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Expression and Functional Characterization of a *Drosophila* Neuropeptide Precursor with Homology to Mammalian Preprotachykinin A*

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Peptides structurally related to mammalian tachykinins have recently been isolated from the brain and intestine of several insect species, where they are believed to function as both neuromodulators and hormones. Further evidence for the signaling role of insect tachykinin-related peptides was provided by the cloning and characterization of cDNAs for two tachykinin receptors from *Drosophila melanogaster*. However, no endogenous ligand has been isolated for the *Drosophila* tachykinin receptors to date. Analysis of the *Drosophila* genome allowed us to identify a putative tachykinin-related peptide prohormone (prepro-DTK) gene. A 1.5-kilobase pair cDNA amplified from a *Drosophila* head cDNA library contained an 870-base pair open reading frame, which encodes five novel *D*rosophila tachykinin-related peptides (called DTK peptides) with conserved C-terminal F*X*G*X*R-amide motifs common to other insect tachykinin-related peptides. The tachykinin-related peptide prohormone gene (*Dtk*) is both expressed and post-translationally processed in larval and adult midgut endocrine cells and in the central nervous system, with midgut expression starting at stage 17 of embryogenesis. The predicted *Drosophila* tachykinin peptides have potent stimulatory effects on the contractions of insect gut. These data provide additional evidence for the conservation of both the structure and function of the tachykinin peptides in the brain and gut during the course of evolution.

Substance P was the first peptide signaling molecule to be identified by virtue of its effects upon blood pressure and smooth muscle contraction (1) and is the archetypal member of the tachykinin family of peptides. Vertebrate tachykinins represent a large family of peptides that elicit a wide range of both central and peripheral responses (2–5). Although these peptides are structurally diverse, all contain a conserved C-terminal F*X*GLM-amide motif. Like other biologically active peptides, substance P is derived from a larger prohormone polypeptide (preprotachykinin A (PPT-A)[1]) that also allows the production of several other biologically active peptides (neurokinin A, neuropeptide K, and neuropeptide γ) (6). Three different isoforms of preprotachykinin can be produced as a result of alternative splicing of the PPT-A mRNA, which, in conjunction with alternative post-translational processing of the prohormone, allows the production of these peptides in a tissue-specific manner (7–9). A fifth mammalian tachykinin, neurokinin B, is derived from a separate gene product, preprotachykinin B (10).

The tachykinin family is not confined to vertebrates, and a large number of tachykinins have now been isolated from a variety of invertebrate species such as the cockroach *Leucophaea maderae* (11, 12), the mosquito *Culex salinarius* (13), and the echiuroid worm *Urechis unicinctus* (14). In contrast to the vertebrate tachykinins, almost all of the invertebrate tachykinins contain a conserved C-terminal F*X*G*X*R-amide motif and, for this reason, have been termed tachykinin-related peptides (TRPs). Notable exceptions are eledoisin, sialokinin I, and sialokinin II, which were isolated from the salivary glands of the cephalopod *Eledone moschata* and the mosquito *Aedes aegypti*, respectively, and which contain a C-terminal F*X*GLM-amide like the vertebrate tachykinins (2, 16, 17). Antisera to insect TRPs display immunoreactivity in both the gut and the nervous system of a variety of insect species (18, 19), suggesting that, like the mammalian tachykinins, these peptides function as both neuromodulators and endocrine signaling molecules. A tachykinin receptor (STKR) has recently been cloned and characterized from the stable fly (20), and two tachykinin receptors have been characterized from *Drosophila melanogaster*: NKD and DTKR (21–23). Although expressed NKD and DTKR show responses to the locust peptide LomTK-II and to substance P and physalaemin, respectively, to date, no tachykinins or tachykinin-related peptides have been isolated from *D. melanogaster*. As a consequence, the endogenous ligands for NKD and DTKR are not known. A tachykinin precursor cDNA

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF248040.

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[1] The abbreviations used are: PPT-A, preprotachykinin A; TRP, tachykinin-related peptide; DTK, *Drosophila* tachykinin-related peptide; BAC, bacterial artificial chromosome; RACE, rapid amplification of cDNA ends; bp, base pair(s); LemTRP, *L*eucophaea *m*aderae tachykinin-related peptide; LomTk-II, *Locusta migratoria* tachykinin-II; TAP, tachykinin-associated peptide; LTK-LI, *L*ocusta tachykinin-like immunoreactive.

has recently been cloned from the mosquito *A. aegypti* that contains only a single tachykinin-like peptide, sialokinin I (24). Sialokinins are thought to be released into mosquito saliva and thus enter the host at a blood meal. However, as described above, the sialokinins are atypical compared with other invertebrate TRPs and may be the result of convergent evolution to exploit vertebrate tachykinin receptors (17). It therefore remains unclear whether other insect TRPs are synthesized from precursor molecules containing single or multiple copies of TRPs. Here we demonstrate for the first time the existence of an insect TRP prohormone gene (*Dtk*) that, like the mammalian PPT-A gene, codes for multiple copies of novel *Drosophila* tachykinin-related peptides referred to as DTK peptides. In addition, we show that this gene is expressed in a restricted number of cell bodies in the brain and ventral nerve cord and in midgut endocrine cells, a site at which we demonstrate potent biological activity using the mature peptides. Sequence alignment showed that *Dtk* displays 31% sequence identity to human PTT-A in its substance P and neurokinin A progenitor sequences.

EXPERIMENTAL PROCEDURES

Materials—Unless otherwise stated, reagents were of molecular biology grade and purchased from Sigma. DTK peptides were synthesized via Fmoc (*N*-(9-fluorenyl)methoxycarbonyl) solid-phase methodology on an Applied Biosystems 433A peptide synthesizer according to previously described conditions (25) and were quantified by amino acid analysis.

