Identification of the Gene Encoding Bursicon, an Insect Neuropeptide Responsible for Cuticle Sclerotization and Wing Spreading

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Summary

To accommodate growth, insects must periodically replace their exoskeletons. After shedding the old cuticle, the new soft cuticle must sclerotize. Sclerotization has long been known to be controlled by the neuropeptide hormone bursicon [1, 2], but its large size of 30 kDa has frustrated attempts to determine its sequence and structure. Using partial sequences obtained from purified cockroach bursicon [3], we identified the *Drosophila melanogaster* gene *CG13419* as a candidate bursicon gene. *CG13419* encodes a peptide with a predicted final molecular weight of 15 kDa, which likely functions as a dimer. This predicted bursicon protein belongs to the cystine knot family, which includes vertebrate transforming growth factor-β (TGF-β) and glycoprotein hormones [4]. Point mutations in the bursicon gene cause defects in cuticle sclerotization and wing expansion behavior. Bioassays show that these mutants have decreased bursicon bioactivity. In situ hybridization and immunocytochemistry revealed that bursicon is co-expressed with crustacean cardioactive peptide (CCAP). Transgenic flies that lack CCAP neurons [5] also lacked bursicon bioactivity. Our results indicate that *CG13419* encodes bursicon, the last of the classic set of insect developmental hormones. It is the first member of the cystine knot family to have a defined function in invertebrates. Mutants show that the spectrum of bursicon actions is broader than formerly demonstrated.

Results and Discussion

We identified *CG13419* using a modified protein BLAST search [6] of the *Drosophila* genome [7] using the *P. americana* partial bursicon peptide sequences obtained by microsequencing [3] (Figure 1). Sequence analysis of the genomic clone and its corresponding cDNA revealed that the coding sequence is 522 nucleotides long. The gene contains three short exons (130, 125, and 267 nucleotides, respectively) and two introns

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(64 and 58 nucleotides, respectively). The *CG13419* gene product is predicted to be a 173 amino acid preprotein (19 kDa). Removal of the predicted N-terminal signal sequence of 33 amino acids [8] would result in a mature protein of 140 amino acids (Figure 1), approximately 15 kDa. CG13419 is a member of the 10-membered cystine knot protein family [4], which typically forms dimers. Members of this family contain six cysteine residues that form the knot and an optional additional cysteine that may be important for dimerization [4, 6] (Figure 1). They include the glycoprotein hormones, TGF-β, platelet-derived growth factor, and the mucins. The sequence of *CG13419* is most similar to that of the mucin subfamily, which also includes signaling molecules such as the Bone Morphogenic Protein antagonists [4]. Previous data indicate that bursicon functions as a 30 kDa dimer in the cockroach [9]. The available sequence information for the homologous partial peptide sequences from the *Anopheles gambiae* and *Apis mellifera* genomes show 83% identity to CG13419 (Figure 1). The cysteines within the cystine knot domain are conserved in these species.

In the course of a genetic screen for flies that failed to expand their wings, we identified five mutants that formed a single complementation group that mapped to the vicinity of *CG13419*. Genomic sequencing of these mutants showed that each has an independent single base pair change at *CG13419*. All have wing spreading and sclerotization defects when either homozygous or heterozygous over deficiencies for the region. Four mutants have sequence changes that are predicted to cause significant alterations of the mature protein structure (Figure 1). The *burs^Z4410* mutation is a G to A conversion at the splice acceptor site for the second exon, which likely prevents normal splicing. This mutation would lead to read-through of the first intron, resulting in premature termination due to the presence of two consecutive stop codons. Since the mutation is located 194 bp from the translation initiation site, the resulting protein product would be comprised of the signal sequence and only 12 additional amino acids (one mutated) and is likely nonfunctional. Three other alleles have mutations within the coding region and either remove or introduce a cysteine residue and thus are likely to disrupt disulfide bridge formation, a key structural feature of cystine knot proteins. The *burs^Z1091* allele results in a conversion of cysteine residue 82 to tyrosine, removing a cysteine that is conserved among all members of the mucin subfamily of cystine knot proteins. The *burs^Z1140* mutation results in a conversion of threonine residue 97 to cysteine. The *burs^Z5569* mutation results in a conversion of glycine residue 115 to cysteine. The fifth mutation, *burs^Z2803*, appears to be a mutation within the regulatory region. It results in a C to T change at −203 base pairs from the translation start site.

