Journal of Cell Science 107, 1817-1824 (1994)
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Evidence that the 16 kDa proteolipid (subunit c) of the vacuolar H^+-ATPase and ductin from gap junctions are the same polypeptide in *Drosophila* and *Manduca:* molecular cloning of the *Vha16k* gene from *Drosophila*

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SUMMARY

The 16 kDa proteolipid (subunit c) of the eukaryotic vacuolar H^+-ATPase (V-ATPase) is closely related to the ductin polypeptide that forms the connexon channel of gap junctions in the crustacean *Nephrops norvegicus*. Here we show that the major protein component of *Manduca sexta* gap junction preparations is a 16 kDa polypeptide whose N-terminal sequence is homologous to ductin and is identical to the deduced sequence of a previously cloned cDNA from *Manduca* (Dow et al., *Gene*, 122, 355-360, 1992). We also show that a *Drosophila melanogaster* cDNA, highly homologous to the *Manduca* cDNA, can rescue *Saccharomyces cerevisiae*, defective in V-ATPase function, in which the corresponding yeast gene, *VMA3*, has been inactivated. Evidence is presented for a single genetic locus (*Vha16*) in *Drosophila*, which in adults at least contains a single transcriptional unit. Taken together, the data suggest that in *Drosophila* and *Manduca*, the same polypeptide is both the proteolipid subunit c component of the V-ATPase and the ductin component of gap junctions. The intron/exon structure of the *Drosophila Vha16* is identical to that of a human *Vha16* gene, and is consistent with an ancient duplication of an 8 kDa domain. A pilot study for gene inactivation shows that transposable P-elements can be easily inserted into the *Drosophila* ductin *Vha16* gene. Although without phenotypic consequences, these can serve as a starting point for generation of null alleles.

Key words: ductin, 16 kDa proteolipid, *Drosophila*, *Manduca*, *Vha16*, gap junction, vacuolar ATPase

INTRODUCTION

The vacuolar H^+-ATPase (V-ATPase) is a proton pump required for acidification of many types of eukaryotic vacuole. These include lysosomes, plant and fungal vacuoles, synaptic vesicles, coated vesicles and Golgi (Nelson, 1992). A component of the V-ATPase is a phylogenetically conserved 16 kDa polypeptide (subunit c), called the 16 kDa proteolipid, and is thought to form the membrane pore through which protons translocate. It appears to have evolved by tandem duplication of an 8 kDa domain with similarity to an 8 kDa proton pore component of the F_1F_0 ATP synthase (Mandel et al., 1988). The 16 kDa proteolipid of the yeast *Saccharomyces cerevisiae* is encoded by the *VMA3* gene, inactivation of which results in loss of V-ATPase activity and inability to grow at pH 7.5 (Nelson and Nelson, 1990a,b; Umemoto et al., 1990).

A 16-18 kDa polypeptide, recently called ductin, has also been found to be the principal protein component of gap junctions isolated from the hepatopancreas of *Nephrops norvegicus* (Finbow et al., 1992; Holzenburg et al., 1993; Finbow and Pitts, 1993). Gap junctions are aggregates of paired connexon channels that allow the intercellular movement of cytoplasmic solutes up to M_r 1,000 within tissues of metazoan animals. Ductin has been sequenced in its entirety (Finbow et al., 1992) and is very similar to the 16 kDa proteolipid encoded by the *VMA3* gene (70% identity). Moreover, a cDNA encoding ductin rescues *VMA3*-defective yeast and the rescued cells contain a hybrid V-ATPase in which ductin of *Nephrops* is the principal membrane component (Holzenburg et al., 1993; Harrison et al., 1994). Ductin-related polypeptides are present in other crustacean gap junction preparations (Finbow et al., 1984; Holzenburg et al., 1993), and in vertebrate gap junction preparations (Finbow et al., 1984, 1993; Buultjens et al., 1988).