Identification of a Tachykinin-like Prohormone in the D. melanogaster Genome—A TBLASTN search (26) of *D. melanogaster* genomic sequence (Berkley Drosophila Genome Project, Release Date September 1999) was carried out using the human PPT-A amino acid sequence (Swiss-Prot accession number P20366). Several BACs were identified that appeared to contain sequence coding for a tachykinin-like progenitor, and *ab initio* gene prediction was performed for a 5-kilobase pair region around these sequences using a *Drosophila*-specific version of FGENESH. Signal peptide prediction was performed using SignalP Version 1.1 (27).

cDNA Cloning and Sequencing—Using the FGENESH-predicted coding sequence of *Dtk*, 5′- and 3′-RACE primers were designed to amplify two overlapping fragments of the *Dtk* cDNA from an adult head cDNA library (Berkley Drosophila Genome Project GH Library): 5′-RACE primers, 5′-AATTAACCCTCACTAAAGGG-3′ and 5′-AGCTTGCTCATGATCGTCAC-3′; and 3′-RACE primers, 5′-GTAATACGACTCACTATAGGGC-3′ and 5′-ACGTCTCCCACCAGCACTAC-3′. Touchdown polymerase chain reaction was performed using the following program: one cycle of 5 min at 95 °C; two cycles of 30 s at 95 °C, 1 min at 66 °C, and 2 min at 72 °C; two cycles of 30 s at 95 °C, 1 min at 64 °C, and 2 min at 72 °C; two cycles of 30 s at 95 °C, 1 min at 62 °C, and 2 min at 72 °C; two cycles of 30 s at 95 °C, 1 min at 60 °C, and 2 min 72 °C; two cycles of 30 s at 95 °C, 1 min at 58 °C, and 2 min at 72 °C; two cycles of 30 s at 95 °C, 1 min at 56 °C, and 2 min at 72 °C; 30 cycles of 30 s at 95 °C, 1 min at 55 °C, and 2 min at 72 °C; one cycle of 10 min at 72 °C. The largest polymerase chain reaction products from each reaction were cloned into a TA vector (Promega pGemTeasy TA vector) and sequenced.

In Situ Hybridization—A 1.2-kilobase pair fragment of the *Dtk* cDNA, derived by 5′-RACE and containing the first two exons and 126 bp of the third exon, was cloned into a TA vector. This plasmid was linearized using either *Sal*I (for the sense probe) or *Nco*I (for the antisense probe), and digoxigenin-labeled sense and antisense riboprobes were synthesized using T7 and SP6 RNA polymerases (Promega), respectively, and digoxigenin-labeled dNTPs (Roche Molecular Biochemicals). *In situ* hybridization was performed on wild-type (Oregon R strain) embryos, larvae, or adults using a modification of the method described previously (28). Dechorionization, devitellinization, and fixation of embryos were performed as described previously (29). Embryos were stored in ethanol at −20 °C until required. Prior to fixing, embryos were rinsed in 1:1 ethanol/xylene; soaked for 2 h in xylene; and then rinsed successively in 1:1 ethanol/xylene, ethanol, and methanol and 1:1 methanol/postfix (10 mM potassium phosphate buffer (pH 7.0) containing 140 mM NaCl, 0.1% Tween 20, and 5% formaldehyde). Adult or third instar larval tissues were dissected and stored in *Drosophila* saline (0.7% NaCl and 0.03% Triton X-100) for up to 30 min before use. Samples were fixed in postfix for 20 min at room temperature, followed by three washes with PBT (10 mM potassium phosphate buffer (pH 7.0) containing 140 mM NaCl and 0.1% Tween 20), and were incubated with proteinase K in PBT (4 μg/ml) for 3 min at room temperature. The reaction was stopped by two washes with PBT containing 2 mg/ml glycine, and the samples were washed twice with PBT before being incubated with postfix for a further 20 min at room temperature. The tissues were washed with five changes of PBT, followed by one wash with hybridization buffer (5× SSC containing 50% formamide, 10 mM KPO_4, 140 mM NaCl, 1 mg/ml glycogen, 0.2 mg/ml sheared salmon sperm DNA, and 0.1% Tween 20 (pH 7.0)) diluted 1:2 with PBT and a further wash with hybridization buffer prior to a 1-h preincubation with hybridization buffer at 55 °C. Samples were then incubated for 43 h at 55 °C with 100 μl of hybridization buffer containing 17 ng of either the sense or antisense riboprobe. Following hybridization, the samples were washed four times with hybridization buffer at 55 °C, followed by a final wash overnight with hybridization buffer at 55 °C. Samples were washed once with 50% hybridization buffer and 50% PBT, followed by four washes with PBT, and then incubated for 2 h at room temperature with 1.0 ml of preadsorbed alkaline phosphatase-conjugated anti-digoxigenin Fab fragment (Roche Molecular Biochemicals) diluted 1:2000 with PBT. Unbound antibody was removed by extensive washing with PBT, and bound antibody was visualized by incubating the samples with staining buffer (1× phosphate-buffered saline, 100 mM NaCl, 9.4% formaldehyde, 50 mM $MgCl_2$, 50 mM EGTA, 100 mM Tris-HCl (pH 9.5), and 0.1% Tween 20) either for 1 h at room temperature or for 16 h at 4 °C with one change of staining buffer. Development was stopped by extensive washing with PBT containing 50 mM EDTA.

Immunocytochemistry—Mixed-stage embryos and tissues of larval (second and third instar) and adult wild-type *Drosophila* (Oregon R strain) were used for immunocytochemistry. Tissues were fixed in 4% paraformaldehyde in 0.1 M sodium phosphate buffer for 3–4 h. Central nervous systems and intestines were used for whole-mount immunocytochemistry as described (30) with an antiserum raised against the locust TRP LomTK-I (31) at a dilution of 1:1000. Either the peroxidase/anti-peroxidase technique or immunofluorescence with a secondary antiserum tagged with Cy3 was used. A number of adult brains and intestines were used for immunocytochemistry (peroxidase/anti-peroxidase method) of cryostat sections. Preabsorption controls were made with antiserum (at 1:1000) incubated with 50 nmol of LomTK-I. The specificity of the antiserum has been established previously (30, 31).

L. maderae Hindgut Contraction Assay—The DTK peptides were tested for their myostimulatory action in an *in vitro* hindgut muscle contraction assay utilizing the gut of the cockroach *L. maderae* (32, 33). The increases in amplitude of spontaneous contractions of the hindgut were monitored at different concentrations of peptides and plotted in percent of maximum contraction for each intestine. Dose-response curves were fitted to a four-parameter logistic equation (GraphPAD Prism Version 2.0). Each peptide was tested on three to five intestines, and the *L. maderae* peptide LemTRP-1 was used as a reference. Results are presented as mean ± S.E.

