Several insect peptides have significant sequence similarity to those of vertebrates. One such is the corticotropin-releasing factor (CRF)-like insect diuretic peptide family, described in 15 species of insect to date (Audsley et al., 1995; Baldwin et al., 2001; Clark and Bradley, 1998; Clark et al., 1998a,b; Clottens et al., 1994; Coast and Kay, 1994; Furuya et al., 2000, 1998). In locust, as in all insects studied so far, CRF diuretic-related peptides stimulate fluid secretion via cyclic AMP (cAMP) (Coast et al., 1991). The ultimate target of cAMP signalling is thought to be the apical V-ATPase (Clark et al., 1998b).

We describe in this work the Dh gene that encodes Drome-DH44, the first member of the CRF-like family to be found in *Drosophila melanogaster*. Functional analysis confirms that this peptide is active on the Malpighian (renal) tubule, and acts through cAMP. The peptide is expressed in six neuroendocrine cells in the pars intercerebralis. We also show for the first time that CRF directly activates its cognate phosphodiesterase, so limiting its own signal.

### Materials and methods

**Materials**

Neuropeptide Drome-DH44 was synthesised at the Emory University Microchemical Facility in Atlanta, GA, USA. Schneider’s medium was obtained from Gibco Life Technologies. *Drosophila* saline (pH 6.7, in mmol l⁻¹; NaCl, 117.5; KCl, 20; CaCl₂, 2; MgCl₂, 8.5; NaHCO₃, 10.2; NaH₂PO₄, 4.3; Hepes, 15; Glucose, 20) was made using products from Sigma, Fisher Scientific and BDH Laboratory supplies. Zaprinast (cyclic GMP-dependant phosphodiesterase inhibitor) was obtained from Calbiochem. IBMX (non-specific phosphodiesterase inhibitor) was purchased from Sigma. Cyclic GMP (cGMP) and cAMP RIA kits (Amerlex-M) were obtained from Amersham Pharmacia plc. Coelenterazine was obtained from Molecular Probes and dissolved in ethanol before use. Primers designed for sequencing were purchased from MWG Biotech AG. All other chemical products were acquired from Sigma.

**Drosophila methods**

*Drosophila melanogaster* Meig were maintained in a 12h:12h light:dark cycle on standard corn meal-yeast-agar medium at 25°C. Strain Oregon R (wild type), c42-aeq and c710-aeq fly lines were those described previously (O’Donnell et al., 1998; Rosay et al., 1997; Terhzaz et al., 1999). Briefly, c42-aeq and c710-aeq are lines that express an aequorin gene under the control of the yeast UAS₉ promoter and appropriate
P{Gal4} insertions, which drive expression in principal and stellate cells of the main segment, respectively (Sözen et al., 1997).

**Fluid secretion assays**

Fluid secretion assays were performed as described previously (Dow et al., 1994). Malpighian tubules from adult flies were dissected under Schneider’s medium and isolated into 10 µl drops of a 1:1 mixture of Schneider’s medium:Drosophila saline. All values are means ± S.E.M.

**Measurement of intracellular cyclic nucleotide concentrations**

Intracellular cAMP and cGMP concentrations were measured by radioimmunoassay as described previously (Davies et al., 1995). Briefly, 20 tubules per sample were dissected and incubated under Schneider’s medium and incubated for 10 min in the presence of 10⁻⁴ mol l⁻¹ isobutyl methyl xanthine (IBMX) for cAMP measurements and 10⁻⁴ mol l⁻¹ zaprinast for cGMP measurements, prior to stimulation with 10⁻² mol l⁻¹ Drome-DH₄₄ for a further 10 min. The reaction was interrupted with ice-cold ethanol and samples homogenised, dried and resuspended in 0.05 mol l⁻¹ sodium acetate buffer before being assayed. Samples were assayed for cyclic nucleotide content by competitive radioimmunoassay following the manufacturer’s instructions (Amersham Pharmacia, plc).