The phenotypes of two bursicon mutants, *burs^Z1091* and *burs^Z5569*, were examined in detail. Flies homozygous for either mutation, heteroallelic *burs^Z5569*/*burs^Z1091* or heterozygous over deficiencies for the region, failed to spread their wings following eclosion and showed a

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                                                    Z4410
                                                    G I * *
P.a.  - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -   0
D.m.  M L R H L L R H E N N K V F V L I L L Y C V L V S I L K L C T A Q P D S S V A A T D N D I T H L G D D C Q V T P V I H   59
A.g.  - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - A S E D G G S H Y S S D D C Q V T P V I H   21
A.m.  - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - D E C Q A T P V I H   10

                                                              ▼                          Z1091
                                                                                         Y
P.a.  - - - - - - - - - - - - - - - - - - - - - - D G S S Y - - L Q V S G S K I W Q M E R - - - - - - - - - E A S V N N V - - -   25
D.m.  V L Q Y P G C V P K P I P S F A C V G R C A S Y - - I Q V S G S K I W Q M E R S C M C C Q E S G E R E A A V S L F C P   116
A.g.  V L Q Y P G C V P K P I P S F A C I G R C A S Y - - I Q V S G S K I W Q M E R S C M C C Q E S G E R E A S V S L F C P   78
A.m.  F L Q Y P G C V P K P I P S Y A C R G R C S S Y L F L Q V S G S K I W Q M E R S C M C C Q E S G E R E A S V S L F C P   69

             Z1140                          Z5569
             C                              C
P.a.  - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -   25
D.m.  K V K P G E R F K K V L T K A P L E C M C R P C T S I E E S G I I P Q E I A G Y S D E G P L N N H F R R I A L Q   173
A.g.  K A K N G E K K F R K V - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -   90
A.m.  R A K P G E K K F R K V K L L L - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -   85
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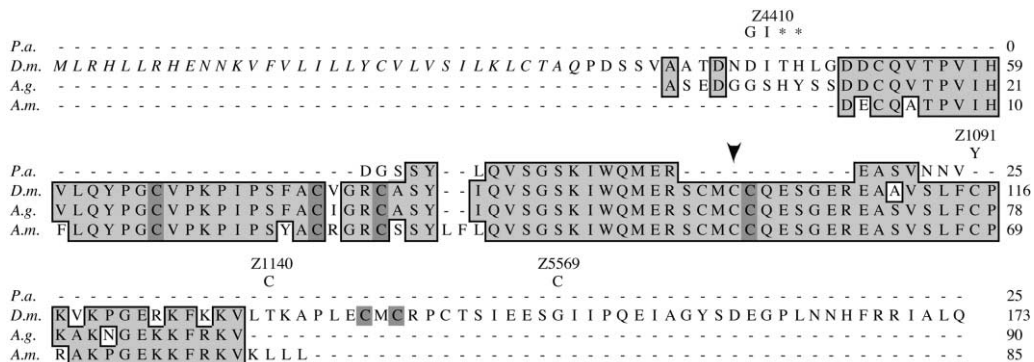
Figure 1. Sequence of the *Drosophila melanogaster* Bursicon Protein and Alignment with Other Insect Bursicon Sequences

The predicted 33 amino acid signal sequence is in italics. The lightly shaded boxed residues indicate identical amino acids between species, and the six highly conserved cysteine residues within the cystine knot domain [4] are in darker shading. An arrowhead indicates the additional cysteine residue predicted to be necessary for covalent dimer formation. The lesions present in mutant alleles *burs^{Z4410}*, *burs^{Z1091}*, *burs^{Z1140}*, and *burs^{Z5569}* are indicated. The three *Periplaneta americana* (*P.a.*) sequences used to identify the CG13419 gene are DGSSYLQVSGSK, IWQMER, and EASVNNV [13]. *A.g., Anopheles gambiae*; *A.m., Apis mellifera*; *D.m., Drosophila melanogaster*.