Drosophila Bioassay for Myotropic Activity—DTK-1–5 were tested for myotropic activity on isolated midgut from adult insects. The gut was severed anterior to the crop and posterior to the midgut-hindgut junction and dissected free of tracheal connections and malpighian tubules. The entire midgut was then transferred to a small chamber (~250-μl volume) and perfused with saline of the following composition: 105 mM NaCl, 5 mM KNO_3, 5 mM $CaCl_2$, 3 mM $MgSO_4 \cdot 7H_2O$, 10 mM L-histidine, and 28 mM glucose. The pH was adjusted to 6.8 with 0.1 M NaOH (34). Contractions were recorded using an optical transducer as described (35, 36). The chamber was perfused at ~800 μl/min, and peptides dissolved in saline were added to the perfusate using a 0.5-ml injection loop and rheodyne valve. Each peptide was tested on five to six midguts at concentrations from 0.1 nM to 1 μM, preliminary studies having found no evidence for receptor desensitization. Data were normalized by expressing contraction height as a percentage of the maximum response obtained with the TRP under investigation, and dose-response curves were fitted to a four-parameter logistic equation (GraphPAD Prism Version 3.00) with the maximum and minimum responses set at 100 and 0%, respectively.

RESULTS

Identification of a Tachykinin Prohormone Gene in D. melanogaster Genomic Sequence and Cloning and Sequencing of the cDNA—TBLASTN searches using the human PPT-A amino acid sequence identified several BACs (GenBank™/EBI acces-

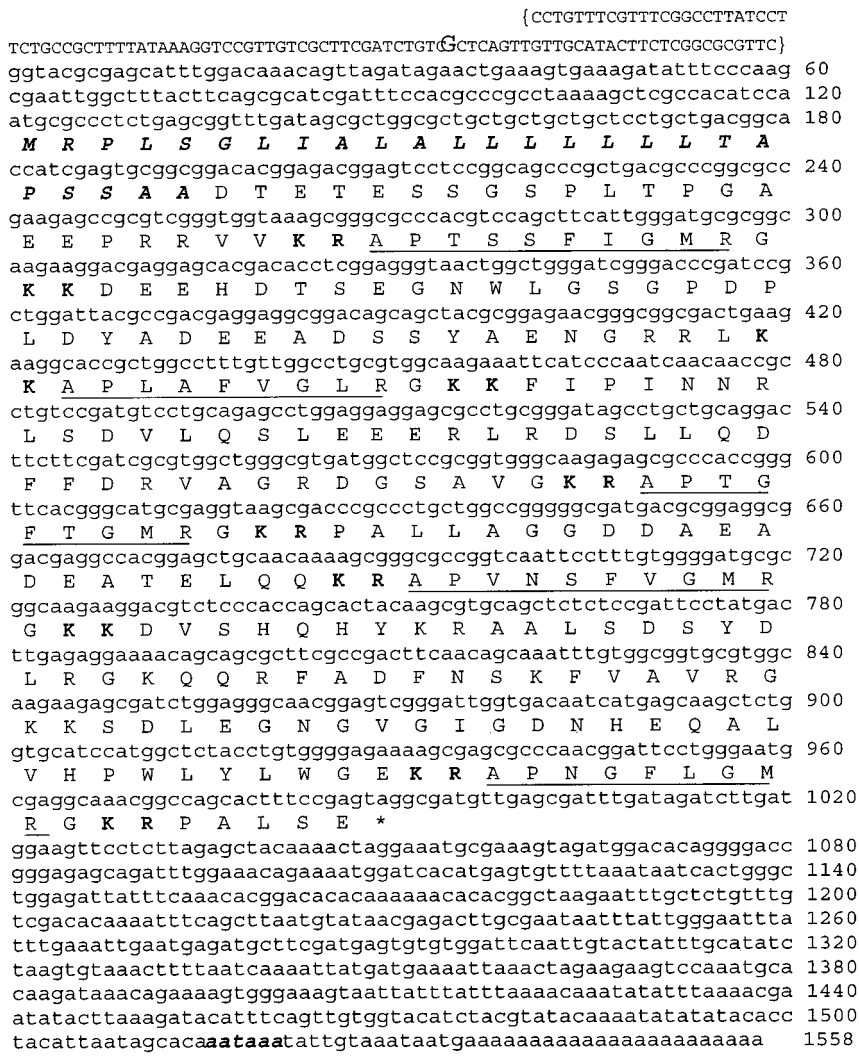

FIG. 1. **Nucleotide and deduced amino acid sequences of prepro-DTK.** A BAC genomic sequence (GenBank™/EBI accession number AC020418) upstream of the sequenced cDNA is included in *braces*. This region contains the predicted transcriptional start site (NNPP Version 1.2) shown in *large font* and a TATA box. The 870-bp *Dtk* open reading frame begins at nucleotide 121 of the sequenced cDNA and finishes at nucleotide 990 (including the stop codon). Predicted mature DTK peptides are *underlined*, and flanking pairs of basic residues representing processing sites are shown in *boldface*. The predicted N-terminal signal peptide is indicated in *boldface italics*. A consensus polyadenylation site is shown in *bold lower-case italics*, and the first stop codon is marked by an *asterisk*. Nucleotides 1–76, exon 1; nucleotides 77–771, exon 2; nucleotides 772–976, exon 3; nucleotides 977–1535, exon 4; and nucleotides 1536–1558, poly(A) tail.

sion numbers AC007692, AC007889, and AC007890) containing genomic sequence coding for part of a tachykinin-like peptide progenitor sequence that aligned with the neurokinin A progenitor sequence of human PPT-A. Subsequently, a fourth BAC was also identified (GenBank™/EBI accession number AC020418). *Ab initio* gene prediction revealed a putative three-exon gene. The coding sequence of this predicted *Drosophila* tachykinin gene (*Dtk*) contains five copies of glycine-extended tachykinin-like peptides, each flanked by pairs of basic residues, and a predicted N-terminal signal peptide for targeting of the prohormone to the secretory pathway.

A 1558-bp cDNA was amplified by polymerase chain reaction from an adult *Drosophila* head cDNA library as described under "Experimental Procedures." The cDNA contains an 870-bp open reading frame, 120 bp of 5′-untranslated region, and a 568-bp 3′-untranslated region that includes a 23-bp-long poly(A) tail (Fig. 1). Translation of the open reading frame confirmed the prediction of sequences corresponding to five tachykinin-related peptides (DTK-1–5) (Table I) flanked by pairs of basic residues, which presumably serve as processing sites for prohormone convertases. In addition to the DTK peptides, an additional six possible peptide products, called tachykinin-associated peptides (TAP-1–6) (Table I), exist in the prohormone sequence. Three of these peptide sequences (TAP-1, TAP-2, and TAP-5) have a C-terminal Gly residue and therefore have the potential to be processed to amidated peptides, as shown in Table I. TAP-5 has a structure at the C terminus similar to a recently isolated tachykinin-related peptide from another dipteran, *Stomoxys calcitrans* (37). All the other TAPs are novel sequences showing little homology to known bioactive peptides.

