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Characterization of the *Drosophila melanogaster* alkali-metal/proton exchanger (NHE) gene family

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Summary

The NHE family of Na^+/H^+ exchangers is believed to play an essential role in animals, but may play an additional, specialised epithelial role in insects. The pharmacological sensitivity of the *Drosophila melanogaster* Malpighian tubule to a range of amiloride derivatives was shown to be consistent with an effect on an exchanger, rather than a Na^+ channel. Consistent with this, no degenerin/epithelial Na^+ channel (ENaC) genes could be detected in Malpighian tubules by reverse transcriptase/polymerase chain reaction (RT-PCR). Using a low-stringency homology searching, three members of the NHE family were identified in the genomic sequence of *Drosophila melanogaster*, although only two genes were represented as expressed sequence tags. All three genes (*DmNHE1* at cytological position 21B1, *DmNHE2* at 39B1 and *DmNHE3* at 27A1) were found by RT-PCR to be widely expressed, and one (*DmNHE2*) was shown to have multiple transcripts. The putative translations of the three genes mark them as distantly related members of the family, inviting the possibility that they may serve distinct roles in insects.

Key words: V-ATPase, epithelial transport, Malpighian tubule, Na^+/H^+ exchanger, *Drosophila melanogaster*.

Introduction

NHE (Na^+/H^+ exchanger) family

The presence of cation/proton exchangers in eukaryotic cells was first proposed in 1961 by Mitchell as part of his chemiosmotic hypothesis, and these exchangers are now known to be important in pH homeostasis, cell volume regulation and transepithelial Na^+ transport (Wakabayashi et al., 1997). NHEs are electroneutral and exchange 1 Na^+ for 1 H^+. The exchange is reversible and driven by the electrochemical gradients for the two cations. Under physiological conditions, the inwardly directed Na^+ current produced by the Na^+/K^+-ATPase provides a constant force that extrudes H^+ from the cell (Wakabayashi et al., 1997). There are at least six human NHE genes, NHE1–NHE6; five are plasma membrane NHEs (Klanke et al., 1995; Malakooti et al., 1999; Noel and Pouyssegur, 1995; Orlowski and Grinstein, 1997; Wakabayashi et al., 1997) and one (NHE6) is a mitochondrial exchanger (Numata et al., 1998). The NHE1 gene (Noel and Pouyssegur, 1995) appears to be the ubiquitously expressed, 'house-keeping' type of the exchanger that plays a major role in controlling the intracellular pH of nearly all animal cells. Similar genes have been identified in various other organisms, such as the rat, mouse, rabbit, pig, trout, *Caenorhabditis elegans*, *Amphiuma tridactylum*, yeast and bacteria (Attaphitaya et al., 1999; McLean et al., 1999; Noel and Pouyssegur, 1995; Padan and Schuldiner, 1994; Wakabayashi et al., 1997), and an NHE family member has been implicated in salt tolerance in the plant *Arabidopsis thaliana* (Shi et al., 2000).

The Wieczorek model for ion transport

In insects, NHEs may play a significant additional role. Some animal plasma membranes, including most insect epithelia, are energised by proton-motive forces instead of the basolateral Na^+/K^+-ATPase that energises most animal epithelia (Harvey and Wieczorek, 1997; Klein et al., 1991; Wieczorek et al., 1991). In insect epithelia, apical plasma membrane H^+ V-ATPases generate transmembrane electrochemical gradients, which in turn drive other processes such as acidification, fluid secretion and sensory signalling. According to the Wiezcorek model, the electrogenic V-ATPase drives one or more alkali-metal/proton exchangers, resulting in a net transepithelial transport of Na^+ or K^+. It has been established that the two transport functions are pharmacologically distinct because the V-ATPase is bafilomycin-sensitive (Wieczorek et al., 1991) and the antiport is sensitive to amiloride (Wieczorek, 1992). Although there is no reason *a priori* to assign such V-ATPase-partner antiporters to the NHE family [indeed, in *Manduca sexta* midgut, the exchanger may be electrogenic (Azuma et al., 1995)], both Na^+ and K^+ transport in insect epithelia are amiloride-sensitive

(Hegarty et al., 1992; Wieczorek, 1992). It is therefore particularly interesting to characterise the insect NHE exchangers, both as possible candidates for the Wieczorek exchanger and as potential components of animal cell ionic regulation. Surprisingly, although there are some preliminary reports, no paper describing a sequence for insect cation/proton exchangers has been published.

The Drosophila melanogaster *Malpighian tubule*

The fruit fly *Drosophila melanogaster* is a useful genetic model with a completed genome sequence (Adams et al., 2000), powerful transgenic technology (Rubin, 1988; Spradling and Rubin, 1982; Spradling et al., 1995). It also serves as a good experimental model, permitting the use of biochemical, cell biological and physiological techniques in disciplines such as developmental biology, neurobiology (Rubin, 1988) and integrative physiology (Dow et al., 1998). The Malpighian tubule of *D. melanogaster* is known to be sensitive to both bafilomycin and amiloride (Dow et al., 1994), consistent with the V-ATPase/antiporter in that it has been shown to be energised by an apical V-ATPase confined to the principal cells (Davies et al., 1996). However, amiloride is a relatively non-specific probe for NHE function because it also inhibits a range of Na^+ channels (Kleyman and Cragoe, 1988). In the present paper, we show that fluid secretion in the Malpighian tubules is inhibited by amiloride derivatives that are consistent with inhibition of NHEs rather than Na^+ channels. Furthermore, no expression of epithelial Na^+ channels (ENaCs) could be detected by reverse transcriptase/polymerase chain reaction (RT-PCR) in Malpighian tubules. In contrast, the *Drosophila* NHE family is shown to consist of three genes, called *DmNHE1*, *DmNHE2* and *DmNHE3*, that encode distant relatives of the NHE exchanger family, all of which are expressed in Malpighian tubules.

Materials and methods

Drosophila *methods*

Oregon R (wild-type) *Drosophila melanogaster* were maintained on a 12 h:12 h light:dark cycle on standard corn meal/yeast/agar medium at 25 °C. All manipulations, unless stated otherwise, were carried out at room temperature (22–25 °C).

Fluid secretion assays

Malpighian tubules were dissected from adult female and male flies, and fluid secretion assays were performed as described previously (Dow et al., 1994). The bathing medium was a mixture of Schneider's insect culture medium and *Drosophila* saline (1:1 v/v). *Drosophila* saline (pH 6.7) consisted of (in mmol l^{-1}): NaCl, 117.5; KCl, 20; $CaCl_2$, 2; $MgCl_2$, 8.5; $NaHCO_3$, 10.2; NaH_2PO_4, 4.3; Hepes, 15; glucose, 20. Volumes of secreted fluid were determined at 10 min intervals. The data were analysed using an Apple Macintosh computer and Excel 4.0. All data are reported as means ± S.E.M. Statistical significance of differences between treatments was assessed using Student's *t*-test for unpaired samples, taking the critical value of P to be 0.05 (two-tailed).

Cardioacceleratory peptide 2b (CAP_{2b}) and *Drosophila* leucokinin were custom-synthesised by Research Genetics, Inc. and added to tubules at 10^{-7} mol l^{-1}. This combined treatment powerfully stimulates diuresis, acting both on active cation transport and on the Cl^- shunt conductance (Dow and Davies, 2001; Dow et al., 1998), and so was expected to unmask any inhibition by amiloride. Amiloride (Sigma-Aldrich A7410) and 5-*N*,*N*-dimethyl amiloride (DMA) (Sigma-Aldrich A4562) were dissolved to 10–500 mmol l^{-1} in dimethylsulphoxide (DMSO), then 1:100 in Schneider's/saline, and used at a range of concentrations together with 1:100 (final dilution) DMSO in Schneider's/saline as the vehicle. Benzamil (Sigma-Aldrich B-2417), 2′,4′-dichlorobenzamil (DCB; Molecular Probes D-6898) and 5-*N*-ethyl-*N*-isopropyl amiloride (EIPA; Sigma-Aldrich A3085) were dissolved in methanol to 50–100 mmol l^{-1}, then diluted 1:100 in Schneider's/saline and used at a range of concentrations, together with 1:100 (final concentration) methanol in Schneider's/saline as the vehicle. Neither DMSO nor methanol vehicles had any effect on Malpighian tubules at these final concentrations (data not shown). The amiloride analogue was added to half the tubules after 30 min, and all the tubules were then treated with *Drosophila* leucokinin and CAP_{2b} at 60 min. Secretion assays were performed at a range of concentrations from 10^{-4} mol l^{-1} to 10^{-8} mol l^{-1}, and dose/response curves were plotted. For each experimental set of at least 10 tubules, the response to amiloride was defined as the mean maximum secretion rate (controls) minus the mean maximum secretion rate (amiloride-treated). This value was expressed as a percentage of the control maximum secretion rate.