prolonged retention of the elongate abdomen shape characteristic of a newly eclosed fly (Figures 2A–2C). By the day after eclosion, their abdomens shortened but failed to taper into the normal adult shape, consistent with a failure to sclerotize properly. The two alleles differed in their effects on the timing of cuticle pigmentation and the expansion of the thoracic cuticle. The *burs^{Z5569}* mutants were delayed in melanization, with only 40% completing it during the 3 hr following eclosion (n = 35), but they inflated their thoracic cuticle normally. In contrast, *burs^{Z1091}* mutants pigmented normally (n = 36) (Figures 2B and 2C) but failed to complete thorax expansion, resulting in the postscutellar bristles remaining crossed (Figures 2D–2F). Flies that were heteroallelic for the two mutations (*burs^{Z5569}*/*burs^{Z1091}*) showed characteristics of both mutants. They resembled *burs^{Z5569}* mutants in being slow to pigment, with only 18% completing pigmentation within 3 hr of eclosion (n = 22). However, they had crossed postscutellar bristles like *burs^{Z1091}* homozygotes. Both bursicon mutations are recessive, displaying no wing expansion or sclerotization defects when in heterozygous combinations with complementing deficiencies, nonallelic mutants, or balancer chromosomes (data not shown).

The levels of bursicon produced by mutant larvae were determined using bursicon bioassays based on neck-ligated *Sarcophaga bullata* [10, 11] or *Drosophila* [12]. In the *Sarcophaga* bioassay, extracts of CNSs from control *bw; st* larvae stimulated tanning with an average score of 2.50 ± 0.18. By contrast, CNS extracts from *burs^{Z1091}* mutant larvae yielded an average score of 0.27 ± 0.08, similar to that obtained with phosphate buffer controls (Figure 3). (In the *Sarcophaga* bioassay, bursicon activity below 10% of maximum activity cannot be detected [11]. Thus, the level of bursicon in *burs^{Z1091}* flies is less than 10% of that of wild-type.) *burs^{Z5569}* flies gave a low but measurable average score of 0.85 ± 0.63. Extracts from *burs^{Z5569}*/*burs^{Z1091}* and *burs^{Z5569}*/*Df (3R) e-GC3* yielded low scores (0.81 and 0.75, respectively), consistent with the hypothesis that the mutations in CG13419 are solely responsible for decreased bursicon bioactivity. Extracts from *burs^{Z5569}*/*TM6B Tb* (essentially *burs^{Z5569}*/+) larvae gave scores of 2.15 and 2.20, which are similar to those of the *bw; st* control.

We also assayed the blood of newly emerged flies for bursicon activity using a *Drosophila* test system [12]. Injection of hemolymph from control *bw; st* flies into neck-ligated control *bw; st* or bursicon mutant flies resulted in tanning. In contrast, the hemolymph from homozygous *burs^{Z5569}* or *burs^{Z1091}* mutants of the same age failed to cause neck-ligated control flies to tan (Table
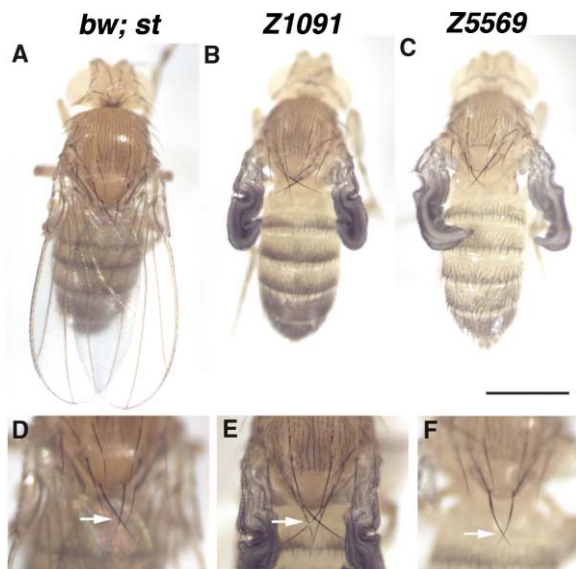

Figure 2. Phenotype of *Drosophila melanogaster* Wild-Type and Mutant Male Flies 2–2.5 hr Posteclosion

(A) A control *bw; st* fly showing normal wing expansion and melanization.

(B and C) Flies from both bursicon mutant strains *burs^{Z1091}* (B) and *burs^{Z5569}*(C) fail to expand their wings and retain an elongated abdomen. Note also defects in abdominal melanization in (C).