TABLE I
Amino acid sequences of DTK-1–5 and TAP-1–6

Each of the DTK peptides contains a conserved FX_1GX_2R-amide motif common to other invertebrate tachykinin-related peptides. With the exception of DTK-2, X_2 is a methionine residue. The conserved N-terminal AP motif in each DTK is likely to protect against degradation by aminopeptidases. Of the TAPs, TAP-5 possesses a C-terminal FVAVR-amide motif and is reminiscent of a tachykinin.

Peptide	Sequence
DTK-1	APTSSFIGMR-amide
DTK-2	APLAFVGLR-amide
DTK-3	APTGFTGMR-amide
DTK-4	APVNSFVGMR-amide
DTK-5	APNGFLGMR-amide
TAP-1	DEEHDTSEGNWLGSGPDPLDYADEEADSSYAEN-amide
TAP-2	FIPINNRLSDVLQSLEEERLRDSLLQDFFDRVAGRDGSAV-amide
TAP-3	PALLAGGDDAEADEATELQQ
TAP-4	DVSHQHY
TAP-5	AALSDSYDLRGKQQRFADFNSKFVAVR-amide
TAP-6	SDLEGNGVGIGDNHEQALVHPWLYLWGE

Genomic Organization of the Drosophila Prohormone Gene Dtk—BACs containing the *Dtk* sequence (GenBank™/EBI accession numbers AC007692 and AC007889) map to region 87A4–87B2 of the right arm of chromosome 3. *Dtk* spans 11.2 kilobase pairs of genomic sequence and contains four exons and three introns, the first intron being 9292 bp in size (Fig. 2).

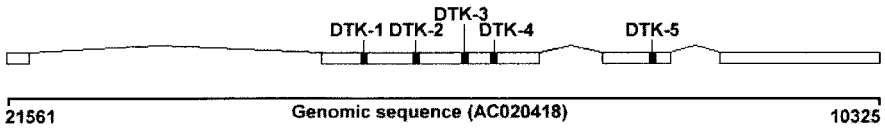

FIG. 2. **Schematic diagram showing the genomic organization of the *Dtk* gene (not to scale).** Exons are represented by *boxes*, with the first exon shown to the left. The position of the gene relative to the BAC genomic sequence (GenBank™/EBI accession number AC020418) is shown on the *bar* below. The positions of each of the exons in relation to the BAC genomic sequence (AC020418) are as follows: exon 1, nucleotides 21561 to 21486; exon 2, nucleotides 12194 to 11500; exon 3, nucleotides 11296 to 11092; and exon 4, nucleotides 10883 to 10325. DTK progenitor sequences are labeled and indicated by *black boxes*.

Sequences coding for DTK-1–4 are contained in exon 2, whereas the DTK-5 progenitor sequence is contained in the third exon. The first exon contains entirely 5′-untranslated region. Analysis of the genomic sequence upstream of *Dtk* (NNPP Version 2.1 (38, 39)) revealed a predicted transcriptional start site 38 bp upstream of the 5′-end of the sequenced *Dtk* cDNA and a TATA box 30 bp farther upstream. The cDNA that we have sequenced therefore appears to be almost full-length.

Expression Pattern of the DTK Prohormone—The central nervous system and the intestines of dipteran insects are known to be rich sources of tachykinin-related peptides; and therefore, these tissues, along with 0–24-h embryos, were selected for *in situ* hybridization studies. Strong staining was observed in a group of 14 cells plus one isolated cell in the midgut of stage 17 embryos (Fig. 3). Staining in the midgut was not observed at earlier stages. In addition, at the same stage, *Dtk* was expressed in a pair of medially located unidentified cells, just posterior to the brain, and in two lateral groups of cells that may be associated with tracheae. Expression of *Dtk* in the larval gut was restricted to cells with endocrine cell-like morphology in the posterior midgut, just anterior to the malphigian tubules (Fig. 4, *A* and *B*). Expression in the adult female gut was also restricted to the midgut, with no *Dtk* transcript detected in the hindgut (Fig. 4*C*). However, in contrast to the third instar larval midgut, *Dtk* expressing-cells were detected throughout the length of the adult female midgut, with the exception of a region toward the middle of the midgut (one-third of the midgut length) in which we have not been able to detect *Dtk*-expressing cells in any of the samples examined (more than five specimens). The cells showing a hybridization signal in the female adult midgut also appeared to be endocrine cells; however, they were more numerous, smaller, and more elongated than those staining in the larval midgut (Fig. 4*C*). In the brain of third instar larvae, the *Dtk* transcript was detected in a restricted number of neuronal cell bodies (Figs. 4, *E* and *F*; and 6*A*). There were nine pairs of cell bodies displaying a strong hybridization signal in the brain and another four pairs displaying a weaker signal (12 well labeled central nervous systems were analyzed). In the ventral nerve cord, two pairs of cell bodies were detected in the metathoracic neuromere and eight pairs in abdominal neuromeres (Figs. 4*G* and 6*A*).

Tissue Distribution of Drosophila Tachykinin-related Peptides—Immunocytochemistry was performed on *Drosophila* brain, midgut, and hindgut with an antiserum to the locust peptide LomTK-I. This antiserum is known to cross-react with peptides of the family of invertebrate TRPs, but with no other known peptides. In the larval central nervous system, 22 neurons were consistently labeled with this antiserum. Of these, 18 were in the brain and subesophageal ganglion (Figs. 5, *A* and *B*; and 6*B*). Two pairs of *Locusta* tachykinin-like immunoreactive (LTK-LI) neurons (*DN 1 and DN 2*) with cell bodies in the subesophageal ganglion formed varicose axons that descended throughout the ventral nerve cord (thoracic and abdominal ganglia) (Figs. 5*A* and 6*B*). Two pairs of tritocerebral neurons (*Tr 1 and Tr 2*) formed branches in tritocerebral neurophils (Fig. 5*B*). The remaining five pairs of neurons (*Pr 1–5*) formed processes locally in the protocerebrum (Figs. 5, *A* and *B*; and 6*B*). Two pairs of LTK-LI neuronal cell bodies were seen in the metathoracic part of the ventral nerve cord (Fig. 6*B*). Immunolabeling of cell bodies in the abdominal neuromeres was

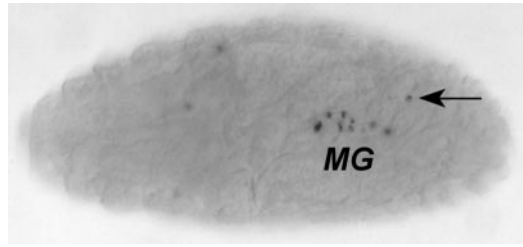

FIG. 3. **Dorsal view of a stage 17 embryo.** Strong hybridization was seen in a group of 14 cells in the midgut (*MG*) plus an isolated cell, also apparently part of the midgut (*arrow*).