The question arises as to whether the V-ATPase 16 kDa proteolipid and ductin of gap junctions are the same polypeptide, or merely closely related polypeptides. If the former, one might expect a single gene. cDNAs representing homologues of the 16 kDa proteolipid and ductin are available for a number of species, including several vertebrates, and the insects *Manduca sexta* (Dow et al., 1992) and *Drosophila melanogaster* (Meagher et

al., 1990). Even under conditions of high stringency, Southern blot analysis of vertebrate DNA has been taken to suggest up to three or four genetic loci (Nezu et al., 1992; Hasebe et al., 1992). Under similar conditions, though, only a single genetic locus is detected in *Manduca* (Dow et al., 1992). However, it is not known if ductin is a component of gap junctions in insects.

Here we show that gap junction preparations from *Manduca* larval midgut contain a 16 kDa polypeptide, the N-terminal sequence of which is identical to that predicted from the *Manduca* cDNA. We also show that the *Drosophila* cDNA, like the *Nephrops* cDNA, can rescue VMA3-defective yeast. A molecular genetic analysis shows that only a single *Drosophila* locus is detected under conditions of high stringency hybridisation, and this appears to give rise to a single species of transcript. The evidence is consistent with the 16 kDa proteolipid and ductin being one and the same polypeptide at least in the two insect species. As a route to the generation of mutations in the *Drosophila* gene, we have used the technique of 'site-selected' P-element mutagenesis (Kaiser and Goodwin, 1990; Kaiser, 1990) to isolate lines in which the gene is tagged by transposon insertion.

MATERIALS AND METHODS

Characterisation of *Manduca* gap junctions

Midguts of 50 fifth instar larvae of *M. sexta* were removed and washed in a small volume of 1 mM $NaHCO_3$, 0.5 mM $CaCl_2$ (isolation solution, IS). Ten midguts at a time were homogenised by 15 strokes of a size 'B' Dounce pestle in a volume of 40 ml of IS. Gap junctions were isolated as described by Finbow et al. (1984, 1992). Electron microscopy was carried out as described by Lane and Dilworth (1989). SDS-PAGE was carried out as described by Finbow et al. (1984). N-terminal analysis of the 16 kDa polypeptide was carried out on a solid-phase sequencer (Findlay et al., 1989).

Expression of a *Drosophila* cDNA (pDm16k) in *S. cerevisiae*

Growth and routine maintenance of *S. cerevisiae* were carried out as described by Guthrie and Fink (1991). W303-1B (*MATα, leu2, his3, ade2, trp1, ura3)* is a haploid strain in which the *VMA3* gene is disrupted by insertion of *LEU2* (*LEU::vatc*; Nelson and Nelson, 1990b). YPH500 (*MATα, leu2, his3, ade2, trp1, ura3, lys2*), also haploid, was used as a growth control.

The cDNA insert of pDm16k encodes a *Drosophila* polypeptide of similar length and high identity to the *VMA3* gene product and ductin of *Nephrops* (Meagher et al., 1990). It is a 1.1 kb *Eco*RI fragment cloned in the vector pIC20H (Marsh et al., 1984), allowing excision with the enzymes *Hin*dIII and *Xho*I. It has an apparently complete open reading frame, flanked by 116 bp of 5′ untranslated DNA and 561 bp of 3′ untranslated DNA. The 1.1 kb fragment, and also a PCR-generated cDNA fragment containing only the coding region, were subcloned into pYes 2 (Invitrogen) at its *Hin*dIII (5′) and *Xho*I (3′) restriction sites. The PCR product was generated by amplification with the following primers (restriction sites underlined):

5′-GCCAAGCTTAAAATGTCTTCTGAAGTGAGCAGCGAC and
5′-GCCCTCGAGTTATTTCGTGTACAGGTAAATGGCCAC.

The PCR product was sequenced in its entirety and was identical over the coding sequence to the cDNA insert of pDm16k. pYes 2 contains the selectable marker *URA3*, and allows expression of an inserted cDNA under control of the *GAL1* promoter. The latter is functional only in the presence of galactose. Transformants were selected, and subsequently maintained, on minimal medium (2% glucose, 0.67% yeast nitrogen base without amino acids)/agar supplemented with histidine, adenine and tryptophan.

