:** 5’ RACE products were isolated from adult flies using the Invitrogen 5’ RACE system or the First Choice RLM-RACE kit (Ambion, Austin, TX), which is designed to recognize only full-length, capped messages. Adult 3’ RACE products were isolated with the Invitrogen 3’ RACE system and the First Choice RLM-RACE kit. 5’ RACE primers were designed to exons B (5’-GATCCAAAAGACACAAAAATCAGAGC AATCGGATTG-3’ and 5’-AAAAAGACACACAAATCAGCAG AATCGGTAGT-3’), and D (5’-AATCGG
GAAGACTGTGTTG-3' and 5'-TTGCGGAGCGCACTGTGA-3'). 3' RACE primers were designed to exons C (5'-AAGGAGGAC TGCTACCTAC-3'), E (5'-CACCCTTGGCCACTGGAAG-3'), and F (5'-TCCAAAATGTGCTAACAG-3'). All products were purified and cloned into the pPCR-Script Amp SK(+) cloning vector (Stratagene, La Jolla, CA) for sequencing. Out of 29 3' RACE clones chosen for sequence analysis, a total of 17 class I clones were sequenced (see Figure 3 for clone structures). Three class II, two class III, three class IV, and four class V clones were sequenced as well.

Real-time PCR and standard reverse transcriptase-PCR: For real-time PCR, whole-body total RNA was extracted from a homogenate of 15–40 adult females of the genotypes Canton-S, loj00898, loj04026/Df(3LR)Z1N47, loj00898/TM3, loj00898, loj00898/Df(3LR)Z1N47, and loj00898/TM3. cDNA was prepared with the Superscript first-strand synthesis system for reverse transcriptase (RT)-PCR (Invitrogen). For relative quantitative detection of products (real-time PCR), cDNAs were amplified with primers directed to a region common to all known loj transcripts (forward primer 5'-AAGGAGGACTGCTACCA TCA-3' and reverse primer 5'-AATCCGGGAGCTGTGA-3') using the SYBR green PCR master mix for RT-PCR (PE Biosystems, Foster City, CA). An ABI PRISM 7700 sequence detection system (PE Biosystems) was used to detect products. Amplification of rp49 (forward primer 5'-TGACCTACGCGCCAGCA TACA-3' and reverse primer 5'-TCTCGGCGAGTAAAC-3') in independent reactions served as the endogenous control for normalizing the amount of cDNA in each reaction. Controls for both sets of reactions included reactions that lacked cDNA template or primers as well as no reverse transcriptase reactions to test for genomic DNA contamination.

Results from each primer set were used to generate a standard curve for statistical comparisons of the samples. Samples were run in quadruplicate and were analyzed for differences in transcript levels and statistical significance using the SAS program (Version 8.01) for ANOVA analysis. The average from multiple, independent runs is reported for each genotype. All values were normalized to loj00898/TM3 heterozygotes. The slight difference between loj00898/TM3 and loj04026/TM3 females' transcript levels was not significant, but transcript levels in both of these control female genotypes were significantly different from results obtained with all mutant combinations tested (a = 0.05, Tukey's Studentized range). To show that the observed decrease in loj transcript levels in mutant females was specific to this locus, we designed primers (forward primer 5'-TGACCTACGCGCCAGCA TACA-3' and reverse primer 5'-TCTCGGCGAGTAAAC-3') to a nearby open reading frame (ORF; CG10467) that is located 0.5–1.0 kb upstream of the loj00898 P element. Transcription from this gene is not affected in either loj00898 or loj04026 homozygous females.

For standard RT-PCR, whole-body or tissue-specific total RNA was extracted from a homogenate of 5–25 male or female wandering third instar larvae, pupae, or adult animals or from adult tissues (head, abdomen, abdomen without ovaries, and ovaries). cDNA was generated as described above. The cDNA was amplified with primers specific to the class I transcript (forward primer 5'-CCACCTTAAAGCAACAGAA-3' and reverse primer 5'-TGATGGTAGCAGTCCTCCT-3') using Taq polymerase.

Immunohistochemical and enhancer trap analyses: Tissues from loj00898/TM3, loj04026/TM3, loj00898, and loj04026 larvae, pupae, and adult animals were dissected in PBS. For antibody detection of β-galactosidase (β-gal) expression, tissues were fixed in 3.5% formaldehyde, rinsed with PBST (PBS, 0.1% Triton X-100), and incubated with rabbit anti-β-gal (1:10,000; Cappell, Malvern, PA) overnight at 4°C. Tissues were rinsed with PBST, incubated with biotinylated anti-rabbit secondary antibody (Vector, Burlingame, CA) at room temperature, and developed with diaminobenzidine (Sigma, St. Louis) in the presence of the ABC reagent (Vector). Signal was not detected in female abdomens (data not shown).