Cyberscreening

Cyberscreening was performed using Netscape Communicator 4.5 on an Apple Macintosh computer and searching NCBI (http://www.ncbi.nlm.nih.gov/) and BDGP (http://www.fruitfly.org) databases with BLASTN, BLASTP, BLASTX or TBLASTN searches, as appropriate. Sequence alignments were performed and displayed using MacVector 6.5.1 or 7.0, AssemblyLIGN, SeqVu 1.0.1, ClustalW PPC and TreeView PPC.

RT-PCR

mRNA was prepared using the Dynabeads Oligo $(dT)_{25}$ kit according to the manufacturer's protocol (Dynex Technologies) and reverse-transcribed with SUPERSCRIPT II RNase H^- reverse transcriptase (Life Technologies) to produce a solid-state cDNA library. For each PCR reaction, 1 µl of beads, corresponding to cDNAs derived from one Malpighian tubule or approximately 0.2 head, was used. RT-PCR was performed on cDNA from whole male flies, whole female flies, heads, bodies, tubules, larvae and pupae using primers designed to bracket introns as a guard against genomic DNA contamination.

PCRs were performed as follows: 94 °C for 1 min; followed

by 30 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 2 min (depending on the length of the DNA template), and finally one cycle of 72 °C for 5 min. PCR reactions were analysed on 1 % agarose gels, stained with ethidium bromide and photographed according to standard protocols (Sambrook and Russell, 2001).

cDNA clones

Low-stringency searches of the *Drosophila* genome allowed three candidate genetic loci to be identified. cDNA clones were identified by BLAST searching against expressed sequence tags (ESTs), using genomic sequence for each gene as a probe. All available clones were obtained from Research Genetics. No EST hits were obtained for *DmNHE2*, implying that it was not widely expressed. EST clones HL05853, AT11019 and LP03712, identified as the longest available 5′ clones for *DmNHE1*, *DmNHE2* and *DmNHE3*, respectively, on the basis of available EST information, were sequenced in full on both strands. To survey for possible alternative 3′ splicing, the 3′ ends of the other EST clones were also sequenced.

Primers used were as follows:

DmNHE1-2306R, CCCCACAACAGCCATTTAAT; DmNHE1-F1, AGCGACCACGTCACGTTTTGTC; DmNHE3-1943F, TACGAATGGCAGTTTGGG; DmNHE3-2700R, CATTTTCGATTTCAGTTGAGACC; DmNHE2-F2, TCTACATGCTTCCACCGATTATCC; DmNHE2-R2, AGTGAGGCAAATAGAAACACGTCC; DmNHE2-1684F, TTGGCGTGGTGCTCTATTTC; DmNHE2-3509F, CCTGCGGAAAGATGGGAATTTAC; DmNHE2-3803F, TGTGATGTACCACATGATGGAG; DmNHE2-3849R, TGTCCAAGCCAATCTCATTGTAGG; DmNHE2-4088R, AAATGGGTTCTATGACACGCAC; DmNHE2-4859F, TCACTTGATGGCTGGAATTGAG; DmNHE2-5324F, GAGCTGAGCCGAAGATCATC; DmNHE2-5367R, CATCGTGAGTTTGGAGTACGTC; DmNHE2-59554R, TCAGAGATCAGAGAGACAGAGAGAG; PM001, CGTTAGAACGCGGCTACAAT; M13 Forward, CTGGCCGTCGTTTTAC; M13 Reverse, CAGGAAACAGCTATGAC.

Results

Tubule sensitivity to amiloride

It has been shown previously that *Drosophila* Malpighian tubules are sensitive to amiloride (Dow et al., 1994). Amiloride inhibits Na^+ channels, Na^+/H^+ exchangers and Na^+/Ca^{2+} exchangers (Kleyman and Cragoe, 1988; Orlowski and Grinstein, 1997). However, work carried out on mammalian NHEs and Na^+ channels has shown that different amiloride analogues specifically inhibit channels or exchangers. Channels are inhibited more effectively by amiloride or by 2-carbonylguanido substituents, such as benzamil, whereas NHEs are much more sensitive to R5 group substituents, such as 5-*N*-ethyl-*N*-isopropyl amiloride (Kleyman and Cragoe, 1988; Orlowski and Grinstein, 1997). In Malpighian tubules, it was therefore imperative to study amiloride pharmacology with fluid secretion assays using a number of amiloride analogues that had previously been shown to be mammalian NHE- or Na^+-channel-specific. Dose/response curves for the five analogues are shown in Fig. 1. All analogues inhibited fluid secretion, but at different concentrations. By comparison with results obtained in vertebrate systems (Table 1), it is clear that the relatively low sensitivity to amiloride and 2-carbonylguanidino substituents compared with R5 substituents (particularly EIPA) is diagnostic for an NHE, rather than a channel, target for amilorides in Malpighian tubules.

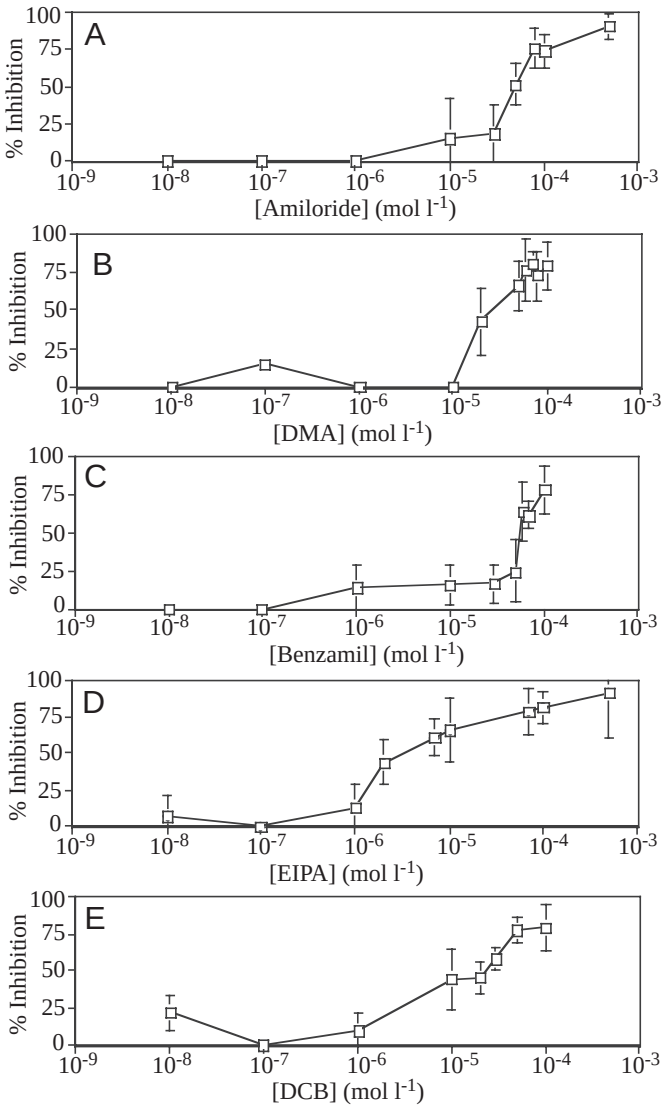

Fig. 1. Sensitivity of secretion by the *Drosophila melanogaster* Malpighian tubule to inhibition by amiloride and its derivatives. Dose/response curves for amiloride, 5-*N*,*N*-dimethyl amiloride (DMA), benzamil, 5-*N*-ethyl-*N*-isopropyl amiloride (EIPA) and 2′,4′-dichlorobenzamil (DCB). The upper limits of each graph are determined by the solubility of the compounds. Values are means ± S.E.M. (*N*=10).