(D–F), Crossing of postscutellar bristles (arrows). The pronounced crossing in *burs^{Z1091}* (E) indicates a failure in thorax expansion. Scale bar equals 0.5 mm. The flies were raised, collected, aged, and photographed in parallel.
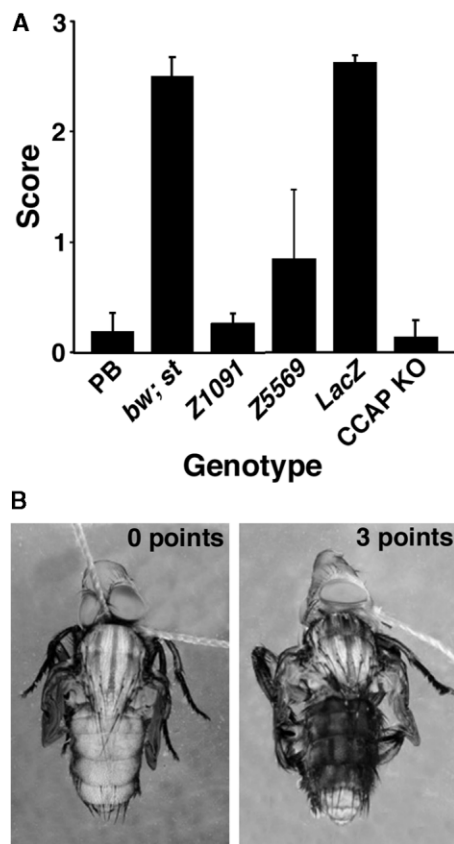
Figure 3. Results of the Neck-Ligated *Sarcophaga* Bioassay

(A) Bursicon bioactivity in homogenates from positive control (*bw; st* and *LacZ*), bursicon mutant (*burs^Z1091* and *burs^Z5569*), or CCAP KO third instar larval nervous systems. PB, phosphate buffer (negative control). At least ten flies were injected per experiment and then averaged for three experiments ± standard deviation. Abbreviations: *LacZ, CCAP-GAL4/UAS-LacZ* [20]; CCAP KO, *CCAP-GAL4/UAS-rpr, UAS-LacZ* [11].

(B) Neck-ligated *Sarcophaga* injected with either PB (left; 0 points) or a homogenate of wild-type *Drosophila melanogaster* third instar larval nervous systems (right; 3 points).
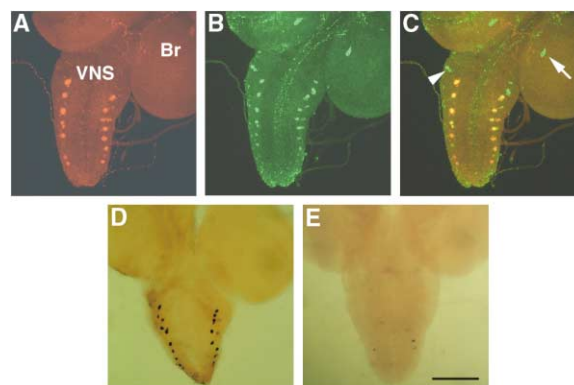

Figure 4. Bursicon mRNA Localization in the Central Nervous System of Third Instar Larval *Drosophila melanogaster*

(A–C) Confocal images of bursicon RNA expression in CCAP-immunoreactive neurons.

(A) In situ labeling of bursicon mRNA. Bursicon is found in distinct bilateral neurons in the ventral nervous system (VNS).

(B) Corresponding pattern of CCAP immunoreactivity in the same preparation.

(C) Superimposition of (A) and (B) showing that bursicon RNA is expressed in all CCAP-immunoreactive neurons except for two pairs in the brain and two pairs in the first thoracic neuromere (arrow and arrowhead, respectively).

(D and E) Detection of bursicon mRNA in the CNS of control (D) and CCAP KO (E) third instar larvae. In this CCAP KO example, three surviving neurons show weak staining. Br, brain. Scale bar equals 75 μm.

1). These results are consistent with decreased bursicon activity in the mutants. We do not currently understand why *burs^Z1091* fails to cause pigmentation in these bioassays despite the fact that when homozygous or in heterozygous combinations with deficiencies, *burs^Z1091* mutants pigment normally. The amino acid changes in the two mutants may result in differences of their interaction with the receptor and/or in aspects of transport, metabolism, and stability of the mutant peptides.