**Measurement of intracellular calcium**

The effect of Drome-DH₄₄ on intracellular [Ca²⁺] levels in Malpighian tubules was assessed using a P{Gal4}/UAS-Aequorin system as described previously (MacPherson et al., 2001; O’Donnell et al., 1998; Rosay et al., 1997; Terhzaz et al., 1999). Briefly, Malpighian tubules were dissected and incubated in Schneider’s medium containing 2.5 mmol l⁻¹ MgCl₂, pH 7.4) and positive controls carried out using pCDNA-bovPDE5 transformed cell lysates (Corbin et al., 2000).

**Localisation of expression of the Dh gene**

**Antibodies**

Approximately 2 mg of Drome-DH₄₄ was conjugated to 5 mg of thyroglobulin using difluorodinitrobenzene as the conjugating agent, as described elsewhere (Kean et al., 2002). A single New Zealand white female received a total of four injections at 5–6 week intervals. The first injection was performed with complete Freund’s adjuvant and for the subsequent injections incomplete Freund’s adjuvant was used. The rabbit was bled 10 days after each booster. Three antisera to other insect CRF-like diuretic hormones were used, the N- and C-terminal specific antisera to Manduca sexta diuretic hormone (Veenstra and Hagedorn, 1991) and a previously unpublished antiserum to Culex salinarius CRF-like diuretic hormone. The latter was produced by J.A.V. while at the University of Tucson using the same protocol as that described here.

**Immunocytochemistry**

The protocol used for peptide immunocytochemistry was the same as that described recently (Kean et al., 2002). The third and fourth bleedings of the rabbit used to raise antiserum to Drome-DH₄₄ gave very similar results and were used at 1:2000 dilution. The antiserum to the other insect CRF-like diuretic hormones were used at a dilution of 1:500. For double-labelling experiments the anti-CRF-like antibodies were purified from the serum by octanoic acid precipitation, dialyzed, lyophilized and labelled with carboxytetramethylrhodamine, as described elsewhere (Veenstra et al., 1995). For double labelings the tissues were first incubated with the leucokinin receptor antibody (Radford et al., 2002) at a dilution of 1:1000, followed by a fluorescein-labeled Fab fragment of goat-anti-rabbit IgG (Jackson Immunologicals) to visualize the leucokinin receptor antibody, and then the rhodamine-labeled IgGs to CRF-like diuretic hormone, following a protocol described elsewhere (Veenstra et al., 1995).

In situ hybridisation

Adult and larval brains were dissected, fixed for 25 min in
5% paraformaldehyde in PBT (PBS with 0.1% Tween 20), washed five times in PBT and digested for 9 min in 1 ml PBT containing 4 μg ml⁻¹ proteinase K. The digestion was stopped by washing the tissue twice in 1 ml cold 0.2% glycine in PBT under agitation, and once in PBT. The tissue was then fixed again for 25 min in 5% paraformaldehyde in PBT, washed five times in PBT, once with a 1:1 mixture of PBT and hybridisation solution, and thrice in hybridisation solution. The hybridisation solution consisted of 50% formamide, 5× SSC, 100 μg ml⁻¹ heparin, 100 μg ml⁻¹ denatured salmon sperm DNA and 0.1% Tween 20 in diethyl pyrocarbonate-treated distilled water (Sambrook and Russell, 2001). After prehybridisation for 1 h at 55°C, digoxigenin-labelled RNA probes were added and allowed to hybridise overnight. Digoxigenin-labelled RNA probes were prepared using a commercial kit from Roche Molecular Biochemicals following the instructions of the manufacturer. The probe was reduced by alkaline treatment to a size of approximately 300 nucleotides. After washing the tissue five times for 15 min at 55°C in hybridisation solution, tissues were washed at room temperature, once in 1:1 mixture of hybridisation solution and PBT, and four times in PBT. An alkaline-phosphatase-labelled antibody was used to localise the probes.