Are there Na^+ channels in Drosophila *Malpighian tubules?*

Although the pharmacology of the response to amiloride of Malpighian tubules was consistent with an effect on an NHE,

Table 1. *Comparison of IC_{50} responses in vertebrates and in* Drosophila melanogaster *Malpighian tubules*

Analogue	IC_{50} (mol l^{-1}) Vertebrate NHE[1]	Vertebrate Na^+ channel[1]	*D. melanogaster* Malpighian tubule[2]
Amiloride	8×10^{-5}	4×10^{-7}	8×10^{-5}
2-Carbonylguanidino substituents			
Benzamil	10^{-3}	4×10^{-8}	7×10^{-5}
2′,4′-Dichlorobenzamil	8×10^{-5}	10^{-7}	3×10^{-5}
R5 substituents			
Dimethyl amiloride	7×10^{-6}	$>10^{-5}$	5×10^{-5}
5-*N*-ethyl-*N*-isopropyl amiloride	4×10^{-7}	$>10^{-5}$	7×10^{-6}

[1]Kleyman and Cragoe, 1988; Orlowski and Grinstein, 1997; [2]this study.
NHE, Na^+/H^+ exchanger.

rather than a channel, a genomic/RT-PCR strategy was employed to establish whether Na^+ channels are present in Malpighian tubules that could explain this inhibition of fluid secretion by amiloride.

Amiloride-sensitive Na^+ channels previously described in *Drosophila* include two *Drosophila* degenerin/ENaC family genes, *pickpocket* (*ppk*) and *ripped pocket* (*rpk*) (Adams et al., 1998). *Pickpocket* appears to be abundantly transcribed in early-stage embryos and is possibly involved in early development, whereas *ripped pocket* is found in a subset of neurons of the peripheral nervous system and is amiloride-sensitive (Adams et al., 1998). *Ripped pocket* is identical to *dGNaC1* (Darboux et al., 1998a) and *pickpocket* is identical to *dmdNaC1* (Darboux et al., 1998b), genes found in the peripheral nervous system and the gonads, respectively. *dGNaC1* has been shown to be amiloride-sensitive by expression in *Xenopus laevis* oocyte, whereas the amiloride sensitivity of *dmdNaC1* is inferred from sequence similarity.

Our search of the Berkeley *Drosophila* genome project for Na^+ channels led to the identification of nine genes, *rpk*, *ppk*, *CG10972*, *CG14398*, *CG4805*, *CG8546*, *CG9499* and *Nach*. These genes are divergent in sequence, being at least as related to the human search sequence as to each other (Fig. 2A). By RT-PCR, none of these genes was detected in Malpighian tubules: some appeared to be expressed in heads or whole flies only, and for others, no expression could be detected (Fig. 2B). This is consistent with recent data implicating *Drosophila* ENaCs in very specialised roles in thermoreception (Zinkevich et al., 2001) and salt taste perception (Liu et al., 2001).

Identification of Drosophila *NHEs by cyberscreening*

A low-stringency BLAST search of the Berkeley *Drosophila* genome database using the human NHE1 protein sequence revealed three putative *Drosophila* members of the NHE family of exchangers, termed *DmNHE1*, *DmNHE2* and *DmNHE3* in the order in which they were identified. Three EST hits have been described for *DmNHE1*: HL05853, GH04225 and GH04168, with HL05853 being the longest. Nine EST hits were identified for *DmNHE3*: LP03712, GH16168, GH025044, GH27182, LD37666, LD07057, LD20719, LP02917 and SD07542, the last being the longest. *DmNHE2* was identified in genomic clone DS02919 from cytological position 39A3–39B1, but there were no EST clones available for this gene. HL05853, the EST clone for *DmNHE1*, and LP03712, the EST clone for *DmNHE3*, were sequenced and the cDNA and inferred protein sequences determined (Fig. 3, Fig. 4). *DmNHE1* (GenBank accession number AF142676) or CG12178, localised to cytological position 21B1, encodes a 649-amino-acid protein (AAD32689) with a predicted relative molecular mass of 71 277, whereas *DmNHE3* (AF199463) or CG11328, localised to 27A1, encodes a 687-amino-acid protein (AAF13702) with a predicted relative molecular mass of 71 276.

For *DmNHE2*, the story is more involved. Partial cDNA sequencing of *DmNHE2* (which was not available as an EST) was achieved with an RT-PCR-based strategy, designing primers against putative exons flanking introns and sequencing the cDNA fragments obtained from Malpighian tubule cDNA. This partial sequence was deposited in GenBank (accession AF239763). However, another cDNA sequence corresponding to *DmNHE2* has been deposited in GenBank (X. Lin, D. C. Huang, W. Yan and D. L. Barber, unpublished: accession number AF235935). Although longer than our sequence, it is clearly incomplete at both the 5′ and 3′ ends, lacking a 5′ untranslated region (UTR) and finishing on an exon boundary without a credible polyadenylation site. Since then, the Berkeley *Drosophila* Genome project (BDGP) has produced an automated annotation of the locus, which erroneously splits the AF235935 transcript into two genes, named *NHE2* and *CG9255*. Very recently, four new ESTs from an adult testes library have been deposited in GenBank (clones AT08048, AT04839, AT11019 and AT12693). They all start within 15 bases of each other and extend the available sequence by nearly 400 bases 5′ to the AF23595 sequence. There was, therefore, no single sequence that describes the very large *DmNHE2* transcript. Accordingly, we sequenced the longest available testes clone (AT11019) to resolve the issue. Our sequence (Fig. 5) is clearly a novel splice variant of *DmNHE2*. Neither the AF23595 nor Gadfly sequences had a credible signal peptide (PSORT II), which is essential for this integral membrane protein. Our longer cDNA had several novel potential methionine initiator sites, only one of which read into a