The neurons that express the bursicon gene were identified using in situ hybridization. As seen in Figures 4A and 4D, labeling using the bursicon antisense probe revealed one to two pairs of neurons in each of the thoracic and abdominal neuromeres of the larval CNS. No labeling was seen with the sense probe (not shown). Since bursicon is co-expressed in some of the neurons that express the neuropeptide CCAP in a number of other insects [3, 13], we examined whether this relationship also held for *Drosophila*. By double labeling with an antiserum directed against CCAP, we found that bursicon transcripts are indeed expressed in a set of the neurons that contain CCAP (Figures 4B and 4C). However, as in other insects [3], not all CCAP neurons expressed bursicon. Bursicon transcripts were not detected in two pairs of CCAP-immunoreactive (ir) neurons in the brain or in two pairs in the first thoracic neuromere (Figure 4C). Antibodies directed against amino acids 91–108 of the bursicon sequence also labeled the CCAP-ir cells in the ventral nervous system (VNS). Using

	Donor Strain							
	bw; st		*burs^Z5569*		*burs^Z1091*		Uninjected Control	
Recipient Strain	# inj	Tanned	# inj	Tanned	# inj	Tanned	#	Tanned
bw; st	21	20	12	0	11	0	18	0
burs^Z5569	15	15	nd	nd	nd	nd	12	0
burs^Z1091	14	13	nd	nd	nd	nd	15	0

Table 1. *Drosophila* Hemolymph Injections

Abbreviations: nd, test not performed; # inj, number injected

this antibody, we detected a wild-type pattern of bursicon-ir neurons in the *burs^Z5569* and *burs^Z1091* mutants (data not shown), indicating that these mutants produce bursicon protein, although it has greatly reduced biological activity.

Transgenic *Drosophila* bearing targeted ablations of CCAP neurons (CCAP KO flies) are defective in wing expansion and tanning and have low survival to adulthood [5]. In situ hybridization of the CNS of CCAP KO larvae using a bursicon antisense probe showed a significant reduction in the number of labeled neurons (n = 10), as would be expected if CCAP neurons expressed bursicon. In some preparations, a few surviving neurons weakly expressed the CG13419 message in the posterior VNS (Figure 4E), consistent with previous findings [5]. Similarly, we predicted that homogenates of CNSs from the CCAP KO flies would not contain bursicon bioactivity if tested in the neck-ligated *Sarcophaga* bioassay. Figure 3 shows that the homogenates from CCAP KO flies contained little or no bursicon activity in the *Sarcophaga* bioassay (0.14 ± 0.07), whereas homogenates of the relevant control flies *(LacZ)* produced a score (2.62 ± 0.15) similar to that of wild-type flies.

The phenotype of *burs* mutants is similar to that of *rickets* (*rk*) mutants, which also fail to sclerotize, pigment, and expand their wings [12]. The *rk* gene encodes a G protein-coupled receptor with a large, leucine-rich extracellular domain and was proposed to be a bursicon receptor. It belongs to a subfamily of receptors whose ligands include the vertebrate glycoprotein hormones [14], which are also cystine-knot proteins. The similarity in phenotype between *burs* and *rk* mutants suggests that they may represent the ligand and receptor for this hormonal signaling pathway. Nevertheless, flies that were transheterozygous for mutant alleles of both genes (*rk^4*/+; *burs^Z1091*/+ and *rk^4*/+; *burs^Z5569*/+) showed no defects in wing expansion or tanning. Additional detailed experiments will need to be carried out to determine whether these two molecules interact.

Conclusions

The homology to peptide sequences from cockroach bursicon and other insect species, the phenotypes and reduction in bursicon activity in mutants of this gene, and the localization of its transcripts to neurons known to produce bursicon in other insect species make a convincing case that *CG13419* encodes the tanning hormone bursicon. Hence, 42 years after its discovery [1, 2], the structure of the last of the classic insect developmental hormones has finally been elucidated. Bursicon is the first member of the cystine knot family of signaling molecules to have a defined hormonal function in invertebrates. Knowledge of the molecular identity of bursicon now provides tools for the analysis of its function, including the mechanism of its tightly timed action in triggering the biochemical events [15] that lead to cuticle hardening. The colocalization of bursicon with CCAP, a peptide that activates the ecdysis motor program [5, 16–18], poses the fascinating problem of how these two hormones are differentially regulated to produce two temporally distinct behaviors. The understanding of the neuroendocrine control of cuticle sclerotization may

also have potential for the development of novel insect-specific pest control agents and measures.