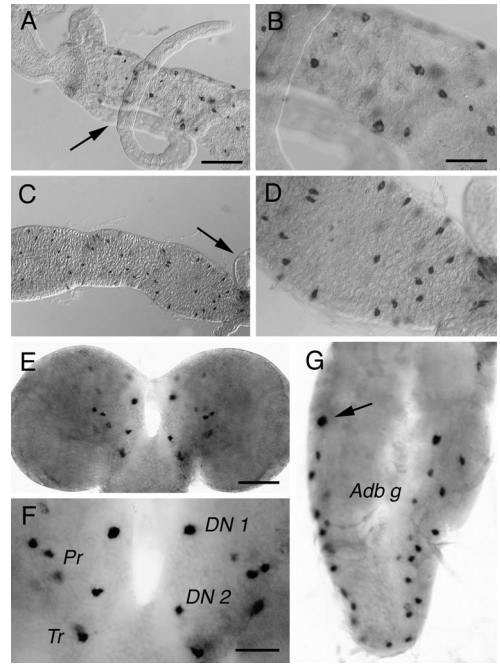

FIG. 4. **Micrographs showing the *Dtk* mRNA transcript in *Drosophila* tissues detected by *in situ* hybridization.** *A*, posterior midgut of third instar larvae. Strong hybridization signals are visible in large endocrine cells. Hybridization was also seen in a number of smaller cells. Malphigian tubules are labeled with an *arrow*. *Bar* = 50 μm. *B*, detail of third instar larval midgut endocrine cells. *Bar* = 25 μm. *C*, adult midgut where a larger number of smaller endocrine cells are seen to contain *Dtk* mRNA compared with the third instar larval midgut. Malphigian tubules are labeled by an *arrow*. *Bar* = 50 μm. *D*, detail of adult midgut endocrine cells expressing *Dtk* mRNA. *Bar* = 25 μm. *E*, distribution of *Dtk* mRNA in neuronal cell bodies in larval brain. *Bar* = 50 μm. *F*, detail of *Dtk*-expressing neuronal cell bodies. *Pr*, protocerebral cell bodies; *Tr*, tritocerebal cell bodies; *DN 1* and *DN 2*, large descending neurons. *Bar* = 25 μm. *G*, distribution of *Dtk* mRNA in the ventral nerve cord of a third instar larva. The *arrow* indicates the metathoracic cell body. Segmentally distributed cell bodies are seen in abdominal ganglia (*Abd g*). *Bar* = 25 μm.

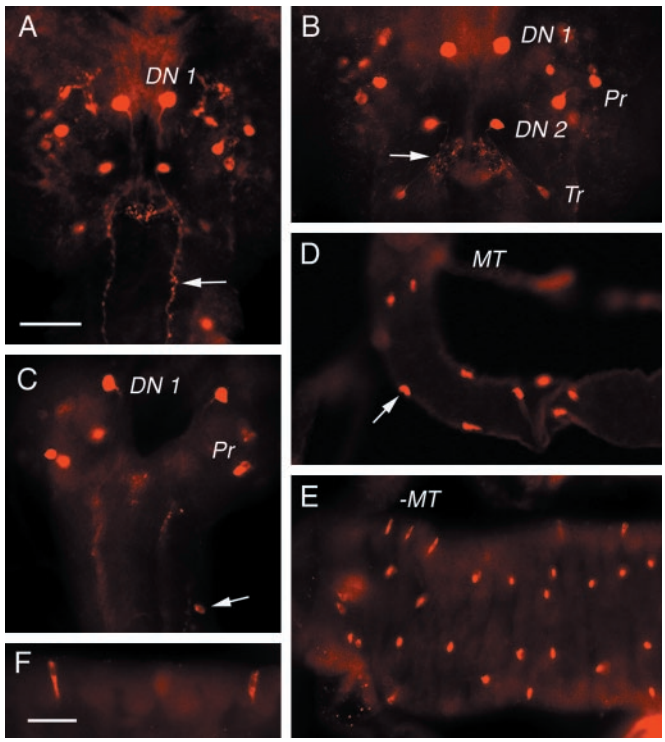

FIG. 5. **Micrographs of tachykinin immunoreactivity in *Drosophila* tissues (using antiserum to LomTK-I).** The tissues were whole-mounted, and a Cy3-tagged secondary antiserum was used. *A*, LTK-LI neurons in the brain and anterior thoracic ganglia of a third instar larva. The anterior large descending neurons (*DN 1*) of the subesophageal ganglion are in focus, and one of their descending axons can be seen at the *arrow*. *B*, detail of LTK-LI cell bodies in the brain of a third instar larva. Focus is on arborizations in tritocerebral neurophils. *DN 1* and *DN 2* (large descending neurons), *Pr* (protocerebral cell bodies), and *Tr* (tritocerebal cell bodies) are identifiable neuronal cell bodies. *C*, LTK-LI neurons in the central nervous system of a second instar larva. In focus are *DN 1* and a few protocerebral cell bodies. *D*, posterior midgut of a third instar larva (just anterior to the Malpighian tubules (*MT*)). Note a few large endocrine cells displaying immunoreactivity (*e.g.* at the *arrow*). *E*, posterior midgut of an adult fly. A larger number of smaller LTK-LI endocrine cells can be seen. *F*, detail of two endocrine cells in side view. The gut lumen is toward the bottom of figure, and the gut surface to the body cavity is at the top. Note the unstained nuclei toward the apical end of the cells.