For analysis of pH dependence of growth, transformants were grown overnight in minimal (glucose-free) medium supplemented as above and containing 2% galactose. They were then streaked on YPD agar plates (2% bactopeptone; 1% yeast extract) supplemented with either 2% glucose or 2% galactose. Both 50 mM MOPS and 50 mM MES were used to buffer the medium, pH being adjusted to 7.5 or 5.5 with NaOH.

Cloning and characterisation of the *Drosophila* gene

A *Drosophila* genomic DNA library (Canton-S strain), constructed in the vector EMBL3 (Kaiser et al., 1994) was screened with the *Drosophila* cDNA, pDm16k. Two clones with overlapping inserts were identified (1A1 and 2B2; see Fig. 7). Direct sequencing of phage DNA, and of sub-clones, generated the 7.2 kb contig shown in Fig. 7A. That subclones represented adjacent regions of the genome was verified by sequencing of appropriate PCR products (data not shown).

Drosophila genomic DNA was prepared essentially as described by Ashburner (1989). Cleaved DNA was separated by electrophoresis on a 0.8% agarose gel, and blotted onto Hybond N membranes as described by the manufacturer (Amersham). High-stringency hybridisation was carried out at 65°C in 5× SSC (1× SSC is 150 mM NaCl, 15 mM sodium citrate), 100 mM NaH_2PO_4, pH 6.8, 0.04% bovine serum albumin, 0.04% polyvinylpyrrolidone, 0.04% Ficoll, 0.5% SDS. The blot was washed in 0.2× SSC, 0.5% SDS at 65°C. Low-stringency hybridisation was carried out at 42°C in 20% formamide, 7× SSC, 100 mM NaH_2PO_4, pH 6.8, 0.04% bovine serum albumin, 0.04% polyvinylpyrrolidone, 0.04% Ficoll, 0.5% SDS. The blot was washed in 2× SSC at 50°C. A ^{32}P-probe, prepared by random priming of linearised pDm16k, was used to probe both Southern and Northern blots.

In situ hybridisation to *Drosophila* polytene chromosomes (Canton-S strain) was performed as described by Pardue (1986). The probe was pDm16k, labelled with Bio-16-dUTP using the conditions recommended by the manufacturer (Boehringer Mannheim).

Total adult *Drosophila* RNA was isolated by the method of Chomczynski and Sacchi (1987). Poly(A)$^+$ RNA was purified by the poly-ATtract™ method (Promega). A 5 μg sample of poly(A)$^+$ RNA was separated on a formaldehyde/agarose gel, and was blotted onto a Hybond-C$^+$ membrane as described by the manufacturer (Amersham). Hybridisation was carried out at 42°C in 50% formamide, 5× SSPE, 0.04% bovine serum albumin, 0.04% polyvinylpyrrolidone, 0.04% Ficoll and 0.1% sodium dodecyl sulphate (SDS). The blot was washed in 0.1× SSC, 0.1% SDS at 65°C.

Sequencing of double-stranded DNA was carried out using the Sequenase Version 2.0 protocol (United States Biochemical Corporation). Direct sequencing of PCR products was by the modification of Winship (1989). DNA sequence analysis was performed with the assistance of the GCG suite of computer programmes (Program Manual for the GCG Package, version 7, April 1991, Genetics Computer Group, 575 Science Drive, Madison, Wisconsin, USA). Exon 1, 2 and 3 sequences were obtained on both strands, the rest of the 7.2 kb contig on one strand. Other methods were as described by Sambrook et al. (1989).