Experimental Procedures

Identification and Isolation of *CG13419*

Partial amino acid sequences from *P. americana* [3] were used in a protein BLAST search against the *Drosophila melanogaster* genome database ("search for short nearly exact matches") [6, 7] to identify the CG13419 gene product. PCR primers (B3F 5′-ATGCTGCGC CACCTGCTCCGC-3′ and B3R 5′-CTATTGCAGAGCAATGCGCCG-3′) were used to amplify the complete *CG13419* genomic and cDNA sequences using the polymerase chain reaction (PCR) and reverse transcription PCR (Roche), respectively. The resulting PCR products were cloned using a TOPO TA cloning kit (Invitrogen) and sequenced using Big Dye chemistry (Applied Biosystems) with an ABI PRISM 377 DNA sequencer. The genomic and cDNA clones were identical to the CG13419 sequence except for two silent polymorphisms (both C to T) at positions 292 and 228, respectively.

Genetic Identification of Bursicon Mutants

Flies bearing mutations in CG13419 were identified among a collection of viable EMS-mutagenized second and third chromosome strains induced in a *bw; st* background [19, 20]. We screened a subset (that were noted as having wing defects by E. Koundjakian, K. Lunde, and E. Bier) for wing expansion defects that resembled those of transgenic flies lacking eclosion hormone neurons [21]. Strains of interest were tested for complementation with each other to make a preliminary assessment of allelism, and the mutations were mapped by recombination with the *ru cu ca* or *Pr ru cu ca* chromosome. Mutations that mapped to the region of CG13419 were tested for complementation using deficiencies that corresponded to that region, roughly 93–94 on the cytological map. The mutations were mapped between 93E1 and 93F5 based on their complementation of these deficiencies (breakpoints): *Df (3R) e-F1* (93C6-D1; 93E1) [22] and *Df (3R) 93Fx2* (93F5; 94A8) [22] and their failure to complement *Df (3R) e-BS2* (93C3; 93F14) [22], *Df (3R) e-GC3* (93C6; 94A1-4) [22], and *Df (3R) e-H4* (93D1; 93F6-8) [22].

Sequencing the *bursicon* Mutants

Genomic DNA from homozygous mutant or *bw; st* control flies was used to PCR amplify the bursicon coding sequence using external primers burs1 5′-TGAAAGGACACTCGCAGTCG-3′ and burs2 5′-ATG GGCATGGGTATGAGTGC-3′. Amplified DNA was sequenced directly with an ABI 3700 high-throughput capillary DNA Analyzer using Big Dye chemistry (Applied Biosystems) using the external primers and two internal primers, burs3 5′-ACGCATCTTGGCGAC GATTG-3′ and burs4 5′-AGGAACGCTCCATTTGCCAG-3′. DNA from each mutant and the control *bw; st* strain was amplified and sequenced at least twice independently in both directions. DNA sequences were assembled and compared using Sequencher (Gene Codes Corporation).

Identification of *A. mellifera* and *A. gambiae* Homologs

The *CG13419* cDNA was used in a nucleotide tBLASTx search against the *A. gambiae* database [6]. A sequence that encompassed the *Drosophila* exon 2 and part of exon 3 was obtained. The finding that this gene encodes bursicon was cited [23] upon our agreement. An *A. mellifera* brain expressed sequence tag (est) was identified by searching the est database (tBLASTn) [6] with the CG13419 translated nucleotide sequence. Sequences were aligned using ClustalW [24].

Bursicon Bioassays

Flies that are neck-ligated immediately after adult ecdysis do not release their own bursicon but can sclerotize and melanize in response to injection of bursicon-containing homogenates. Bursicon levels in central nervous system (CNS) homogenates were determined using the *Sarcophaga bullata* (blowfly) bioassay as described previously [10, 11]. 80–100 CNSs from *Drosophila* third instar larvae were collected on dry ice, homogenized in phosphate buffer (PB) (pH 7.4) at 1.5 μl per CNS, and centrifuged at 10,000 rpm for 10 min. Five microliters of supernatant were injected per neck-ligated fly, and bursicon bioactivity was assigned a score between 0 (no

tanning) and 6 (dark tanning) as previously described [11, 13]. In each experimental series, wild-type and mutant homogenates and buffer controls were tested in parallel two or three times. At least 10 flies were injected and scored in each experiment. The average of three experiments $\pm$ standard deviation is shown to indicate the variance of the data. If two experiments were conducted, the mean score of each experiment is shown. Most flies injected with buffer remained white and soft (average score of 0.25 $\pm$ 0.15).