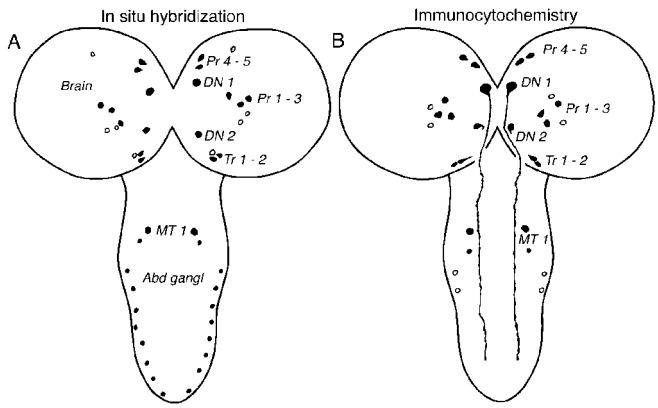

FIG. 6. *A*, tracing of cell bodies expressing *Dtk* mRNA in the central nervous system of a third instar larva. Cell bodies displaying strong hybridization signals are shown in *black*, and weaker signal are depicted by *unfilled* cell bodies. A strong signal is seen in five pairs of smaller protocerebral cell bodies (*Pr 1–5*) and two pairs of larger cell bodies (*DN 1* and *2*) in the subesophageal ganglion as well as two pairs of tritocerebal cell bodies (*Tr 1–2*). In the ventral nerve cord, there are two pairs of cell bodies in the metathoracic ganglion (*MT 1*) and eight pairs in the abdominal ganglia (*Abd gangl*). *B*, tracing of LTK-LI neurons in a third instar larva (strongly labeled neurons shown as *black* and *unfilled* are weakly labeled). The axons are traced only for the large descending neurons (*DN 1*) with cell bodies in the subesophageal ganglion. The other pair of subesophageal descending neurons (*DN 2*) have similar axons, running more laterally than those of *DN 1*. Five pairs of protocerebral (*Pr 1–5*) and two pairs of tritocerebral (*Tr 1–2*) LTK-LI cell bodies were seen in all specimens. In the metathoracic ganglion, two pairs of neurons were detected (*MT 1*). In abdominal ganglia, immunolabeling was variable; often only anterior located cell bodies were seen.

inconsistent, but occasionally, segmentally arranged cell bodies could be seen. The same neurons were also present in the brain and ventral nerve cord of second instar larvae (Fig. 5*C*). In the adult brain of *Drosophila*, there are many more LTK-LI neurons, and they form complex arborizations in brain neuropils (18). The two pairs of descending neurons (*DN 1 and DN 2*) were also seen in adults. In the adult ventral nerve cord, the metathoracic cell bodies were still present, in addition to a pair of large cell bodies in the posterior part of the abdominal neuromeres (data not shown).

In the midgut of third instar larvae, there were ~20 LTK-LI cells with a morphology similar to that of endocrine cells (Fig. 5*D*). These were found only in the posterior region of the midgut, just anterior to the attachment of the Malpighian tubules. The cells were rather large, with a diameter of ~9.5 ± 0.27 μm (mean ± S.E., n = 38; from three intestines). In the adult midgut, there were a much larger number of slightly smaller LTK-LI endocrine cells (Fig. 5*E*). These had an elongated shape and often spanned the gut epithelium (Fig. 5*F*). Their diameters were 4.4 ± 0.13 μm (mean ± S.E., n = 36; from three intestines). As seen by *in situ* hybridization, the LTK-LI endocrine cells were restricted to the anterior and posterior thirds of the midgut.

Biological Activity of the Drosophila Tachykinins—Three of the DTK peptides (DTK-1–3) and LemTRP-1 were tested in the *L. maderae* hindgut contraction assay. This was done since all of the other arthropod TRPs were isolated with the aid of this assay, alone or in combination with enzyme immunoassay, and dose-response curves have been constructed for all (19). Dose-response curves for each of these peptides are shown in Fig. 7. DTK-1 and -3 were about equally bioactive. The concentrations yielding a half-maximum response (EC_{50}) were ~3.6×10^{-8} M for DTK-1 and 5.1×10^{-8} M for DTK-3. DTK-2 was nearly an order of magnitude less bioactive, with an EC_{50} of 1.5×10^{-7} M. In comparison, LemTRP-1 was almost an order of magnitude more bioactive than DTK-1 and -3 ($EC_{50} = 4.9 \times 10^{-9}$ M). The differences in EC_{50} values between LemTRP-1 on one hand and DTK-1 and -3 on the other were statistically significant ($p < 0.01$; analysis of variance), as was that between DTK-1 and -3 and DTK-2 ($p < 0.05$).

The myotropic activity of all five DTK peptides was tested on midguts from adult *D. melanogaster*. This tissue was selected because results from both immunocytochemistry and *in situ* hybridization had revealed high densities of *Dtk*-expressing midgut endocrine cells. The majority of midguts removed from adult flies were not spontaneously active, but responded to the addition of supra-threshold concentrations of DTK-1 to the perfusate with several phasic contractions that lasted for several minutes (Fig. 8). The peak height of the contractions was dose-dependent and was used to generate dose-response curves. Fitted curves are shown in Fig. 9, and EC_{50} values are listed in Table II. When tested on adult midgut, the rank order of potency was DTK-3 > DTK-5 > DTK-1 > DTK-2 = DTK-4. To compare their efficacy, each DTK peptide was tested at 1 μM on the same preparation, and the peak height of contraction was expressed as a percentage of the maximum response. In general, this concentration is sufficient to evoke a maximum response (Fig. 9), although the dose-response curve for DTK-2

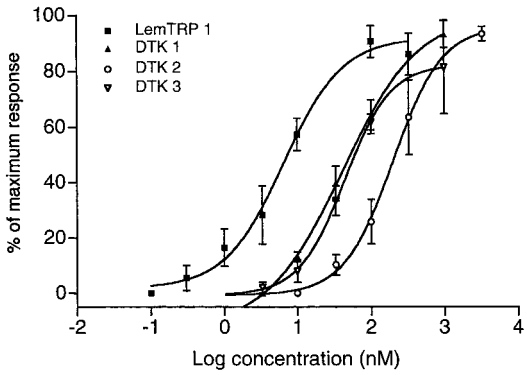

FIG. 7. **Dose-response curves for the myostimulatory activity of three of the DTK peptides on isolated *L. maderae* hindgut.** As a reference, the *L. maderae* peptide LemTRP-1 was tested. Each point represents the mean ± S.E. of three to five replicates. Note that LemTRP-1 is about an order of magnitude more bioactive than the two best DTK peptides.

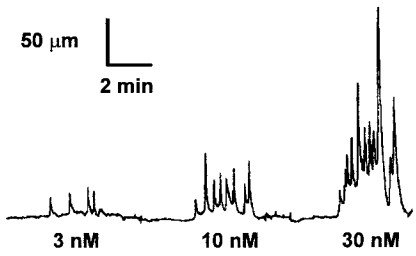

FIG. 8. **Representative trace showing the effect of 3–30 nM DTK-1 upon contraction of isolated *D. melanogster* adult midgut.** *Scale bars* indicate amplitude of contraction (*y axis*) and time (*x axis*).