### Immunocytochemistry for cyclic AMP

Slides were treated with 100 μl of 0.1 mg ml⁻¹ poly-L-lysine solution for 30 min, washed with water and left to dry. Tubules were dissected in Schneider’s *Drosophila* medium and stuck onto slides in 1× PBS solution. A solution with final concentration 10⁻² mol l⁻¹ DromeDH₄₄ and 10⁻⁵ mol l⁻¹ IBMX (phosphodiesterase inhibitor) in 1× PBS was added for 7 min (the same solution without peptide was added to control samples). The tubules were fixed with 4% paraformaldehyde in 1× PBS for 30 min. They were washed 5 times in 1× PBS before permeabilization with 0.2% (v/v) Triton X-100 in 1× PBS for 30 min. Permeabilization solution was changed every 10 min, after which the tubules were blocked for 3 h in PAT [PBS containing 0.5% (v/v) Sigma cold fraction V bovine serum albumin and 0.2% (v/v) Triton X-100]. The tubules were then hybridised overnight in a humidity chamber with the primary antibody [rabbit anti-cyclic AMP polyclonal antiserum (US Biological C8450)], at 1:250 dilution in PAT. Tubules were then blocked with PAT containing 2% (v/v) normal rabbit serum for 2 h. Samples were incubated for 1 h with the secondary antibody (fluorescein-conjugated anti-rabbit) diluted 1:250 in PAT with 2% normal rabbit serum in a humidity chamber. The tubules were then washed 4 times over a period of 2 h in PAT and twice for 10 min in PBS before being mounted in VectaShield medium (Vector labs). Slides were examined under epifluorescence (Zeiss) and photographs were taken with an Axiocam HRc (Zeiss) using appropriate filters, and the same exposure time for all the samples, at 40x/0.75 magnification.

### Statistical analysis

Where appropriate, statistical significance of differences was assessed with Student’s *t*-test (two-tailed) for unpaired samples, taking the critical level for *P*=0.05. Significant differences are marked graphically with an asterisk.

### Results

**Identification of the gene encoding Drome-DH₄₄**

The *Stomoxys calcitrans* DH₄₅ CRF-like peptide was used as a ‘baït’ for a TBLASTN search that was run against the Berkeley *Drosophila* Genome database. The results were hits against three EST sequences: AT 14664 (GenBank accession no. BF499889), GH 27214 (GenBank accession no. AI514143) and HL08001 (GenBank accession no. AA699224), and genomic sequence at region 85D-E of chromosome 3R. This locus had been annotated automatically by the *Drosophila* genome project, and was subsequently identified *in silico* as containing a CRF peptide, and named *Dh* (Vanden Broeck, 2001). AT14664 and GH27214 (Research Genetics) were sequenced partially on the positive strand: GH27214 was found to be chimaeric, and so was rejected. The AT14664 clone was accordingly chosen for full sequencing due to its greater chance of carrying the complete cDNA of the gene. It was also chimaeric, but contained an apparently full-length cDNA for *Dh* with a poly(A) tail and a polyadenylation signal. This cDNA and deduced peptide is shown in Fig. 1A. This cDNA differs significantly from those annotated automatically by the genome project, or by previous workers. The automated Gadfly annotation misses the 5’ end of the gene and includes an intron between bases 1102–1103; and the recent automatic sequencing of the same cDNA by the genome project did not identify the chimaeric nature of the AT14664 clone. Accordingly, we believe that the cDNA sequence in Fig. 1 is authoritative. The cDNA has a poly(A) tail, with a polyadenylation signal centred 23 bases upstream of the first A. The prepropeptide contains only one region with similarity to any other genes, the area corresponding to the mature CRF-like peptide, which we named Drome-DH₄₄, nor are there any other obvious propeptide cleavage sites (Veenstra, 2000). Accordingly, we do not believe that other neuropeptides are encoded by this gene. The prepropeptide also has a signal peptide sequence, as identified with pSortII: this is required for peptides with a secretory fate. The sequence of the Drome-DH₄₄ peptide encoded by *Dh* is very similar to other known insect sequences, and is almost indistinguishable from other Dipteran sequences (Fig. 2). The Drome-DH₄₄ peptide sequence is flanked by dibasic convertase cleavage sites (Veenstra, 2000). The peptide is amided, as are all the CRF-like peptides identified to date. Some studies suggest the importance of amidation for its function (Audsley et al., 1995).