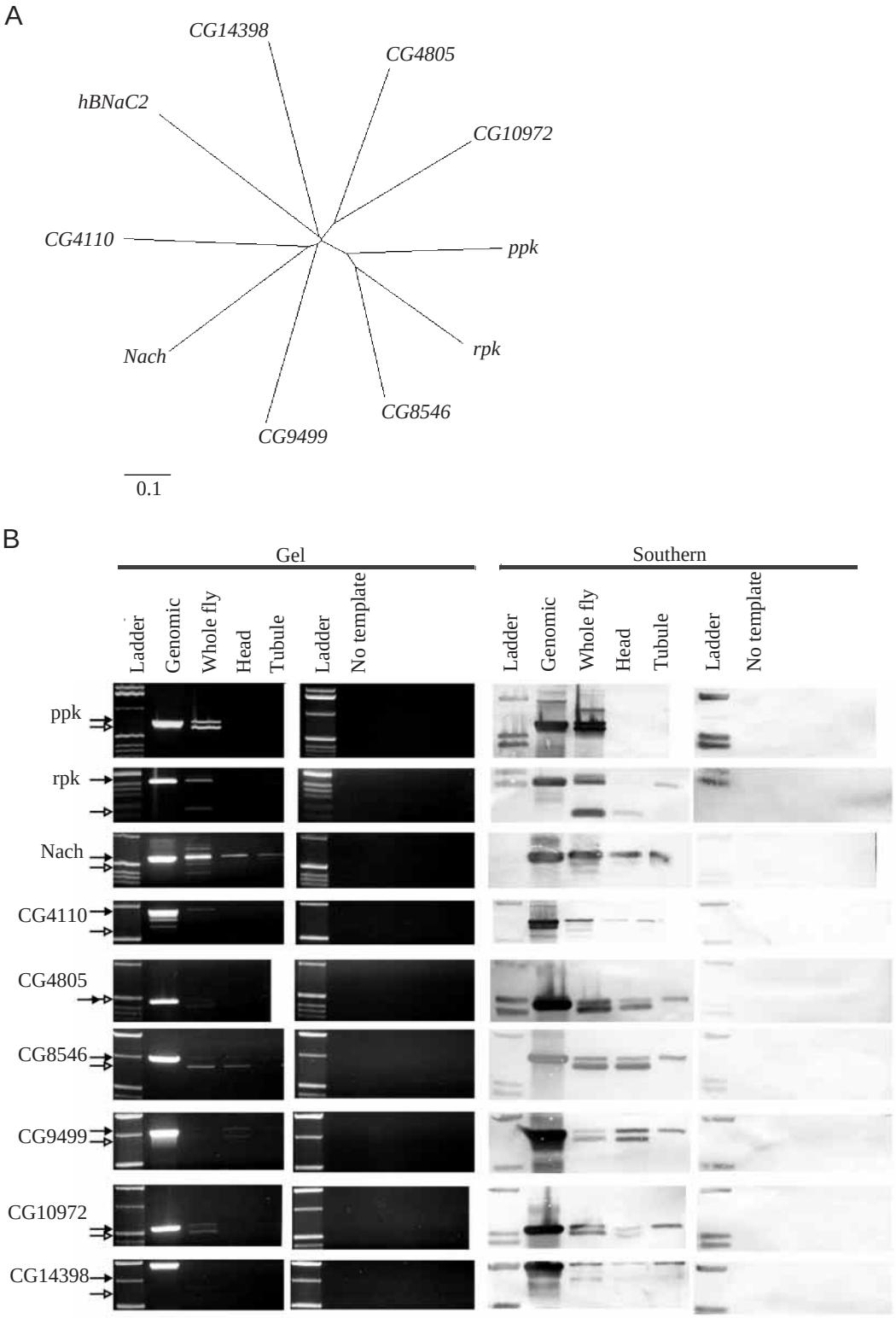

Fig. 2. Malpighian tubules do not express epithelial Na^+ channels (ENaCs). (A) Phylogenetic tree of all *Drosophila* ENaCs identified by BLASTP search using human amiloride-sensitive cation channel 2, neuronal hBNaC2 (GenBank accession number NP 064423) protein sequence as a probe. (B) RT-PCR for putative EnaCs (left-hand panels) and corresponding Southern blots with probes specific to each gene (right-hand panels). Labels refer to known genes or to Gadfly-predicted genes. Size markers denote expected sizes from genomic (black arrows) and cDNA (white arrows) templates. The ladder is a Gibco BRL 1 kb ladder. The templates are as follows: Genomic, genomic DNA; Whole fly, whole-fly cDNA; Head, head cDNA; Tubule, Malpighian tubule cDNA; No template, no template (negative control).

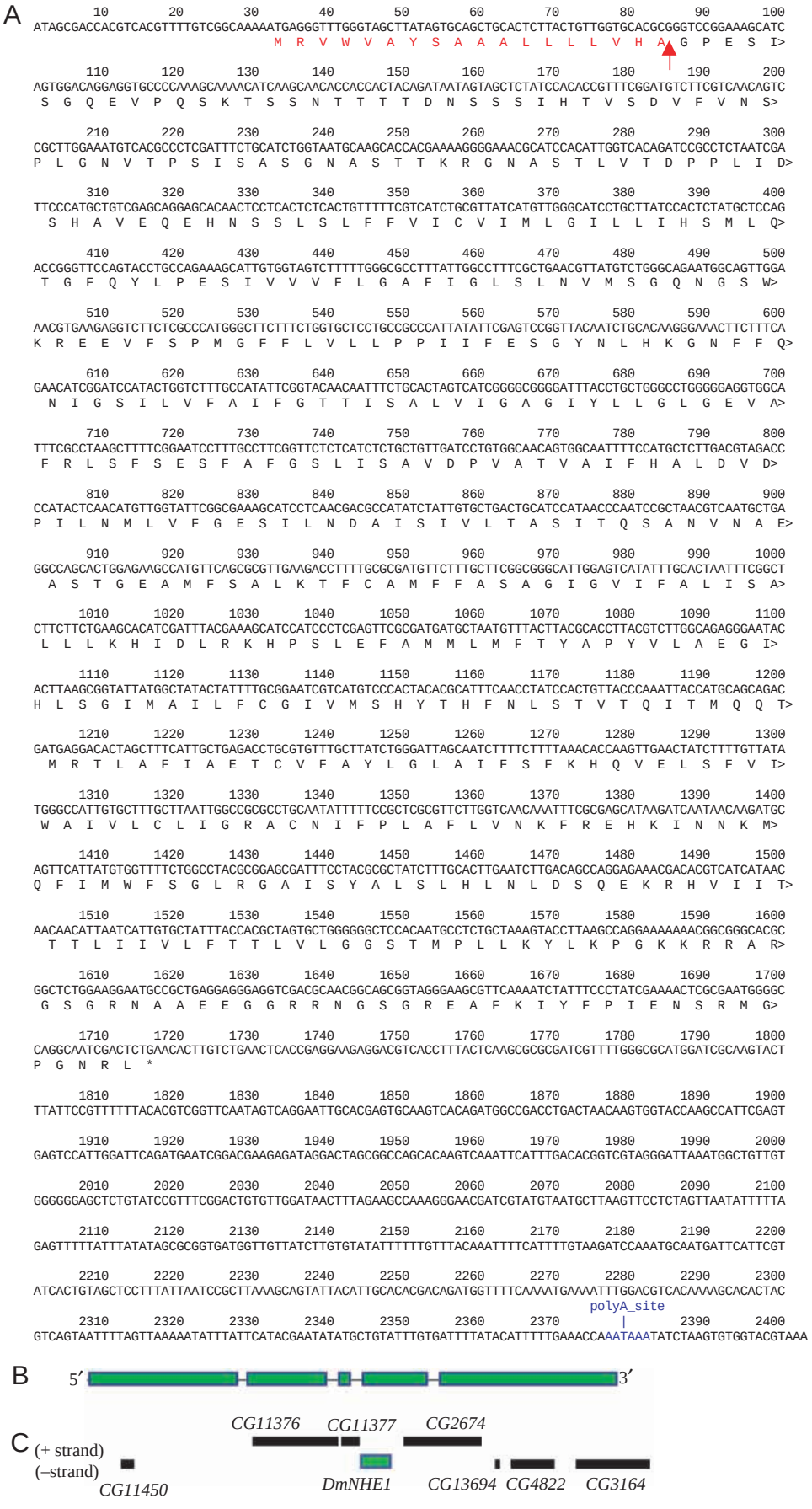

Fig. 3. cDNA, predicted protein sequence (A) and genomic context (B,C) for *DmNHE1*. (A) The signal peptide and putative cleavage sites are marked in red. The polyadenylation signal is marked in blue. (B) Transcript structure. *DmNHE1* is a simple gene, with five exons spanning 2.5 kb of genomic sequence. (C) Genomic context of *DmNHE1* at 21B1 on chromosome 2, showing the surrounding gene-dense region, with seven putative genes within 45 kb (from Gadfly annotation). This sequence has been deposited in GenBank with the accession number AF142676.

plausible signal peptide. Accordingly, we take this to be the likeliest start site (Fig. 5).

Predicted structures of Drosophila *NHEs*

Using the PSORT II (Nakai and Horton, 1999) and PROSITE (Bairoch et al., 1997) protein prediction programmes, it appears that all three *Drosophila* NHEs are plasma membrane integral proteins (61%, 57% and 70% predicted plasma membrane targeting, respectively), with 11 putative transmembrane domains. Although some of the *Drosophila* NHEs appear to sit close to mitochondrial NHEs in the similarity tree (see Fig. 7), they lack mitochondrial targeting sequences (PSORT II). The transmembrane probability plots (von Heijne, 1992) for the three proteins can be seen in Fig. 6. Clearly, the *Drosophila* NHEs share the same organisation (short N terminus, compact 10–12-pass transmembrane domain of approximately 400 residues, long hydrophilic C terminus) with human NHE1, the archetype of the NHE family, and so can be assigned with confidence to the family. *DmNHE1* and *DmNHE3* contain a signal peptide sequence with a putative cleavage site at residue 18/19. For *DmNHE2*, there are three contending sequences. The Gadfly CG9255 *DmNHE2* sequence is clearly incomplete (Fig. 6), so we reject this automated annotation in this case. The translation of the AF23595 sequence for *DmNHE2* lacks an N-terminal signal peptide sequence; however, if translation were to start at the initiator methionine corresponding to bases 628–630 of our sequence, then there would be a clear 21-residue signal peptide (PSORT II prediction), and the disposition of the N terminus of the peptide would be almost identical to that of human NHE1 (Fig. 6). At the C terminus, *DmNHE2* appears to encode an extremely long cytoplasmic C-terminal