Wild-type, loj00898, loj04026/TM3, loj00898/Df(3LR)Z1N47, and loj04026/Df(3LR)Z1N47 adult female genital tracts were prepared as above and incubated with rabbit anti-synaptotagmin (1:1000) for visualization of synaptic innervation patterns (Finley et al. 1997).

For X-gal staining, loj00898/TM3, loj04026/TM3, loj00898, and loj04026 female and male tissues were fixed in 3.7% formaldehyde, rinsed with PBST, and incubated overnight at 37°C with reaction buffer (PBS with 2.5 mM MgCl2, 3 mM K3Fe(CN)6, 3 mM K4Fe(CN)6) in the presence of 8% X-gal (Sigma) in DMSO.

Heat-shock rescue: Constructs for heat-shock overexpression of loj were generated by cloning the class I cDNA (clone LD30746) into the pcasa-act plasmid (DAVINO and THOMMEL 1999). This transcript was chosen because it is widely expressed and is expected to encode the same protein as class Ia, II, and III messages. The construct was injected into yw embryos by the CBRC Core Facility at Massachusetts General Hospital. More than 26 independent transgenic lines containing pCaSpeR-loj were established.

Expression from pCaSpeR-loj was sufficient to rescue the oviposition phenotype. Three independent insertions on the X chromosome (H1b, H1a, and C10e) were introduced into the loj04026 background. Mutant and control females were collected at 22°C and placed in food vials at 25°C with wild-type males. Eggs were counted at 5–11 days for each genotype. For the heat-shock treatment, females within 12 hr of eclosion were placed at 37°C for 1 hr.

Sequence analysis: LOJ motifs were predicted using the following programs: SignalP (Center for Biological Sequence Analysis, The Technical University of Denmark, Lyngby, Denmark; http://www.cbs.dtu.dk/services/SignalP/; Nielsen et al. 1997) to detect the location of potential signal peptide cleavage sites; COILS (European Molecular Biology Network, Swiss program (Version 8.01) for ANOVA analysis. The average for both sets of reactions included reactions that lacked cDNA template or primers as well as no reverse transcriptase reactions to test for genomic DNA contamination.

RESULTS

The loj00898 and loj04026 P element causes female sterility and an egg-retention phenotype: Our interest in identifying genes that are required for female reproductive behaviors led us to screen candidate female-sterile mutants for deficits in mating and oviposition. A female-sterile P insert strain, designated here as loj00898, produces homozygous females that do not lay eggs. We examined these females for the retention of mature eggs in the female genital tract, a defect characteristic of females that are mutant for the sex-determining gene dsy (Finley et al. 1997) and of females mutant for TBH, which encodes a biosynthetic enzyme that produces the neurotransmitter octopamine from tyramine (Monastirioti et al. 1996). In females homozygous for the P insert or trans-heterozygous for loj00898 and a deficiency (Df) chromosome that uncovers the mutation...
To determine if function in adult females: G. E. Carney and B. J. Taylor

**TABLE 1**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Frequency of mating (N) (%)</th>
<th>Average latency to mating ±SEM</th>
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<tbody>
<tr>
<td><em>loj</em>&lt;s&gt;po88&lt;/s&gt;/TM3</td>
<td>90.91 (11)</td>
<td>7.55 ± 1.82</td>
</tr>
<tr>
<td><em>loj</em>&lt;s&gt;po22&lt;/s&gt;/TM3</td>
<td>100.00 (14)</td>
<td>5.07 ± 0.75</td>
</tr>
<tr>
<td><em>loj</em>&lt;s&gt;po88&lt;/s&gt;</td>
<td>100.00 (6)</td>
<td>10.92 ± 2.08</td>
</tr>
<tr>
<td><em>loj</em>&lt;s&gt;po88&lt;/s&gt;/Df(3L)ZN47</td>
<td>88.24 (17)</td>
<td>6.67 ± 0.94</td>
</tr>
<tr>
<td><em>loj</em>&lt;s&gt;po22&lt;/s&gt;</td>
<td>100.00 (4)</td>
<td>5.00 ± 1.17</td>
</tr>
<tr>
<td><em>loj</em>&lt;s&gt;po22&lt;/s&gt;/Df(3L)ZN47</td>
<td>66.67 (3)</td>
<td>8.00 ± 4.89</td>
</tr>
</tbody>
</table>

Control and *loj* females were tested for frequency of and latency to mating with wild-type males. Frequency is the percentage of females tested that successfully mated within a 30-min observation window. N, no. of females tested. Latency is the time in minutes to mating and was determined only for females that mated in the observation period. These numbers are not significantly different from one another by ANOVA analysis (P = 0.13).