Assays of hemolymph from recently emerged *Drosophila* in the process of wing expansion were performed using neck-ligated *Drosophila* as described previously [12] with the following modifications: recipient flies were ligated within 5 min of eclosion. Hemolymph from control *bw; st* donors was taken at the time of wing expansion. Since the bursicon mutants do not expand their wings, hemolymph was taken 30–40 min following eclosion. Hemolymph from one fly was injected into as many as four ligated flies.

In Situ Hybridization and Immunocytochemistry
In situ hybridizations were performed on *Drosophila* third larval instar CNSs as previously described [25] using a digoxigenin (DIG)-labeled mRNA probe (1:500) derived from the entire *CG13419* coding sequence. Anti-DIG Fab fragments conjugated to alkaline phosphatase (Roche) were used to detect labeling. Alkaline phosphatase activity was visualized using either NBT/BCIP (Sigma) or Fast Red (Sigma). Nervous systems were processed for immunocytochemistry as described [3] using an anti-CCAP antibody (1:2000) and a Cy2-conjugated anti-rabbit secondary antibody (1:500) and observed under a confocal microscope. IgYs were harvested from egg yolks of chicken immunized and boosted with synthetic *Drosophila* CG13419 peptide (sequences 91–108) coupled to keyhole limpet hemocyanin. Specificity of the IgYs for the partial bursicon peptide was determined by noncompetitive ELISA [3]. *Drosophila* third larval instar nervous systems were treated as described [3]. Bursicon immunoreactivity was visualized using a Cy3-conjugated rabbit anti-chicken secondary antibody using confocal microscopy.

Additional Fly Strains
Larvae lacking CCAP neurons were produced by expressing the cell death gene *reaper* (*rpr*) in the CCAP neurons in transgenic flies by driving a UAS-*rpr* transgene using a *CCAP-GAL4* driver, as previously described [5]. The chromosome bearing the UAS-*rpr* insert also carried the marker UAS-LacZ, which provided an independent label for the CCAP neurons. The controls were progeny from a cross between the same *GAL4* driver and flies carrying only the UAS-LacZ transgene.

The *w^{1118}; rk^4* strain was previously described by molecular and genetic criteria; *rk^4* appears to be a null allele [12].

References

1. Fraenkel, G., and Hsiao, C. (1962). Hormonal and nervous control of tanning in the fly. Science *138*, 27–29.

2. Cottrell, C.B. (1962). The imaginal ecdysis of blowflies. Detection of the blood-borne darkening factor and determination of some of its properties. J. Exp. Biol. *39*, 413–430.

3. Honegger, H.W., Market, D., Pierce, L.A., Dewey, E.M., Kostron, B., Wilson, M., Choi, D., Klukas, K.A., and Mesce, K.A. (2002). Cellular localization of bursicon using antisera against partial peptide sequences of this insect cuticle-sclerotizing neurohormone. J. Comp. Neurol. *452*, 163–177.

4. Vitt, U.A., Hsu, S.Y., and Hsueh, J.W. (2001). Evolution and classification of cystine knot-containing hormones and related extracellular signaling molecules. Mol. Endocrinol. *15*, 681–694.

5. Park, J., Schroeder, A.J., Helfrich-Förster, C., Jackson, F.R., and Ewer, J. (2003). Targeted ablation of CCAP neuropeptide-containing neurons of *Drosophila* causes specific defects in execution and circadian timing of behavior. Development *130*, 2645–2656.

6. Altschul, S.F., Gish, W., Miller, W., Myers, E.W., and Lipman, D.J. (1990). Basic local alignment search tool. J. Mol. Biol. *215*, 403–410.

7. Adams, M.D., Celniker, S.E., Holt, R.A., Evans, C.A., Gocayne, J.D., Amanatides, P.G., Scherer, S.E., Li, P.W., Hoskins, R.A., Galle, R.F., et al. (2000). The genome sequence of *Drosophila melanogaster*. Science *287*, 2185–2195.

8. Nakai, K. (1991). Predicting various targeting signals in amino acid sequences. Bull. Inst. Chem. Res. Kyoto Univ. *69*, 269–291.

9. Kostron, B., Market, D., Kellermann, J., Carter, C.E., and Honegger, H.W. (1999). Antisera against *Periplaneta americana* Cu,Zn-superoxide dismutase (SOD): separation of the neurohormone bursicon from SOD, and immunodetection of SOD in the central nervous system. Insect Biochem. Mol. Biol. *29*, 861–871.