The genomic context of *Dh* is shown in Fig. 1B. The gene is in a gene-dense area, and sits between *Scm*, an RNA polymerase II transcription factor, and CG9492, a dynein ATPase, within the genomic sequence. *Dh* is punctuated by four introns, one large enough to contain an entire gene (*frost*, encoding a cold-induced gene).
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A

Fig. 2. Alignment of insect corticotropin-releasing factor-related peptides. The sequences producing significant alignments against Drome-
DH44 peptide after a protein Blast search are shown below in red. Mac Vector clustal analysis was performed to align the sequences. Residues
highlighted in yellow have ≥50% identity. Swissprot accession numbers for each peptide are as follows: Musca domestica (house fly),
DIUH_MUSDO; Periplaneta americana (American cockroach), DIUH_PERAM; Zootermopsis nevadensis (termite), DIUH_ZOONE;
Diploptera punctata (Pacific beetle cockroach) class I, DIUH_DIPPU; Locusta migratoria (migratory locust), DIUH_LCOMI; Tenebrio molitor
(yellow mealworm), DIU2_TENMO; Acheta domestica (house cricket), DIUH_ACHDO; Manduca sexta (tobacco hornworm)
precursor 1, DIU1_MANSE; DromeDH, dh gene product (present study); Culex salinarius, CCRF-DP.

B

Fig. 1. The Drosophila melanogaster CRF-like gene. (A) cDNA and deduced peptide sequence of Dh. Putative dibasic
convertase cleavage sites are underlined. The C-terminal amidation signal glycine residue is in italic. The positions of introns in
the genomic sequence are marked with <<. The predicted peptide is indicated in blue. The encoded peptide has a signal peptide, with a predicted
cleavage site between amino acid residues 24 and 25, marked with ^, as predicted by
PSORTII Prediction program (http://psort.nibb.jp/form2.html). The polyadenylation signal is indicated in yellow. Differences
between the genomic sequence and the cDNA are marked in red. (B) Genomic context of Dh. View of
region 8SE of chromosome 3R. The neighbours of Dh are, on the negative strand, Scm, an RNA
polymerase II transcription factor implicated in gene silencing, and
CG9492, a dynen ATPase implicated in microtubule-based
movement. Within an intron of Dh, on the opposite strand, is frost (fst), a gene implicated in response to
cold. Dh (old) denotes the original, incomplete, Gadfly annotation of the Dh gene.
Effect of Drome-DH44 on fluid secretion

Drome-DH44 stimulates fluid secretion rates in Drosophila melanogaster Malpighian tubules at concentrations equal to or higher than 10^{-8} mol l^{-1}, typically by up to 1.5–2.5 nl min^{-1} (Fig. 3A); this is comparable with stimulation rates observed for other native diuretic neuropeptides: DLK (Terhzaz et al., 1999), capa1, capa2 (Kean et al., 2002) in Drosophila melanogaster. The dose–response curve (Fig. 3B) shows that the stimulation is significant when using concentrations greater than 10^{-8} mol l^{-1}.

Cyclic nucleotide assays

In other species, CRF-like peptides have been shown to elevate intracellular cAMP levels. No cGMP increase was observed after stimulation with Drome-DH44 (Fig. 4B). On the other hand, a clear response in cyclic AMP is shown after treatment, as expected for a CRF-like peptide (Fig. 4A). A significant increase, approximately 150% above the basal rate, is observed for concentrations over 10^{-8} mol l^{-1}, consistent with the effect on secretion (Fig. 3). Immunocytochemistry with an antibody against cAMP showed that the levels were selectively increased in principal cells only (Fig. 4C,D), consistent with models for Drosophila tubule function, in which electrogenic cation transport is performed by the principal cells, while stellate cells provide the main route for water and chloride fluxes (Dow and Davies, 1999, 2001; O’Donnell et al., 1996, 1998).