(*loj*<s>po88</s>/Df; see MATERIALS AND METHODS), one or more mature eggs become lodged within the genital tract causing a “logjam” of eggs within the female and preventing the release of eggs (data not shown). While in essentially every case an egg is found in the uterus, eggs are also found within the lateral or common oviducts in many females.

Synaptic connections to the oviduct and uterine muscles are present in *loj* mutants (data not shown); such synapses were previously shown to be absent from the uterine muscles of *dsf* females (Finley et al. 1997). *loj* females mate with latency and frequency similar to that of control females (Table 1) and store sperm within approach to assess whether any gene (see Figure 2) and used a quantitative RT-PCR genomic position of the insert. Inverse PCR (Yeo et al., 1995), providing strong evidence Class Ia (isolated by BDGP and a likely splicing variant of *dsf* uterine muscles of females (see Materials and Methods) corresponding to the CG10733 ORF, we first established the wild-type transcript expression pattern from this region in adult animals. Northern analysis of wild-type adult female or male whole-body RNA revealed a diffuse hybridizing band (Figure 1), potentially containing multiple transcripts. The ~1.15-kb size of the band(s) in both sexes is consistent with the size of clones from the BDGP EST project (e.g., clone LD30746) corresponding to this region. Northern blots of RNA from adult females homozygous for either *P*-element insertion do not produce a hybridization signal when probed with labeled sequences derived from the LD30746 cDNA clone (Figure 1).

To confirm that the *P* elements disrupt expression from the CG10733 ORF in adult females, we designed primers directed to the common region of the predicted gene (see Figure 2) and used a quantitative RT-PCR approach to assess whether any *loj* transcripts are detectable in *loj* mutants. Real-time PCR with primers that recognize all transcript classes (see below) reveals that the *loj*<s>po88</s> and *loj*<s>po22</s> insertions substantially downregulate *loj* transcripts (Table 2) but do not affect a nearby (within 0.5–1 kb) gene (see MATERIALS AND METHODS). These results indicate that *loj* corresponds to the predicted CG10733 ORF.

**Multiple transcripts are produced from the *loj* genetic region:** We used 5’ and 3’ RACE and BDGP database searches to determine the structure of transcripts from females and males, identifying at least six different transcript classes (Figure 2), some of which correspond to cDNAs found in the BDGP database. Class I transcripts are structurally similar to but extended 5’ relative to related BDGP cDNA clones (RE61227, RE18875, GM-12884, LD30746, and LD38695). These class I messages are found at high levels in adults of both sexes and contain a strong consensus ATG translation start site (Cavener and Ray 1991) in the second exon (Figure 2). Class Ia (isolated by BDGP and a likely splicing variant of class I), class II, and class III transcripts are expected to encode the same protein as class I. Two cDNAs from the BDGP database (SD16982 and GM24669) contain sequences that are common to class II and III RACE.
Clones. A fourth transcript (class IV) has a 5' end that begins in the second exon downstream of the consensus translation start site present in class I, Ia, II, and III transcripts (Figure 2).

Class V messages have 5' ends that begin with the first common exon (exon C; see Figure 2) but contain a 9-bp 5' extension that is not present in other transcripts (Figure 2). There is no consensus translation start, and the first ATG in-frame with *loj* is present in the second class V exon (corresponding to exon D of class I messages; see Figure 2).

Results from 3' RACE experiments indicate that all transcript classes likely have the same 3' end. These 5' and 3' RACE studies allowed us to map the positions of the P inserts relative to the different transcripts. *loj* is inserted into the 5' untranslated region of the class I, Ia, and class III transcripts, while *loj* lies in predicted protein coding sequences for classes I–III (Figure 2). Neither P element is inserted into sequences that are first common exon (exon C; see Figure 2) but contain a 9-bp 5' extension that is not present in other transcripts transcribed to class IV or V messages.

**loj** is expressed in the larva, pupa, and adult: We used RT-PCR to analyze the expression pattern of the highly expressed class I transcripts in females and males during multiple developmental stages to understand when and where **loj** is expressed. Our results indicate that **loj** is expressed in female and male larvae, pupae, and adults (Figure 3). We found that in adults class I transcripts are present in heads, abdomens, abdomens from which the ovaries have been removed, and the ovaries themselves (Figure 3).