10. Fraenkel, G., and Hsiao, C. (1965). Bursicon, a hormone which mediated tanning of the cuticle in the adult fly and other insects. J. Insect Physiol. *11*, 513–556.

11. Kostron, B., Marquardt, K., Kaltenhauser, U., and Honegger, H.W. (1995). Bursicon, the cuticle sclerotizing hormone—comparison of its molecular mass in different insects. J. Insect Physiol. *41*, 1045–1053.

12. Baker, J.D., and Truman, J.W. (2002). Mutations in the *Drosophila* glycoprotein hormone receptor, rickets, eliminate neuropeptide-induced tanning and selectively block a stereotyped behavioral program. J. Exp. Biol. *205*, 2555–2565.

13. Kostron, B., Kaltenhauser, U., Seibel, B., Bräunig, P., and Honegger, H.W. (1996). Localization of bursicon in CCAP-immunoreactive cells in the thoracic ganglia of the cricket *Gryllus bimaculatus*. J. Exp. Biol. *199*, 367–377.

14. Eriksen, K.K., Hauser, F., Schiøtt, M., Pedersen, K.-M., Søndergaard, L., and Grimmelikhuijzen, C.P.J. (2000). Molecular cloning, genomic organization, developmental regulation, and a knock-out mutant of a novel leu-rich repeats-containing G protein-coupled receptor (DLGR-2) from *Drosophila melanogaster*. Genome Res. *10*, 924–938.

15. Andersen, S.O., Peter, M.G., and Roepsdorf, P. (1996). Cuticular sclerotization in insects. Comp. Biochem. Physiol. *113B*, 689–705.

16. Gammie, S.C., and Truman, J.W. (1997). Neuropeptide hierarchies and the activation of sequential motor behaviors in the Hawkmoth, *Manduca sexta*. J. Neurosci. *17*, 4389–4397.

17. Gammie, S.C., and Truman, J.W. (1999). Eclosion hormone provides a link between ecdysis-triggering hormone and crustacean cardioactive peptide in the neuroendocrine cascade that controls ecdysis behavior. J. Exp. Biol. *202*, 343–352.

18. Ewer, J., and Reynolds, S. (2002). Neuropeptide control of molting in insects. In Hormones, Brain and Behavior, Vol. 3, D.W. Pfaff, A.P. Arnold, S.E. Fahrbach, A.M. Etgen and R.T. Rubin, eds. (San Diego: Academic Press), pp. 1–92.

19. Niemeyer, B.A., Suzuki, E., Scott, K., Jalink, K., and Zuker, C.S. (1996). The *Drosophila* light-activated conductance is composed of the two channels TRP and TRPL. Cell *85*, 651–659.

20. Tsunoda, S., Sierralta, J., Sun, Y., Bodner, R., Suzuki, E., Becker, A., Socolich, M., and Zuker, C.S. (1996). A multivalent PDZ-domain protein assembles signalling complexes in a G-protein-coupled cascade. Nature *388*, 243–251.

21. McNabb, S.L., Baker, J.D., Agapite, J., Steller, H., Riddiford, L.M., and Truman, J.W. (1997). Disruption of a behavioral se-

quence by targeted death of peptidergic neurons in *Drosophila*. Neuron *19*, 813–823.

22. The FlyBase Consortium. (2003). The FlyBase database of the *Drosophila* genome projects and community literature. Nucleic Acids Res. *31*, 172–175.

23. Riehle, M.A., Garczynski, S.F., Crim, J.W., Hill, C.A., and Brown, M.A. (2002). Neuropeptides and peptide hormones in *Anopheles gambiae*. Science *298*, 172–175.

24. Thompson, J.D., Higgins, D.G., and Gibson, T.J. (1994). ClustalW: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. *22*, 4673–4680.

25. Draizen, T.A., Ewer, J., and Robinow, S. (1999). Genetic and hormonal regulation of the death of peptidergic neurons in the *Drosophila* central nervous system. J. Neurobiol. *38*, 455–465.

Note Added in Proof

A report on the Zuker Collection of autosomal mutants, which includes the mutants described here, was recently published. Koundjakjian, E.J., Cowan, D.M., Hardy, R.W., and Becker, A.H. (2004). The Zuker collection: a resource for the analysis of autosomal gene function in *Drosophila melanogaster*. Genetics *167*, 203–206.