P-element tagging of the *Drosophila* gene

'Site-selected' P-element mutagenesis was carried out essentially as described by Kaiser and Goodwin (1990). Four gene-specific primers (GSPs) were used in combination with two P-element primers, P_L and P_R (see O'Hare and Rubin, 1984, for the structure of P-elements). Locations of the gene-specific primers are indicated in Fig. 7A. Primer sequences and coordinates are shown below:

GSP1 5′-GCTTTTAACGAGCAGCCAGCAG (1094-1115) t_m 68°C
GSP2 5′-TTCAAGTTGTCGCAGCAGCATG (1247-1268) t_m 66°C
GSP3 5′-GTCGCTGCTCACTTCAGAAGAC (2057-2078) t_m 68°C
GSP4 5′-TTGACGTCCATCAGGTGGCAGC (980-1001) t_m 63°C
P_L 5′-GTGTATACTTCGGTAAGCTTCGG t_m 68°C
P_R 5′-AGCATACGTTAAGTGGATGTCTC t_m 66°C

A screen of 3,000 mutagenised chromosomes resulted in the isolation of six independent P-element insertions. The exact location of these was ascertained by direct sequencing of PCR products flanking both ends of each insertion.

RESULTS

Ductin is present in a gap junction fraction isolated from an insect tissue

Previous studies have shown that ductin is the major component of gap junctions isolated from crustacean tissues. Although gap junctions have been isolated from *D. melanogaster* (Ryerse, 1989a,b, 1991) the small amount of protein that can be obtained, together with the impurity of the fractions (Ryerse, 1993), has made biochemical analysis difficult and therefore it is not known if ductin is likewise a component of gap junction preparations isolated from insect tissues. To overcome this problem, the midgut of *M. sexta* larva was used as starting material, being both large and rich in gap junctions (Lane and Dilworth, 1989; Fig 1A).

Fractions enriched in gap junction were prepared using previously described methods based upon the insensitivity of gap junctions to detergent and proteinase. The isolated gap junctions retain their overall morphology as paired membranes (Fig. 1B-D), and in glancing sections cross-striations could be