**TABLE 2**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Relative levels of transcripts (%)</th>
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<tbody>
<tr>
<td><em>loj</em>00898/TM3</td>
<td>100.00</td>
</tr>
<tr>
<td><em>loj</em>04026/TM3</td>
<td>102.50</td>
</tr>
<tr>
<td><em>loj</em>00898</td>
<td>3.64</td>
</tr>
<tr>
<td><em>loj</em>04026</td>
<td>0.38</td>
</tr>
<tr>
<td><em>loj</em>00898/Df(3L)ZN47</td>
<td>14.30</td>
</tr>
<tr>
<td><em>loj</em>04026/Df(3L)ZN47</td>
<td>14.30</td>
</tr>
</tbody>
</table>

Results from real-time PCR with adult female control and mutant cDNA. The data from all samples were normalized to results with *loj*00898/TM3 female controls (see MATERIALS AND METHODS). *loj*00898/TM3 and *loj*04026/TM3 control females have similar levels of *loj* transcripts, while all mutant combinations show a significant reduction in **loj** expression.

**Figure 2.**—Multiple transcripts are produced by the **loj** locus. The structures of the six identified classes of **loj** transcripts are shown as well as their predicted nucleotide lengths. Exons are boxed. Shaded boxes indicate exons predicted to be shared by all six of the transcript classes. Positions of the *loj*00898 and *loj*04026 P-element insertions are shown above the class I message. The class Ia message structure is predicted from a single BDGP cDNA clone (RE44409) derived from embryonic tissue and has a 20-nt 5' extension to the B exon. Arrows indicate PCR primers. a and b are the real-time PCR primers (see Table 2). The forward and reverse primer pair allows transcript-specific amplification of class I messages (see Figure 3). The forward primer spans the A and B exons, and the brackets indicate intronic sequences that were not included in this primer. ATG is the predicted translation start site present in the B exon.

**Figure 3.**—**loj** is expressed at multiple times in development. Class I-specific primers were used to amplify cDNA from sexed, wild-type animals from the wandering third instar larval, pupal, and adult stages. Dissected heads, abdomens, and ovaries from wild-type animals express **loj**, which is consistent with the enhancer trap patterns shown in Figure 4. M, DNA molecular weight marker; f, female; m, male; abd, abdomen; a-ov, abdomens with ovaries removed; ov, ovary; G, genomic DNA control lane (negative control).
To gain further knowledge as to which tissues in the adult express *loj* we took two approaches: (1) an enhancer trap analysis using the *loj*00898 P-element tag to assess expression from the *P* inserts in larvae, pupae, and adult animals and (2) *in situ* hybridization with *loj* riboprobes. Larval expression occurs in the brain and ventral nerve cord (VNC; Figure 4, A and B), and the overall patterns of expression from the enhancer trap elements are similar in pupae and adults of both sexes (Figure 4, C–H). β-Gal activity or expression is limited to the central nervous system (CNS) and ventral epidermis (Figure 4 and data not shown). In larvae signal is present in the CNS (Figure 4, A and B) and ventral epidermis, while in pupae signal is observed in the CNS (Figure 4, C and D). Other pupal tissues were not tested. In the pupal and adult brain, staining is evident in a small number of cells in the antennal lobe, medullar regions of the optic lobe, and the subesophageal ganglion (Fig-

Figure 4.—*loj* is expressed in the larval, pupal, and adult CNS. Composite photographs of expression patterns from the *loj*00898 enhancer trap insertion in larval (A and B), pupal (C and D), and adult (E–H) CNS tissues. Br, brain; VNC, ventral nerve cord; al, antennal lobe; m, medulla; sog, subesophageal ganglion; ab, abdominal ganglion; T1, T2, and T3 delineate positions of the thoracic neuromeres of the VNC. Females are shown in A, G, and H. Males are shown in B, E, and F. For the male in E, we observed weak expression in a pattern similar to that of the female in G. Bar, 100 μm.
Figure 5.—Vitellogenic egg chambers contain loj mRNA. In situ hybridization to sectioned wild-type adult females shows loj expression in vitellogenic eggs (A and B). Arrows point to the high-level staining found in follicle cells and arrowheads to the low-level staining present in the nurse cell compartment. loj<sup>00898</sup> mutant females lack expression in vitellogenic eggs (C and D; see arrows). Wild-type males do not have detectable loj expression in this assay (E and F). Arrows in E and F point to sperm bundles. Bar, 20 μm.

Expression is also present in dorsal and lateral cells of the abdominal and thoracic ganglia of the VNC (Figure 4, D, F, and H).