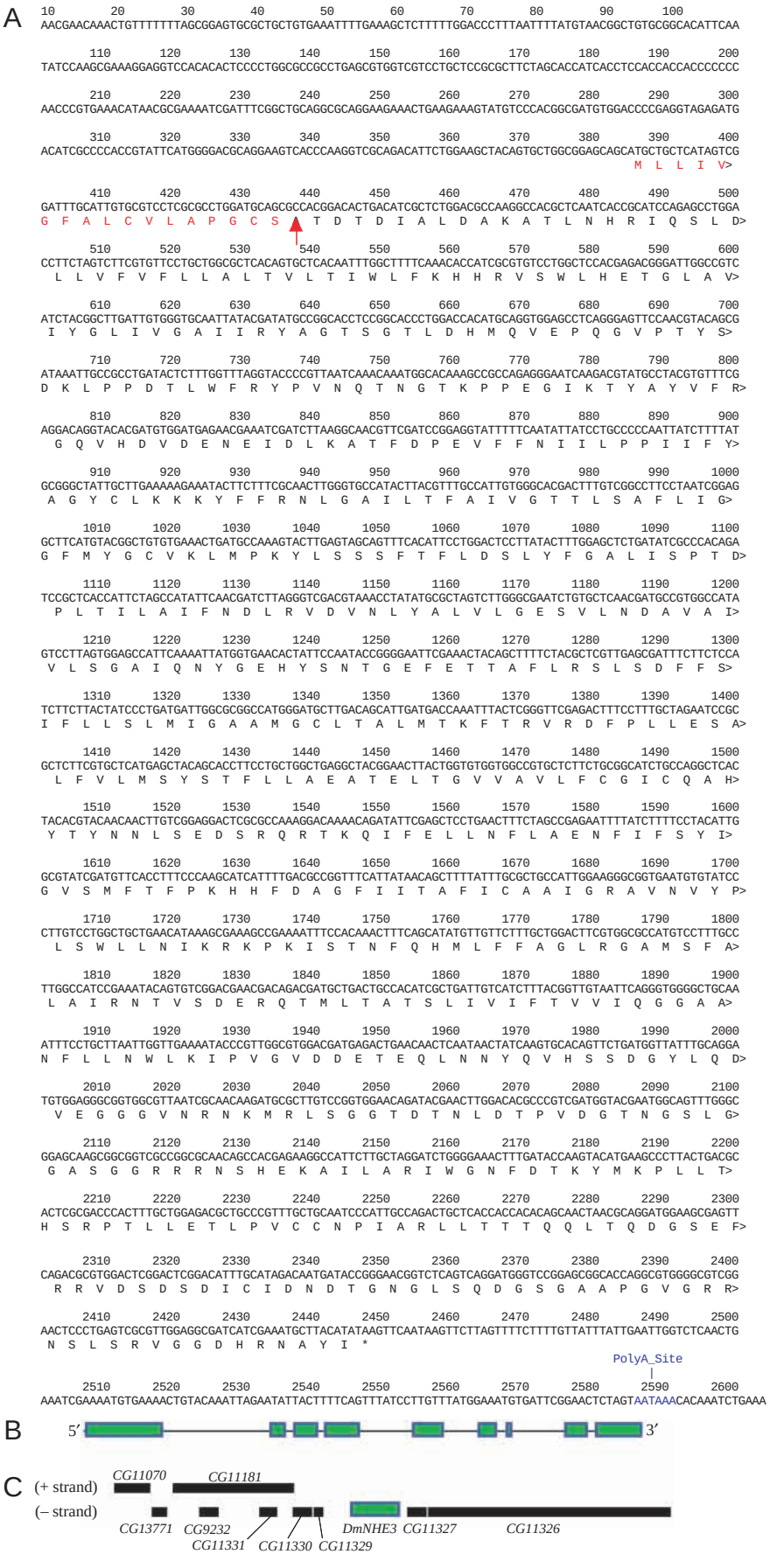

Fig. 4. cDNA, predicted protein sequence (A) and genomic context (B,C) for *DmNHE3*. (A) The signal peptide and putative cleavage sites are marked in red. The polyadenylation signal is marked in blue. (B) Transcript structure. (C) Genomic context of *DmNHE2* at 27A1 on chromosome 2, showing gene-dense region containing nine putative surrounding genes within 50 kb (from Gadfly annotation). This sequence has been deposited in GenBank with the accession number AF199463.

tail, with a very short 3′UTR that lacks a polyadenylation site. However, our confidence in this structure is increased by the conceptual translation of an *Aedes aegypti* sequence which, though lacking an N-terminal signal peptide, has a very similar C-terminal cytoplasmic domain. It it therefore likely to be a partial, but authentic, cDNA. However, the testes cDNA we describe here appears to represent a complete, authentic cDNA that has a 5′UTR, a signal peptide and a polyadenylation site. *DmNHE2* must therefore be considered to have alternative transcripts, both identical at the 5′UTR and the N-terminal and membrane-spanning coding regions, but having very different C termini through facultative read-through of the last exon of our sequence (Fig. 5). As the cytoplasmic C terminus is considered to have control properties, this difference is likely to be functionally very significant.

All three isoforms have multiple putative N-glycosylation sites, putative phosphorylation sites for cAMP- and cGMP-dependent protein kinases, protein kinase C and casein kinase type II sites (Prosite predictions). *DmNHE3* also appears to have two leucine zipper motifs in transmembrane region (TM) 7, which is quite unusual because only human *NHE5* and the *Arapidopsis thaliana* NHEs have leucine zipper motifs (three in human *NHE5* in TMs 1 and 2; one in *Arabidopsis thaliana* NHE in the intracellular domain between TM6 and TM7).

Alignment and phylogenetic relationships

The *Drosophila* NHE sequences were used in further BLAST searches to identify other members of the NHE family. Amongst proteins identified were those from a variety of species, mammalian (human, rat, bovine) and other