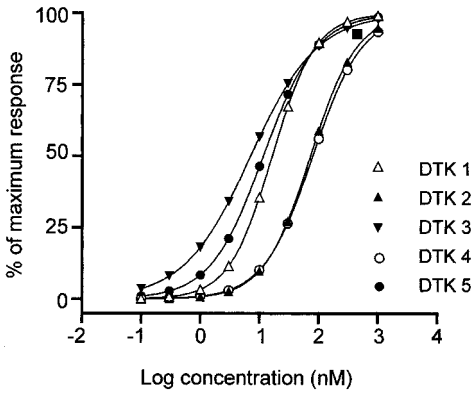

FIG. 9. **Dose-response curves for the myostimulatory activity of each of the five DTK peptides on isolated *D. melanogaster* adult midgut.** The curves presented are the mean results of experiments performed on five to six individual midguts.

does not appear to plateau. The experiment was repeated seven times, varying the order in which the peptides were added to the gut. The results are included in Table II. When tested at 1 μM, the activities of DTK-1, -3, -4, and -5 did not differ significantly, whereas the response to DTK-2 was <50% maximal. The small amount of synthetic DTK-2 available precluded testing this peptide at higher concentrations. However, it is clear that DTK-2 has both low potency and low activity on the midgut of adult *D. melanogaster*. Similar EC_{50} values were also obtained using larval midgut (data not shown).

DISCUSSION

A BLAST search using the human PPT-A amino acid sequence led to the identification of a *D. melanogaster* preprotachykinin gene (*Dtk*). Some minor errors in the *ab initio* prediction of the coding region from the genomic sequence were evident from comparison with the deduced amino acid sequence of a *Dtk* cDNA amplified from a head cDNA library. The genomic region containing the initial exon was not included in the original prediction due to the large size (9.2 kilobase pairs) of the first intron. In addition, the predicted boundary between the second and third exons appears to have been predicted incorrectly.

TABLE II
Effect of Drosophila TRPs on Drosophila *adult midgut*

EC_{50} values for DTK-1–5 were calculated from the dose-response curves shown in Fig 9. The effect of 1 μM of each DTK upon adult midgut is shown in the third column. DTK-2 demonstrated both low potency and low activity compared with the other DTK peptides as judged by its higher EC_{50} value and the poor response to the peptide when tested at 1 μM.

Peptide	EC_{50}	95% confidence limits	Maximum response (mean ± S.E. (*n*))
	nM	*nM*	%
DTK-1	16.3	12.4–21.5	84.5 ± 6.2 (7)
DTK-2	72.4	51.2–102.3	44.2 ± 6.3 (7)
DTK-3	7.0	4.5–11.1	86.9 ± 4.6 (7)
DTK-4	79.1	65.9–95.0	80.7 ± 6.6 (7)
DTK-5	11.5	7.4–18.0	86.8 ± 4.2 (7)

Each of the tachykinin-like sequences in *Dtk* is present in a glycine-extended form flanked by pairs of basic residues. This is a common feature of many insect peptide hormone precursors, where the initial processing step involves hydrolysis of the peptide bond on the C-terminal side of a pair of basic amino acids (40). The presence of multiple members of a peptide family in a single prohormone is common in insects, and this prohormone structure is somewhat similar to that seen in a gene encoding tachykinins from the echiuroid worm *U. unicinctus* (41). This gene contains seven copies of TRPs, and in this respect, *Dtk* appears to be more similar to the echiuroid TRP gene than to the sialokinin gene from the mosquito *A. aegypti*, which encodes a single tachykinin peptide, exclusively expressed in mosquito salivary glands (24).

Tissue-specific splice variants of the mammalian tachykinin prohormone mRNA are known to occur (7), and it is therefore interesting to note that the genomic organization of the *Dtk* gene provides the potential for alternative mRNA splicing to produce all five DTK peptides or just DTK-1–4 since the DTK-5 progenitor sequence is coded for by a separate exon. Evidence for tissue-specific expression of insect tachykinins comes from analysis of the distribution of LemTRPs in the cockroach *L. maderae*, where LemTRP-3 and -4 have been isolated only from the midgut, and LemTRP-6–9 appear to be brain-specific (11, 12). However, preliminary reverse transcriptase-polymerase chain reaction experiments to detect an alternatively spliced *Dtk* transcript lacking the third exon in *Drosophila* adults and third instar larvae have not identified an alternative transcript.[2]

Conserved structural features of the DTK peptides include an N-terminal Ala-Pro sequence and the C-terminal FX_1GX_2R-amide motif, where X_1 is Ile, Leu, Val, or Thr, and X_2 is Met or Leu. This F*X*G*X*R-amide motif is a common feature of almost all invertebrate tachykinins isolated to date (19), and the Arg-amide residue is required for their biological activity in the cockroach hindgut bioassay (42). The N-terminal Ala-Pro residues are likely to protect the peptides from proteolytic degradation by aminopeptidases since few peptidases are capable of hydrolyzing peptide bonds adjacent to prolyl residues due to the structural constraints imposed by the inclusion of this imino acid (43). This N-terminal motif is therefore common among invertebrate peptides (19), and a prolyl residue is also

[2] R. J. Siviter, unpublished results.

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                  *  *  * *  *** *            *          **     ****  **
TKN1_Human  : RRPKPQQFFGLMGKRDADSSIEKQVALLKALYGHGQISHKRHKTDSFVGLMGKR : 54
preproDTK   : KR-APTGFTGMRGKRPALLAGDDDAEADEAT----ELQQKRAPVNSFVGMRGKK : 49
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FIG. 10. **ClustalX alignment of prepro-DTK and human PPT-A amino acid sequences.** The overall sequence identity was 8%. Only the region that aligns with the substance P and neurokinin A progenitor sequences is shown. In this region, sequence identity rises to 31%.

present at an equivalent position in a number of mammalian peptides. However, proline-specific dipeptidyl peptidase activity can selectively cleave the Xaa-Pro-containing TRPs and vertebrate tachykinins (44, 45).