RNA in situ hybridization to sectioned tissues from wild-type adult females revealed expression only in the ovaries, with strong hybridization to follicle cells and weak hybridization to nurse cells of midstage vitelligenic (yolk-containing) eggs (Figure 5, A and B). No hybridization to any tissues was detected in sectioned adult wild-type males (Figure 5, E and F). We did not detect RNA hybridization in loj<sup>00898</sup> homozygous females or males (Figure 5, C and D and data not shown), providing further evidence that, at least in female ovarian tissue, gene function is disrupted in loj mutants.
The oviposition defect can be rescued by *loj* overexpression. We generated transgenic animals containing a heat-inducible *pCaSpeR-loj* plasmid (see MATERIALS AND METHODS). Basal expression from each of three independent *pCaSpeR-loj* constructs is sufficient to partially rescue the egg-laying defect. However, a single heat pulse early in the adult stage provides a greater level of rescue with two of the transgenes (Table 3).

*loj* encodes a predicted protein with homology to EMP24/GP25 cargo receptor proteins: *loj* encodes a predicted protein that shares amino acid homology with the EMP24/GP25 family of putative vesicle cargo receptor proteins (Figure 6). These 24-kD proteins, present in diverse eukaryotic lineages including mustard, yeasts, worm, and mammals, were isolated as components of coat protein (COP)-coated vesicles that shuttle cargo between the endoplasmic reticulum and Golgi membranes for processing (Schimmoller et al. 1995; Stamnes et al. 1995) and may have positive as well as negative functions in mediating molecular transport through the secretory pathway (Schimmoller et al. 1995; Stamnes et al. 1995; Belden and Barlowe 1996; Marzioch et al. 1999; Muniz et al. 2000). The predicted LOJ protein shares important hallmarks of this class of vesicle proteins, including an amino-terminal signal sequence, a large luminal domain containing a coiled-coil protein interaction domain, a single transmembrane region, and a small, carboxy-terminal cytoplasmic tail (Figure 7). The coiled-coil domain of EMP24 proteins likely mediates heteromeric contacts between EMP24 proteins (Belden and Barlowe 1996; Fullekrug et al. 1999; Marzioch et al. 1999; Cuifo and Boyd 2000; Denzel et al. 2000).

### Table 3

<table>
<thead>
<tr>
<th>Genotype</th>
<th>No. females tested</th>
<th>No. eggs laid</th>
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</thead>
<tbody>
<tr>
<td>H1b/+; <em>loj</em>-<em>pass</em></td>
<td>10</td>
<td>15</td>
</tr>
<tr>
<td>H1a/+; <em>loj</em>-<em>pass</em></td>
<td>36</td>
<td>3</td>
</tr>
<tr>
<td>H1a/+; <em>loj</em>-<em>pass</em> + hs</td>
<td>4</td>
<td>6</td>
</tr>
<tr>
<td>C10e/+; <em>loj</em>-<em>pass</em></td>
<td>17</td>
<td>2</td>
</tr>
<tr>
<td>C10e/+; <em>loj</em>-<em>pass</em> + hs</td>
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<td>15</td>
</tr>
<tr>
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<td>0</td>
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<td>4</td>
<td>0</td>
</tr>
<tr>
<td>w/FM6; <em>loj</em>-<em>pass</em></td>
<td>17</td>
<td>1</td>
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*loj* females with or without a *loj* transgene were placed in food vials with at least one wild-type male fly at 25°C and examined at 5–11 days for the presence of eggs laid on the food. Flies were transferred to new food every 3 days. In a separate experiment, *loj* females with or without the transgene were placed in a 37°C incubator within 12 hr of their collection for 1 hr to give a mild heat shock (hs). H1b, H1a, and C10e are independent transgene insertions present on the X chromosome. *w; Sp/CyO*; *loj*-*pass* was the source of the *loj* mutation that was crossed into the transgene stocks and *w/FM6; *loj*-*pass* are negative control animals derived from the stock crosses.

In total, seven predicted EMP24-related products are encoded by the Drosophila genome (Figure 8). Prior to this study, there were no characterized Drosophila mutations in these putative cargo receptors.

### Discussion

A clear understanding of how genetic signals are transduced at the molecular, cellular, and biochemical levels to specify behavioral outcomes is an important goal for behavioral biologists. Drosophila female reproductive behaviors are a good model system for tackling this difficult issue because they provide a strong selective phenotype for mutant screens and because some of the genes that contribute to these behaviors are already known. Using genetic and behavioral screens, we identified a new class of proteins important for oviposition. Our results indicate that *loj* has an essential role in egg-laying behavior and encodes a member of a conserved family of proteins that function in vesicular trafficking in the endomembrane system.