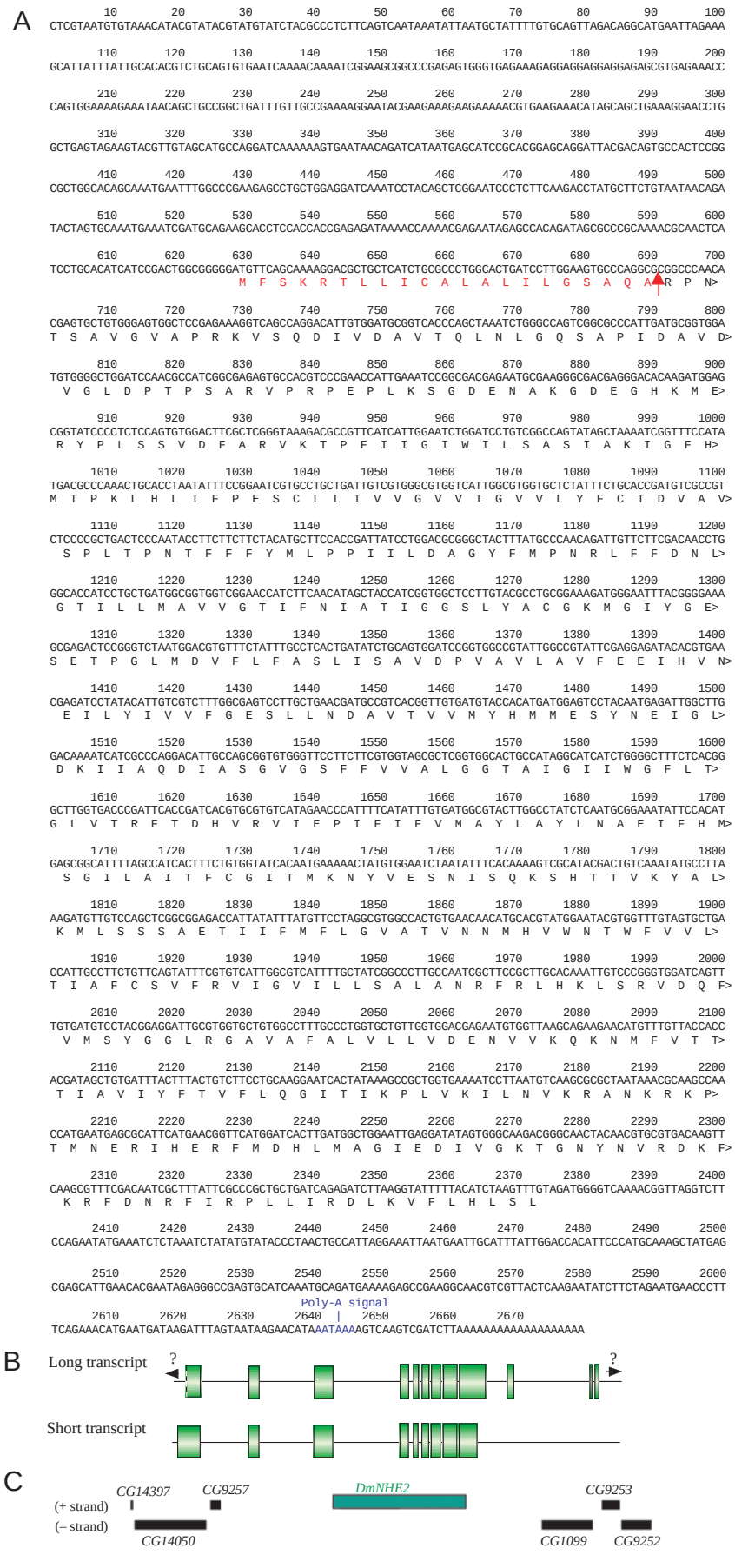

Fig. 5. cDNA, predicted protein sequence and genomic context for *DmNHE2*. (A) cDNA sequence and putative transcript for testes clone AT11019. The putative signal peptide and cleavage site are marked in red. The polyadenylation signal is marked in blue. (B) Transcript structure of *DmNHE2*. The long transcript is that of GenBank accession number AF235935 (X. Lin, D. C. Huang, W. Yan and D. L. Barber, unpublished). The short transcript is our sequence for testes cDNA clone AT11019. (C) Genomic context of *DmNHE2* at 39B1 on chromosome 2, showing the surrounding genes within 53 kb, modified from Gadfly annotation, to reflect the larger size of *DmNHE2*. This sequence has been deposited in GenBank with the accession number AF239763.

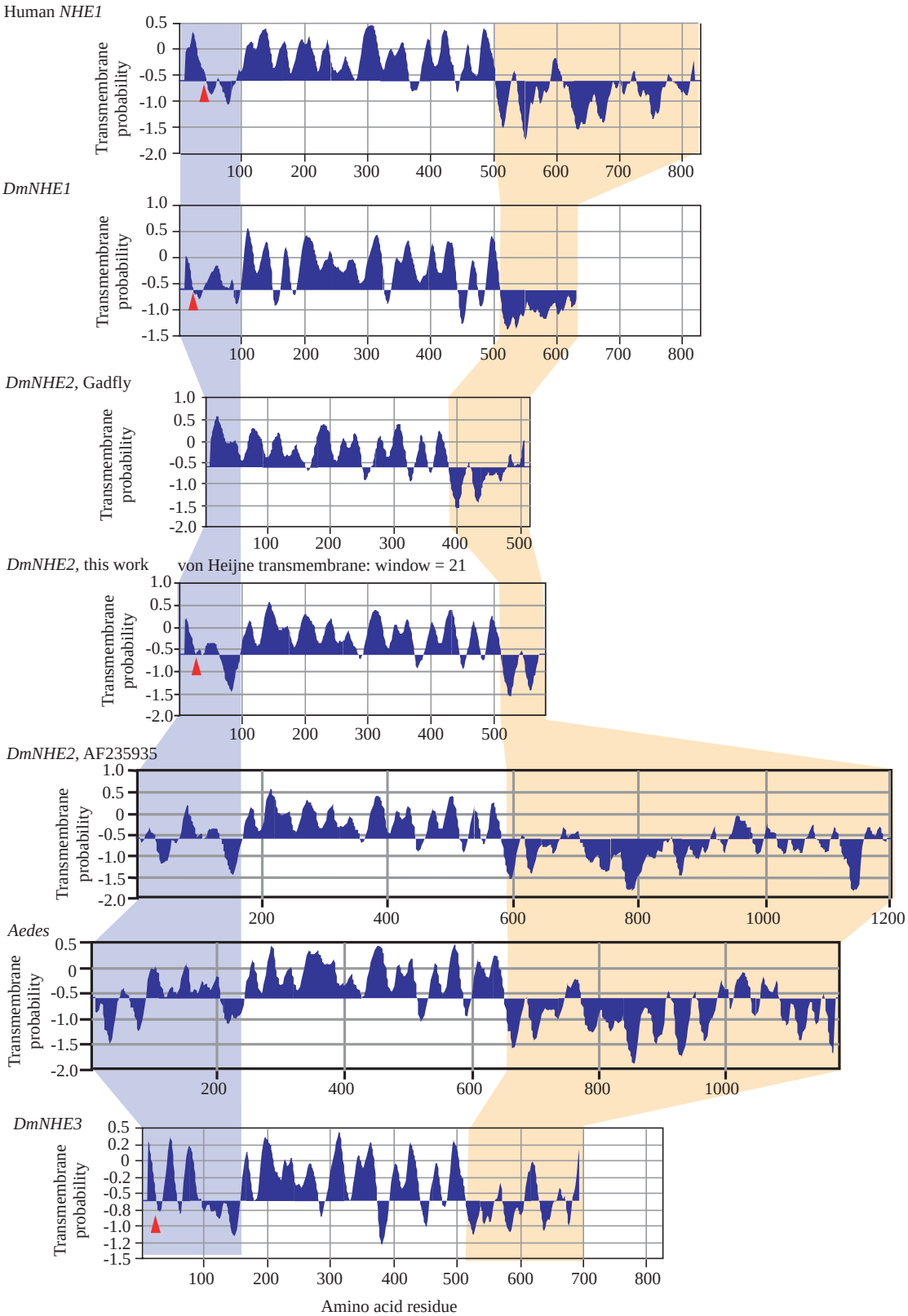

Fig. 6. Structure predictions for human NHE1, *DmNHE1*, *DmNHE2* and *DmNHE3*. Transmembrane probability plots for human NHE1, *DmNHE1*, *DmNHE2*, *Aedes aegypti* NHE3 and *DmNHE3* proteins according to the von Heijne (von Heijne, 1992) algorithm. Predictions were made using MacVector 7.0. The blue background indicates the N-terminal domain; white, the compact transmembrane domain; yellow, the C-terminal cytoplasmic domain. Putative signal peptide cleavage sites are marked with red arrowheads. All three gene models for *DmNHE2* are shown (see text) with the *Aedes aegypti* sequence for comparison.