Although we have presented DTK-1 and -2 as a decapeptide and nonapeptide, respectively (see Table I), and have used these synthetic peptides for assessing biological activity, the presence of an alternative N-terminal dibasic processing site in each case allows the possibility that N-terminally extended forms of both peptides may be produced. The sequences of the resulting peptides would be VVKRAPTSSFIGMR-amide and LKKAPLAFVGLR-amide, respectively. These sequences are reminiscent of the mammalian tachykinins neuropeptide K and neuropeptide γ, both of which are N-terminally extended forms of neurokinin A and contain an unprocessed Lys-Arg motif. Several examples of invertebrate N-terminally extended TRPs also exist, *e.g.* the *Leucophaea* TRPs LemTRP-2 and -3, which contain unprocessed KR and KK sites, respectively (11). Whether these *Leucophaea* extended TRPs represent processing intermediates or are final products in some tissues is not clear, but both are biologically active in a *Leucophaea* hindgut contraction bioassay, and LemTRP-1 and -3 show similar EC_{50} values using this assay (11, 33). It would therefore appear that if similar N-terminally extended DTK peptides exist, they would most likely be biologically active. The ability to produce N-terminally extended peptides therefore seems to be a conserved feature of both vertebrate and invertebrate tachykinin prohormones. Neurokinin A, neuropeptide K, and neuropeptide γ display different affinities for the mammalian NK2 tachykinin receptor (46), and it is possible that N-terminally extended DTK peptides would also show differing affinities for their endogenous receptors.

A number of additional amidated peptides are also potential products of processing of prepro-DTK at pairs of basic residues (see Table I). Of these, TAP-5 seems to bear the most resemblance to a TRP, although there is an alanine residue instead of the glycine in the F*X*G*X*R-amide motif, which represents a conservative change. In this respect, this peptide is similar to the recently characterized stomoxytachykinin from the stable fly *S. calcitrans* (APTGFFAVR-amide), which is capable of inducing contractions in the *Leucophaea* hindgut contraction bioassay (37). It is not clear whether any of the peptides shown in Table I have physiological roles, but the fact that three of them could be produced as amidated peptides suggests that they may possess biological activity.

Immunocytochemistry with an antiserum recognizing the conserved C-terminal pentapeptide of insect TRPs labeled a small number of neurons in the larval central nervous system of *Drosophila*. All of these correspond to neurons displaying the *Dtk* transcript as shown by *in situ* hybridization. The only prominent discrepancy between *Dtk* transcript distribution and TRP immunoreactivity is seen in the abdominal ganglia: segmentally distributed cell bodies always display the transcript, but immunolabeling is variable in these neurons. Two pairs of subesophageal neurons are descending neurons of the subesophageal ganglion with axons supplying varicose processes to all the neuromeres of the thoracic-abdominal nerve cord. The others appear to be local interneurons. By comparison, the blowfly displays a larger number of LTK-LI neurons both in the brain and ventral nerve cord (30). The distribution of LTK-LI cells in the larval and adult midgut of *Drosophila* is very similar to that revealed in the blowfly *Calliphora* (47). In both fly species, the larval endocrine cells are fewer but substantially larger than their adult counterparts. The larval midgut is degraded during metamorphosis, and the adult midgut develops from special anlagen (15). Thus, the endocrine cells of the midgut do not persist during post-embryonic development, in contrast to neurons of the central nervous system. High levels of *Dtk* expression were observed in cells of the midgut from late embryogenesis to the adult stages, which indicates the functional importance of these gut peptides in all feeding stages. As was observed with the immunocytochemistry, *Dtk*-expressing cells in the adult midgut were both smaller and more numerous than those seen in the midgut of third instar larvae.

The distribution of LTK-LI material suggests multiple functions for DTK peptides as both neuroactive compounds and regulators of gut function (brain-gut peptides). In the central nervous system, the LTK-LI neurons are all interneurons, as in most other insects studied so far (19). The DTK peptides tested (DTK-1–3) were all able to stimulate muscle contractions in *L. maderae* hindgut, as has been shown for all known arthropod TRPs (19). Their bioactivities were, however, not as strong as that of LemTRP-1, which showed an EC_{50} value almost an order of magnitude lower than that observed for the best DTK peptides. We were also able to demonstrate biological activity for all five DTK peptides using a *Drosophila* midgut bioassay. In this assay, the biological activity of each of the peptides proved to be considerably better than that demonstrated using the *Leucophaea* hindgut assay. The calculated EC_{50} values ranged from 7 to almost 80 nM and, as such, are similar to those reported for LemTRPs in the *Leucophaea* assay (19). The relatively poor response of the *Leucophaea* hindgut preparation to the DTK peptides is therefore likely to be a consequence of the heterologous nature of the assay. Although we have demonstrated that each of the DTK peptides is capable of stimulating muscle contraction in the midgut of both larval and adult *D. melanogaster*, it will be interesting to investigate what other aspects of midgut physiology are controlled by these peptides. In addition, there is a possibility that the *Dtk*-expressing/LTK-LI midgut endocrine cells might release DTK peptides to act in an endocrine manner at other sites in the organism.

The fact that we were able to identify a stretch of *Drosophila* genomic sequence containing a *Dtk* gene using the human PPT-A amino acid sequence points to the existence of a common evolutionary ancestor for the vertebrate and insect tachykinins. Although the overall sequence identity between human PPT-A and prepro-DTK is low (8%), the identity increases to 31% in the region of prepro-DTK that aligns with the substance P and neurokinin A portion of human PPT-A (Fig. 10). However, the gene structures of *Dtk* and mammalian tachykinin genes appear to be very divergent, *e.g.* six introns are present within the genomic sequence of the rat PPT-A gene (*Tac1*) (6), whereas only three are found in the *Dtk* genomic sequence, none of which are conserved between the two genes. In addition, *Dtk* codes for five copies of TRPs, whereas *Tac1* contains only substance P and neurokinin A plus the two N-terminally extended forms of neurokinin A: neuropeptide K and neuropeptide γ. It therefore seems likely that an ancestral tachykinin prohormone gene contained either a single tachykinin-like se-

quence or two tachykinin-like sequences, which were not separated by an intron in the genomic sequence. In this hypothesis, duplications following the divergence of the arthropod and vertebrate lineages would have increased the number of TRP progenitor sequences present in the arthropod prohormone. TRPs therefore appear to be evolutionarily ancient. Further evidence for this is provided by the fact that NKD and DTKR, the two *Drosophila* tachykinin receptors characterized to date, show high homology to cloned mammalian tachykinin receptors, and expressed DTKR is able to respond to the vertebrate tachykinins substance P and physalaemin (21, 22). As we have demonstrated in this report, the neuronal and gut distribution of mammalian tachykinins and the ability to stimulate gut muscle contraction are also conserved in *Drosophila*. With the identification of *Dtk*, it appears that the entire tachykinin-mediated signaling system is functionally conserved in *Drosophila*.

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Expression and Functional Characterization of a*Drosophila* Neuropeptide Precursor with Homology to Mammalian Preprotachykinin A

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