**Molecular and phenotypic characterization of *loj* in wild-type and mutant animals:** Northern analysis (Figure 1) and RT-PCR (Figure 3) reveal that *loj* transcripts are present in larvae, pupae, and adults. Enhancer trap and *in situ* hybridization experiments suggest that the majority of this signal is derived from *loj* expression in the brain, VNC, and developing eggs (Figures 4 and 5). By *in situ* hybridization to sectioned adult tissues, *loj* expression is detectable only in adult female vitellogenic egg chambers (Figure 5, A and B) and not detectable in the CNS or epidermis. Lack of recognizable *in situ* signal in these tissues in either sex is possibly due to the small number of cells that are likely to be labeled in each section. An alternative possibility is that the difference in the Northern and RT-PCR data vs. data from *in situ* hybridization is that the level of RNA per cell is below detection except in the ovary. In female and male larvae, β-gal staining is present in the CNS (Figure 4, A and B), ventral epidermis, and posterior portion of the proventriculus (data not shown). Midstage pupae also have *loj* CNS expression (Figure 4, C and D).

Females with little *loj* expression are able to mate but do not lay eggs (Tables 1 and 2), and the oviposition defect can be rescued by the expression of a transgene containing a class I cDNA (Table 3). While wild-type females lay more eggs than the rescued *loj* mutants do (data not shown), the numbers of eggs deposited by mutant females containing a *pCaSpeR-loj* transgene are well above those laid by mutants lacking the transgene and indicate that decreased levels of *loj* are responsible for the oviposition phenotype. In one instance, a *loj* mutant female that lacked the transgene laid an egg (Table 3). In our experience this is an extremely rare event because we have observed only a half dozen or
so eggs laid by the many hundreds of mutant females that have been tested in egg-laying assays.

mRNA expression in \textit{loj}^00898 females is significantly reduced relative to other mutant combinations tested (Table 2). The fact that \textit{loj}^00898 females have decreased transcript levels relative to \textit{loj}^0026/Df animals may indicate that this insertion chromosome contains another interacting factor that affects \textit{loj} expression. Two additional Drosophila EMP24 family members are located on the third chromosome. One possibility is that interactions between mutations in one of these two genes and \textit{loj}^0026 account for the further reduction in transcript levels observed in the \textit{loj}^0026 homozygous mutants. Precedence for a similar effect is found in yeast and mice where loss of one EMP24 protein has been shown to affect levels of interacting EMP24 proteins (Marzioch \textit{et al.} 1999; Denzel \textit{et al.} 2000). While this effect in mice is post-transcriptional (Denzel \textit{et al.} 2000), it is possible that an alternate regulatory mechanism occurs in multicellular organisms. Despite the variation in transcript expression levels, there are no obvious phenotypic differences between \textit{loj}^00898, \textit{loj}^0026, or \textit{loj}/Df heterozygous adults.

Interestingly, while \textit{loj} females have a profound behavioral defect, there is no observable deleterious effect on male fertility from loss of \textit{loj} activity. The fact that \textit{loj} mutant adults eclose at the expected Mendelian frequencies and, in the case of males, are able to reproduce suggests that the molecular function supplied by \textit{loj} can be partially supplied by another locus, possibly one of the remaining six Drosophila EMP24/GP25 family members. A second possibility is that \textit{loj} does not function in adult males.

Due to the presence of a strong consensus translational start site in exon B (Figure 2), the class I transcripts highly expressed in both sexes are predicted to encode the same protein as the class Ia, II, and III mRNAs. The class IV and V messages lack an obvious translational start site and are not detectable on Northern blots (see MATERIALS AND METHODS). Theoretical translation of these sequence and database searches did not identify homologies (other than EMP24/GP25) to the predicted peptides. One possibility is that the transcripts are not translated to a functional protein, similar to the sex-nonspecific product of \textit{tra} (Boggs \textit{et al.} 1987) or the female-specific product of \textit{fru} (Usui-Aoki \textit{et al.} 2000). Alternatively, translation may occur by internal ribosome entry or via another mechanism, such as recognition of a less-conserved translation start site. If this is the case, each of these two messages potentially encodes a different version of \textit{LOJ} that has a truncated luminal domain. It is interesting to speculate that truncated proteins produced from the class IV and class V transcripts may have different specificities for cargo molecules or altered interactions with other Drosophila EMP24/GP25 proteins. The class IV and V proteins should contain the same transmembrane and carboxy-
 terminal cytoplasmic domains as all loj products but have shortened amino-terminal luminal domains for interacting with cargo molecules.