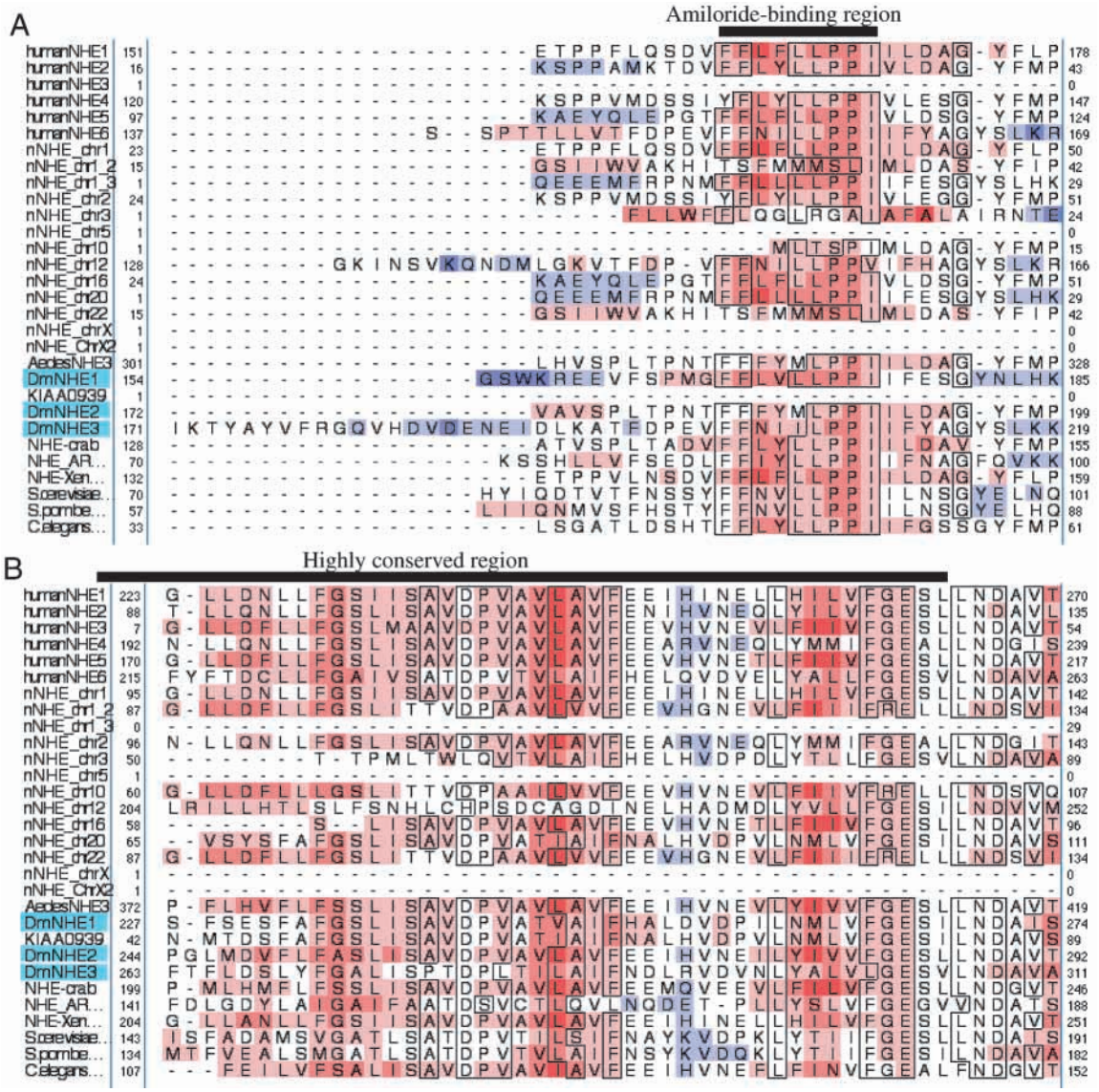

Fig. 7. Alignments of conserved domains in Na^+/H^+ exchangers (NHEs). Alignment of *Drosophila melanogaster* NHEs and other representative NHE family members, produced using ClustalW and SeqVu software. (A) An alignment of the NHEs in the amiloride-binding region and (B) the alignment of NHEs in the highly conserved region. Blue denotes hydrophilic, and red hydrophobic, residues. Nomenclature is as for Fig. 8.

vertebrate (trout, *Cyprinus carpio* NHE) and invertebrate (crab, *Caenorhabditis elegans*) sequences. Amongst these sequences were a number of novel human NHE protein sequences, a protein previously identified as KIAA0939, which is the closest homologue to *DmNHE1* (55% identity at the amino acid level) and 13 sequences newly emerged from the human genome project, partial protein sequences from working drafts of the human genome. KIAA0939 was identified in IMAGE clone 3134373 (GenBank accession number BF222481), from a *Homo sapiens* kidney cDNA library.

The *Drosophila* NHEs were aligned to the human and other members of the NHE family of exchangers (Fig. 7), and a phylogenetic tree was constructed to examine similarities between the different family members (Fig. 8). The alignment shows that the *Drosophila* NHEs are definitely members of the family, with different *Drosophila* NHEs more similar to different branches of the family. *DmNHE1* is most closely related to the novel human NHE KIAA0939 and to some novel sequences from working drafts. *DmNHE3* is more closely related to some new human NHE draft sequences and the human NHE6, which is the mitochondrial NHE isoform (Numata et al., 1998). *DmNHE2*, in contrast, is more closely related to the crab NHE from *Carcinus maenas* and to the exchanger from the yellow fever mosquito *Aedes aegypti*.

The amiloride-binding region, which is present in all known NHE family members (Wakabayashi et al., 1997), is present in all the *DmNHE*s, although it is not perfectly conserved, which could explain the slightly lower amiloride sensitivity we observed (Table 1). The highly conserved region between NHE isoforms, which is thought to be involved in ion exchange

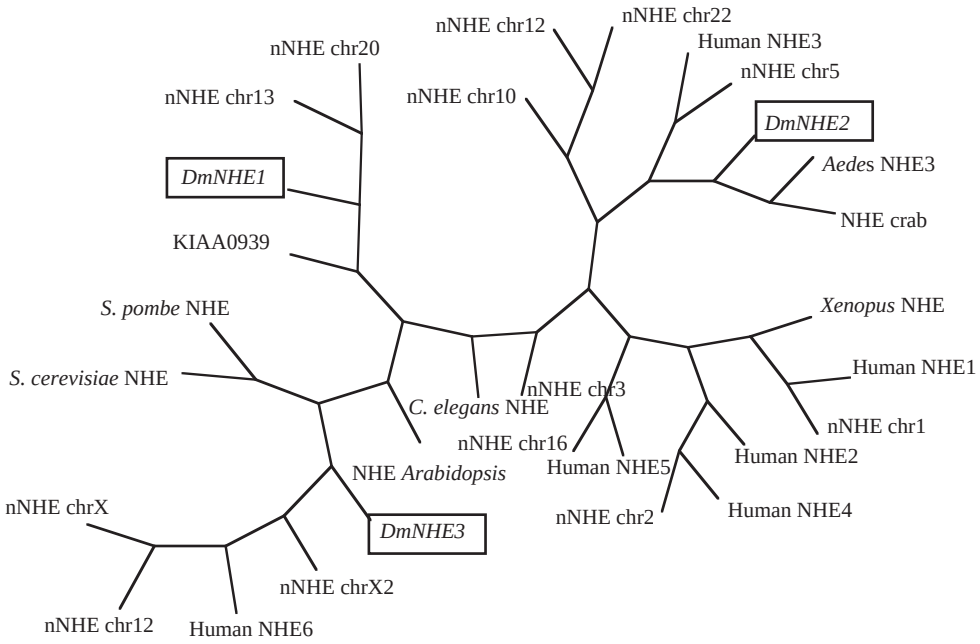

Fig. 8. Phylogenetic tree for selected Na^+/H^+ exchangers (NHEs). Protein sequences are those aligned in Fig. 7, produced using TreeViewPPC and ClustalW software. The boxed names are the *Drosophila* NHEs, and the other abbreviations are as follows: *Xenopus* NHE, *Xenopus laevis*, CAA69925 (Busch, 1997); human NHE1, *Homo sapiens*, AAB59460 (Sardet et al., 1988); nNHE chr1, *Homo sapiens*, Hs1_5070 chromosome 1 working draft sequence; human NHE2, *Homo sapiens*, XP_010884 (NCBI Annotation Project; direct submission, 2001); human NHE4, *Homo sapiens*, P26434 (Orlowski et al., 1992); nNHE chr2, *Homo sapiens*, Hs2_5544 chromosome 2 working draft sequence; human NHE5, *Homo sapiens*, NP_004585 (Klanke et al., 1995); nNHE chr16, *Homo sapiens*, Hs16_10635 chromosome 16 working draft sequence; *C. elegans* NHE, *Caenorhabditis elegans*, P35449 (Marra et al., 1993); nNHE chr3, *Homo sapiens*, Hs3_19514 chromosome 3 working draft sequence; NHE *Arabidopsis*, *Arabidopsis thaliana*, AAF21755 (Quintero et al., 2000); *DmNHE3*, *Drosophila melanogaster*, AAF13702 (this work); nNHE chrX2, *Homo sapiens*, HsX_11991 chromosome X working draft sequence; human NHE6, *Homo sapiens*, AAC39643 (Numata et al., 1998); nNHE chr12, *Homo sapiens*, Hs12_9838 chromosome 12 working draft sequence; nNHE chrX, *Homo sapiens*, HsX_11725 chromosome X working draft sequence; *S. cerevisiae* NHE, *Saccharomyces cerevisiae*, Q04121 (Numata et al., 1998); *S. pombe* NHE, *Schizosaccharomyces pombe*, T37706 (L. Murphy, D. Harris, V. Wood, B. G. Barrell and M. A. Rajandream, unpublished); KIAA0939, *Homo sapiens*, CAB46030 (N. Corby, unpublished); *DmNHE1*, *Drosophila melanogaster*, AAD32689 (J. A. T. Dow, unpublished; and this work; nNHE chr13, *Homo sapiens*, Hs1_22216 chromosome 1 working draft sequence; nNHE chr20, *Homo sapiens*, Hs20_11518 chromosome 20 working draft sequence; nNHE chr10, *Homo sapiens*, Hs10_8739 chromosome 10 working draft sequence; nNHE chr12, *Homo sapiens*, Hs1_22246 chromosome 1 working draft sequence; nNHE chr22, *Homo sapiens*, Hs22_11677 chromosome 22 working draft sequence; human NHE3, *Homo sapiens*, P48764 (Brant et al., 1995); nNHE chr5, *Homo sapiens*, Hs5_7223 chromosome 5 working draft sequence; *DmNHE2*, *Drosophila melanogaster*, AAF53960 (this work); *Aedes* NHE3, *Aedes aegypti*, AF80554 (S. S. Gill, H. Wediak and L. S. Ross, unpublished); NHE crab, *Carcinus maenas*, AAC26968 (Towle et al., 1997). nNHE denotes a novel or undocumented gene in the human genome sequence.

or at least essential for ion translocation (Noel and Pouyssegur, 1995), is also well conserved in the *Drosophila* NHEs, which are, however, more divergent than the other family members. In that respect, *DmNHE2* is the most similar to the other family members. Another domain present in many NHEs is a calmodulin-binding region found in the cytoplasmic C terminus of the exchangers. This region, termed calmodulin-binding region A in NHE1, is also found in the NHE1 isoform of other organisms, such as the mouse, *Xenopus laevis*, bovine NHE1, rat, pig, *Amphiuma tridactylum* and rabbit, in the NHE2 isoform of human and rabbit and the rat NHE4 isoform. It is not, however, found in any of the *Drosophila* NHEs.