Phosphodiesterase assays

For a second messenger to be plausibly implicated in a signalling pathway, there must be a mechanism for terminating the signal, as well as for generating it. In the case of cAMP, a phosphodiesterase is likely to be responsible. Tubules were stimulated with Drome-DH44, and cAMP- and cGMP-directed phosphodiesterase activity separately measured.

Drome-DH44 treatment doubles cAMP-PDE activity and halves cGMP-PDE activity, respectively, though the latter change is not significant (Fig. 5). Drome-DH44 application thus directly stimulates the activity of the enzyme that breaks down its second messenger, and thus provides a feedback mechanism for limitation of the signal. This is the first time that this breakdown pathway has been shown to be modulated by an insect diuretic hormone.

Intracellular calcium

It is conceivable that Drome-DH44 could act indirectly to raise cAMP levels via intracellular calcium, or that it might act through calcium in other cell types. Accordingly, the transgenic aequorin system (Rosay et al., 1997) was used to seek a calcium correlate of hormone stimulation. The neuropeptides CAP2b (Davies et al., 1995) and DLK (Terhzaz et al., 1999) were used as positive controls to generate increases in calcium levels in principal and stellate cells respectively. As described previously, CAP2b and DLK produced elevated intracellular [Ca^{2+}] in principal and stellate cells, respectively, while Drome-DH44 had no effect (Fig. 6).

Localisation of expression of the CRF gene

Both immunocytochemistry (Fig. 7a,b) and in situ hybridisation (Fig. 7c) techniques mark a bilateral triplet of cells in the pars intercerebralis. The two antisera against the Manduca sexta peptide were not immunoreactive in Drosophila, while the two antibodies against the Dipteran hormones recognized the same cells, with the Drosophila...
antibody being clearly more immunoreactive. In both adults and larvae, CRF-like diuretic hormone immunoreactivity was found exclusively in three bilateral pairs of cells in the pars intercerebralis, the axons which could be followed into the retrocerebral complex. These cells are very similar to the B-cells described from other flies (Panov, 1976). The morphology of these cells is thus typical of classical insect neuroendocrine cells. Using in situ hybridization the same number of cells was found in a closely similar location (Fig. 7c). No CRF-like immunoreactive cells were found in the abdominal ganglia, either by immunocytology or by in situ hybridisation.

Interestingly, these cells also express Leucokinin receptor immunoreactivity, both in the cell bodies and the neurohaemal axons and axon terminals in the retrocerebral complex (Fig. 8).

**Discussion**

The primary effect of insect diuretic peptides is considered to be stimulation of fluid secretion by Malpighian tubules.
Three different families of insect neuropeptides have been shown to stimulate fluid secretion: CRF-like diuretic peptides, calcitonin-like peptides (Coast et al., 2001), the kinin family (Holman et al., 1984), and the CAP2b (Davies et al., 1995), capa-1 and capa-2 (Kean et al., 2002) family. Each class of peptides has a distinct mode of action: the capa-like peptides raise intracellular calcium levels and stimulate NOS to raise NO levels and thence cGMP levels; the kinins also act via intracellular calcium levels, to stimulate chloride shunt conductance (Hayes et al., 1989; O’Donnell et al., 1996, 1998; Pannabecker et al., 1993). Both calcitonin-like peptides and the CRF-like peptides are thought to act through cAMP. However, although CRF-like peptides have been reported previously to act through cAMP, this is the first demonstration that they have no effect on calcium. This may differ from the position in Aedes aegypti (another Dipteran), where CRF appears to have two effects: a very weak stimulation of secretion at low concentrations, which may be dependent on calcium, and a strong, cAMP-dependent stimulation of secretion at high concentrations, \(>10^{-8}\) mol l\(^{-1}\) (Clark et al., 1998a,b). In Drosophila, it appears that the response we describe here corresponds closely to the ‘high-concentration’ response described in Aedes. In addition, in Drosophila, Drome-DH\(_{44}\) appears to raise cAMP levels only in the principal cells, implying a clear division of labour between hormones that act to stimulate electrogenic cation transport in principal cells (capa1, capa2, calcitonin-like peptide, CRF-like peptide), and hormones that act to raise chloride shunt conductance in stellate cells (leucokininins).