**Oviposition:** The final deposition of an egg on a substrate is the culmination of a series of events that requires maturation and function of the necessary neural circuitry, ovulation of eggs, and transmittal of the egg through the genital tract to its final release. This passage through the ductwork to the uterus and final release can be defined as “oviposition proper.” The genes that regulate all of these processes (excluding those with strictly developmental functions) can be considered “oviposition genes,” since lack of proper function at any level of this process will lead to an oviposition phenotype.

Little is known about how the oviposition circuitry in Drosophila is generated. In the case of the grasshopper, a large portion of the oviposition neural circuit is completed by the end of embryonic development, well before it is needed for the behavior (Thompson and Roosevelt 1998). Therefore, some oviposition genes must function early in this process. Once the appropriate circuitry has been established, signaling must occur to initiate and maintain the behavior. The activity of motorneurons that directly synapse on the uterine and oviductal muscles is likely to be controlled by descending inputs from the command interneurons in the brain, including the subesophageal ganglion. Local circuit interneurons in the posterior abdominal ganglion and sensory inputs from neurons in the ovaries and internal reproductive tract also are expected to function in activating and modulating oviposition behavior (Thompson 1986a,b).

**Relationship of the loj adult female phenotype to oviposition:** The female response to mating is increased Little is known about how the oviposition circuitry in Drosophila is generated. In the case of the grasshopper, a large portion of the oviposition neural circuit is completed by the end of embryonic development, well before it is needed for the behavior (Thompson and Roosevelt 1998). Therefore, some oviposition genes must function early in this process. Once the appropriate circuitry has been established, signaling must occur to initiate and maintain the behavior. The activity of motorneurons that directly synapse on the uterine and oviductal muscles is likely to be controlled by descending inputs from the command interneurons in the brain, including the subesophageal ganglion. Local circuit interneurons in the posterior abdominal ganglion and sensory inputs from neurons in the ovaries and internal reproductive tract also are expected to function in activating and modulating oviposition behavior (Thompson 1986a,b).

**Figure 7.—**LOJ shares important functional domains with EMP24/GP25 proteins. The sequence of the longest identified class I transcript is shown with the likely translation start and stop sites indicated in boldface type. Predicted amino acids are below the nucleotide sequence. Boldface amino acid residues represent the predicted signal peptide. Residues in boldface italics compose the coiled-coil domain, and boxed residues indicate the transmembrane region.
ing (Chen et al. 1988; Monsma and Wolfer 1988; Aigaki et al. 1991; Kurli 1992; Herndon and Wolfer 1995; Wolfer 1997, 2002; Wolfner et al. 1997; Herndon and Wolfner 1995; Wolfner 1997, 2002; Wolfner et al. 1997; Heifetz et al. 2000, 2001; Chapman et al. 2001; Fleischmann et al. 2001; Saudan et al. 2002). In loj mutants both ovulation and oviposition are affected. We propose that the loss of signals from the CNS as well as the egg are responsible for the lack of oviposition behavior noted for loj mutant females and that disruption of ovulation is a secondary effect of the mutation.

One consequence of the failure of oviposition in loj mutants is that mature eggs are found inside the female genital tract, particularly in the uterus. Interestingly, the genital tracts of loj females are innervated as assayed by detection of synaptotagmin-positive synapses, which suggests that the egg-laying deficit may be due to loss of a required signal rather than simply to loss of motor-neuronal input to the genital tract muscles. This hypothesis is supported by the homology that loj shares with components of COP-coated vesicles, which function in the secretory pathway in both anterograde and retrograde cytoplasmic transport of cellular components between the endoplasmic reticulum and Golgi as well as functioning in shuttling neurotransmitters to their release sites (Rothman and Wieland 1996; Schekman and Orci 1996; Liu et al. 1999). The signaling molecule that is missing in loj mutants may need to mature by passage through the secretory system or may need to be loaded into neuronal vesicles. Such a signal could come from the nervous system, the egg itself, or from both tissues. Hence, the observed CNS and egg chamber expression have important implications for how the loj product functions in oviposition.

**loj function in the CNS:** The brain and VNC are important centers for processing gustatory, olfactory, and visual inputs and transforming this information into an appropriate behavioral outcome such as oviposition. Previous work using mosaic animals, in which male and female tissue was judged only by external cuticular markers, mapped egg-laying behavior to the thorax (Szabad and Fajszi 1982), which contains the VNC. Our enhancer trap data suggest that loj is expressed in numerous cells of the thoracic and abdominal ganglia of the VNC (Figure 4).