Expression patterns of Drosophila *NHEs*

Expression patterns of *DmNHE1*, *DmNHE2* and *DmNHE3* were mapped by RT-PCR using cDNA derived from a variety of tissues using primers designed to bracket introns and using genomic DNA as a positive control for the reaction (Fig. 9). This experiment showed that all three genes are expressed in the head, body and Malpighian tubules and at all developmental stages, indicating that they are widely expressed.

Discussion

Amiloride effects and the Wieczorek model

The *Drosophila* Malpighian tubule is exquisitely sensitive to agents hypothesised to affect the components of the Wieczorek model for insect epithelia, namely the apical V-ATPase and associated exchanger. However, while bafilomycin is agreed to be a selective inhibitor of V-ATPase, amiloride could target a range of molecules on apical or basal surfaces. Here, we show that the Malpighian tubules are blocked by a characteristic range of amiloride derivatives characteristic of exchangers, rather than channels. The order of inhibition of fluid secretion in Malpighian tubules is EIPA⩾2,4-dichloro-benzamil>DMA>amiloride≈benzamil (Fig. 1; Table 1). These results are consistent with those recently obtained in *Aedes aegypti* (Petzel, 2000), increasing our confidence that amiloride targets NHEs in insect Malpighian tubules. This is also consistent with our RT-PCR data showing that *Drosophila* NHE genes, but not ENaC genes (Fig. 9, see also Fig. 2), are expressed in Malpighian tubules. Our failure to identify ENaCs in Malpighian tubules is consistent with electrophysiological analysis, which showed

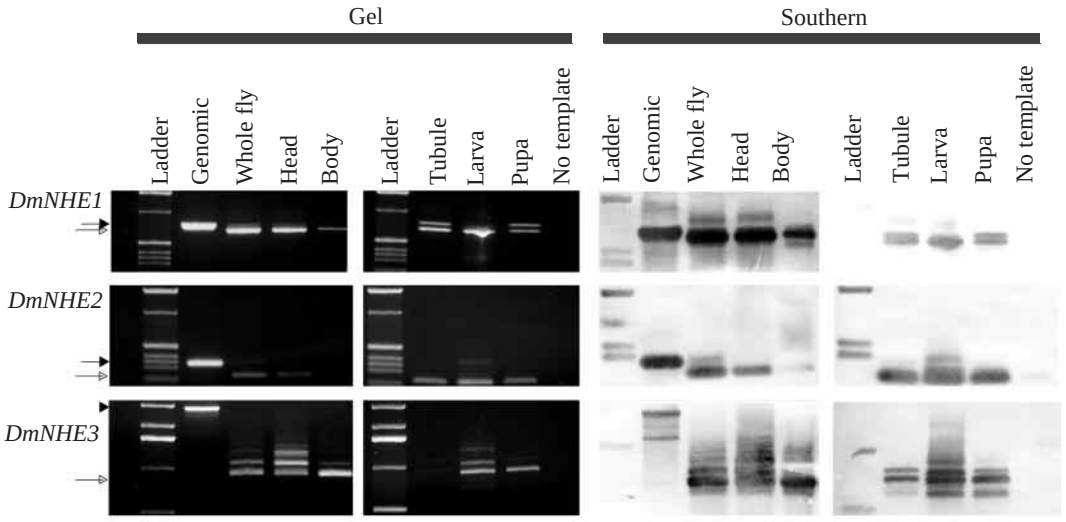

Fig. 9. Expression pattern of *Drosophila* Na^+/H^+ exchangers (NHEs). Left-hand panels: RT-PCR using primers specific for *DmNHE1* (top), *DmNHE2* (middle) and *DmNHE3* (lower panel). Predicted sizes for genomic (black arrow) and cDNA (white arrow) templates are shown. Templates: Genomic, genomic DNA; Whole fly, whole-fly cDNA; Head, head cDNA; Body, body cDNA; Tubule, Malpighian tubule cDNA; Larva, mixed larval cDNA; Pupa, mixed pupal cDNA; No template, no DNA control. Ladder is a 1 kb marker (Promega). Right-hand panels: the identity of the major bands labelled was verified by Southern hybridisation using DIG-labelled cDNA probes derived from an EST clone for *DmNHE1* and *DmNHE3* and from the previously sequenced tubule cDNA RT-PCR product for *DmNHE2*.

that amiloride inhibited transepithelial Na^+ secretion in *Aedes aegypti* Malpighian tubules without any effect on transepithelial and fractional membrane resistance (Hegarty et al., 1992).

The Drosophila *NHE family*

This paper describes three genes that appear to encode the *Drosophila* members of the NHE gene family. Their protein sequences are quite different from the protein sequences of the other members of the family, but there is sufficient similarity to the other NHEs to assign them unambiguously to this group of proteins (Fig. 6). More specifically, *DmNHE1* appears to be very similar to a novel human NHE, KIAA0939, which has been found in kidney (IMAGE 3134373) and brain (GenBank accession number AB023156) (Nagase et al., 1999). *DmNHE2* is most similar to two invertebrate NHEs, the NHE found in *Carcinus maenas* and the newly described NHE3 in *Aedes aegypti* (GenBank accession number AF80554; S. S. Gill, H. Wediak and L. S. Ross, unpublished). DmNHE3 sits near human mitochondrial NHE6 (although *DmNHE3* encodes no mitochondrial targeting sequences) and also close to *Arabidopsis thaliana* and yeast genes.

The three *DmNHEs* described above are predicted to be plasma membrane integral proteins with 10–12 transmembrane domains just like the other members of the family (Fig. 6). All the *Drosophila* NHEs have a putative signal peptide and a possible cleavage site. This is similar to the position in mammalian NHEs, although it is not certain whether the signal peptide is ever cleaved (Zizak et al., 2000); see Shrode et al. (Shrode et al., 1998) and Wakabayashi et al. (Wakabayashi et al., 2000). The presence of distinct messages for DmNHE2, encoding peptides with differing C-terminal domains, has interesting implications for control of the exchanger.

In principle, the elucidation of genes in *Drosophila* would allow the reverse genetic analysis of their function in mutants. However, there are no candidate P-element insertions documented at any of the three loci. The nearest mutation is an insertion, 2 kb beyond the 3′ end of *DmNHE3*, that generates a lethal recessive phenotype. However, this insertion is at the 5′ end of a novel gene (CG11329), and so the lethality is probably attributable to the latter locus.

Are any of these genes candidates for the Wieczorek exchanger? Their relative dissimilarity to cardinal vertebrate NHEs (Fig. 8) would allow them to be ascribed different functional properties. For example, *DmNHE2* sits in a branch of the similarity tree with only invertebrate representatives and so would be a strong candidate. Our data show that, in *Drosophila*, all three exchangers are widely expressed (Fig. 9) and are certainly present in a relevant epithelium (the Malpighian tubule). However, the same general expression pattern would argue against a specialised role in transporting epithelia only, and our pharmacological analysis (Fig. 1) does not distinguish between an apical or basolateral localisation. Recent electrophysiological evidence suggests that amiloride may be acting at the basolateral membrane of *Aedes aegypti* Malpighian tubules (Petzel, 2000), and our results cannot be taken to contradict this view. In insects, it may have been naïve to assume that sensitivity to bafilomycin and amiloride is sufficient proof that an epithelium conforms to the Wieczorek model. However, whether *DmNHE1*, *DmNHE2* or *DmNHE3* transpires to be the elusive apical exchanger, or a vital part of the cell's ion-regulatory machinery, the description of this gene family in a genetic model organism should be useful.

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