Expression of the CRF-like peptide appears to be rather
limited, being confined to only a bilateral triplet of cells in the pars intercerebralis. The number of CRF-like peptide immunoreactive cells reported here is smaller than reported previously in the housefly *Musca domestica* (Iaboni et al., 1998) using an antiserum to locust CRF-like DH. Although we cannot exclude the possibility that the *Locusta* antiserum is more specific than the one we raised to the *Drosophila* hormone, we feel that this is unlikely, as both *in situ* hybridisation and immunocytochemistry techniques identify the same cells. Unlike in the moth *Manduca sexta* (Chen et al., 1994), the locust *Locusta migratoria* (Thompson et al., 1995) or the bug *Rhodnius prolixus* (Te Brugge et al., 2001), there are no abdominal neuroendocrine cells in *Drosophila* producing CRF-like diuretic hormones. This may be typical of Diptera, as in neither the mosquito *Aedes aegypti* (J.A.V., unpublished data) nor the housefly *Musca domestica* (Iaboni et al., 1998) have such abdominal neuroendocrine cells been found. In other species, some of these abdominal neuroendocrine cells express both leucokinins and CRF-like diuretic hormones, and it has been shown for the locust that these hormones have synergistic effects (Thompson et al., 1995). Although *Drosophila* has no neuroendocrine cells that produce both leucokinin and the CRF-like peptide, our observations suggesting expression of the leucokinin receptor in the CRF-like-peptide-producing neuroendocrine cells shows an alternative way of interaction between the two peptides; it is tempting to speculate that release of leucokinin into the haemolymph could induce and/or facilitate release of the CRF-like peptide.

In *Manduca sexta* the number of CRF-like-diuretic-hormone-producing neuroendocrine cells in the pars intercerebralis increases dramatically during metamorphosis. It had been suggested that this increase might be related to the very significant loss of water during metamorphosis when a watery caterpillar is transformed into a much lighter, flying moth (Veenstra and Hagedorn, 1991). The transformation of a maggot to a fly is similarly accompanied by a significant loss of water, but the number of the CRF-like-diuretic-hormone-producing neuroendocrine cells in *Drosophila* does not change during metamorphosis. It will be interesting to see whether this reflects a physiological functional difference of these hormones. Overall, it is not yet clear what role CRF-like peptides play in insect organismal physiology, although in *D. melanogaster* at least, a reverse genetic approach is conceivable. It is also interesting that the cDNA clone that we sequenced came from a testes library; although we have not addressed here the possibility of expression outside the CNS, it would be interesting to investigate whether there was any biological significance to this finding.

It is interesting to note that Drome-DH44 does not act merely to raise cAMP levels, but also to stimulate cAMP-phosphodiesterase twofold. This change would have the effect of terminating the Drome-DH44 signal even faster than would be expected if PDE levels remained constant, and would imply that Drome-DH44 is primarily a short-term modulator of fluid secretion. Technically, we cannot separate the PDE responses of principal and stellate cells in these assays, although as the increase in cAMP concentration is confined to principal cells, it would be reasonable to suppose that the PDE response occurred at least in them. Although statistical significance was not achieved in these experiments (Fig. 5), there was an interesting downward trend in the cGMP-PDE assay, which would have the effect of potentiating stimulation by the capa peptides.

The similarity of action between Drome-DH44 (this work) and calcitonin-like peptide (Coast et al., 2001) might at first seem puzzling. However, there is no guarantee that these peptides act exclusively on Malpighian tubules; it is quite possible that they have distinct actions elsewhere in the fly. The Malpighian tubule, as a prime site for homeostatic regulation, is charged with integrating inputs from a whole range of peptides, many of which may prove not to be primarily diuretic; and so it is not surprising to find multiple peptides with similar modes of action on this tissue (Dow and Davies, 2001; O’Donnell and Spring, 2000; Skaer et al., 2002).
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