The brain also has a role in oviposition regulation, since decapitated or anesthetized Drosophila females lay eggs as a reflex response (Grossfield 1978); loj females not only do not lay eggs voluntarily, but also do not exhibit this reflex response to anesthesia (G. E. Carney and B. J. Taylor, unpublished results). While loj expression in the brain seems at odds with the idea that the brain has an inhibitory function on oviposition that must be released, there are possible explanations. One is that the brain provides excitatory as well as inhibitory signals for oviposition, with positive signaling being mediated by loj. However, releasing this inhibition may not be sufficient if a positive loj signal is needed from the VNC. Therefore, two positive functions of loj may have been lost in female mutants with the net result that oviposition cannot occur.

**loj function in the egg:** A second interesting component of the adult expression pattern is the presence of loj in midstage vitellogenic egg chambers (Figure 5). The majority of this signal is found within the follicle cells that provide nutrients and other components to developing oocytes and produce the outer coverings of the.
egg, the vitelline membrane and the chorion (reviewed in Mahowald and Kambysellis 1980; Spradling 1993). One intriguing possibility is that these loj-positive egg cells provide cues to the female’s genital tract and musculature that aid proper egg release from the ovary and navigation through the genital tract to the uterus. This signaling mechanism would be expected to function prior to formation of the vitelline membrane and chorion or to be a component of these protective coverings of the mature egg. Another possibility is that the observed expression in egg chambers is unrelated to the behavioral phenotype and that loj function is required maternally for proper embryonic development. The second hypothesis is supported by our observation that the eggs from rescued mutant females expressing hs-loj constructs do not hatch (G. E. Carney and B. J. Taylor, unpublished results). However, this observation does not preclude the possibility that loj has an earlier function in the egg that is rescued by loj overexpression.

As noted above, the process of ovulation is affected in loj females. Since a mature egg is found in the uterus of essentially every mutant female, it appears that initial egg release is not affected. However, we have observed partially ovulated eggs in the upper portions of the lateral oviducts as well as multiple eggs in these portions of the genital tract. In wild-type females we have not observed this phenomenon. We theorize that ovulation initially proceeds normally in loj females but that the presence of an un laid egg in the uterus disrupts the feedback loop that regulates ovulation. Therefore, loj mutant females have a weak ovulation defect that is a secondary consequence of loss of oviposition behavior. An alternative hypothesis is that loj functions in both of these processes. The ovulation and oviposition phenotypes may be the consequence of the failure of eggs to signal their intentions to the reproductive tract, suggesting that signaling from the egg is an active process required for oviposition.

Relationship of loj to the somatic sex-determination hierarchy: The genetic members of the somatic sex-determination hierarchy (e.g., tra, fyu, dsx, and dsf) control female or male reproductive behaviors in Drosophila. The lodged-egg mutant phenotype and loj CNS expression pattern suggest the possibility that loj is regulated by or interacts with the sex-determination hierarchy to control female egg-laying behavior. The most likely possibilities are that loj functions downstream of dsx or is one of the determinants of oviposition behavior regulated by dsf. Two reproductive behaviors, receptivity and oviposition, are controlled by dsf, while loj affects only oviposition behavior. Additionally, while dsf mutants lack uterine innervation (Finlay et al. 1997), loj uteruses are innervated. Preliminary real-time PCR experiments in which loj expression was assayed in dsf and dsx mutant females indicate that loj expression is not altered in these females at the whole-animal level (G. E. Carney and B. J. Taylor, unpublished results). This result implies that loj mRNA expression is not controlled directly by these genes and that loj interacts indirectly with the sex-determination hierarchy to regulate female behavior. However, it is possible that loj expression is regulated by sex-determination genes in subsets of cells or tissues and that this regulation is obscured by high levels of loj expression in tissues where loj is not controlled in this manner. An alternative is that certain loj transcripts are regulated by the sex-determination hierarchy while others are not, a possibility that was not tested in our real-time PCR experiments.

Further studies are now necessary to determine how expression and function of loj are related to oviposition behavior. The discovery that a putative cargo receptor is necessary for a behavioral output reveals an important and previously unrecognized use for this class of proteins. The loj mutants provide an opportunity to dissect the functional requirements for loj and other EMP24 proteins in the context of a multicellular organism. Studies of genes such as loj provide a means for understanding the regulation of behaviors in Drosophila as well as in other organisms, since signaling cascades utilized by the fruit fly are likely to be evolutionarily conserved.

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Note added in proof: CG33104 is the current gene designation for CG9443 (see Figure 8). CG31787 encodes an eighth member of the Drosophila EMP24/GP25 protein family.

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