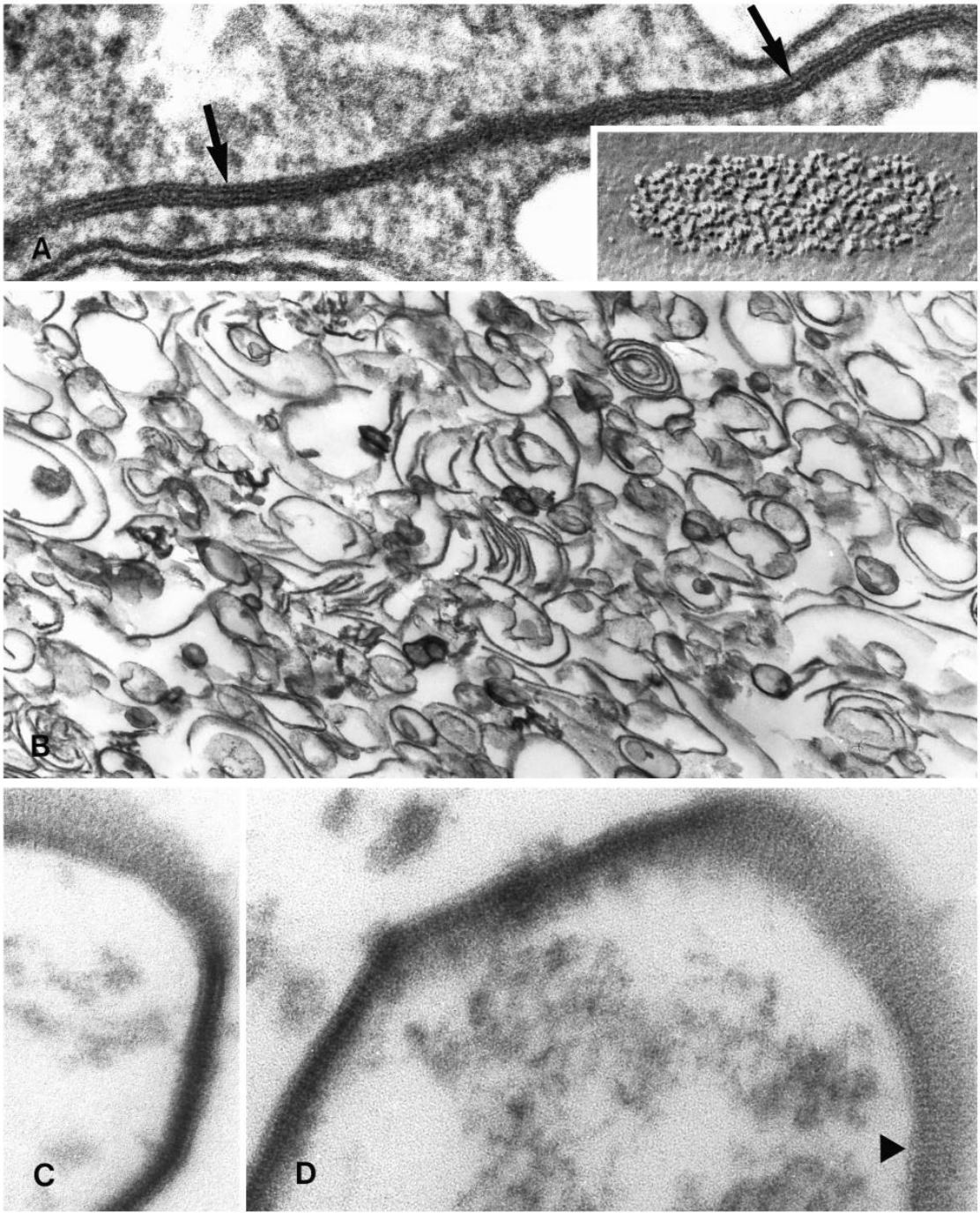

Fig. 1. Electron micrographs of *M. sexta* gap junction preparations. (A) In thin sections, gap junctions between columunar epithelial cells have a characteristic heptalaminar appearance (arrows). Freeze-fracture (inset) shows aggregates of connexon particles in the E-face. (B) Thin section analysis of a preparation enriched in gap junction. (C,D) Higher magnification showing cross-striations (arrowhead), due to the particulate substructure of connexons. Amorphous material can also be seen in (B-D). A, ×230,000; A (inset), ×94,000; B, ×40,000; C, ×280,000; D, ×317,000.

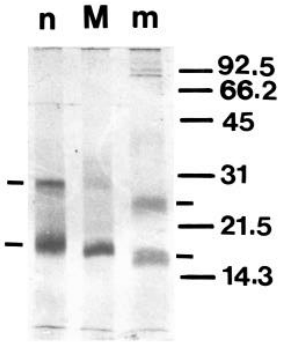

Fig. 2. SDS-PAGE of gap junctions preparations. Monomeric and dimeric species of the 16 kDa polypeptide isolated from *Nephrops* (n) hepatopancreas, *Manduca* (M) midgut, and mouse (m) liver. The indicated sizes are of Bio-Rad low molecular mass markers (in kDa).

seen that are typical of connexon packing. Some amorphous material was also present.

SDS-PAGE (Fig. 2) reveals a polypeptide of similar mobility to the ductin polypeptides present in mouse and *Nephrops* gap junction fractions. N-terminal sequencing of the *Manduca* polypeptide shows that it has high similarity to *Nephrops* and vertebrate ductins (Finbow et al., 1992). The primary structure over the sequenced region is also identical to the deduced sequence of a cDNA from *Manduca* midgut larvae (Fig. 3; Dow et al., 1992), which itself is highly homologous to a previously cloned *Drosophila* cDNA (Meagher et al., 1990). Ductin is present, therefore, in gap junction preparations isolated from an insect tissue.

Expression of a *Drosophila* cDNA (pDm16k) restores growth at pH 7.5 to a *VMA3*-defective strain of *S. cerevisiae*

The deduced amino acid sequences of the cDNAs isolated from *Drosophila* and *Manduca* are very similar in sequence and length to the 16 kDa proteolipid from *S. cerevisiae* encoded by the *VMA3* gene and known to be the subunit c polypeptide of the V-ATPase. The question arises, therefore, of whether the polypeptides encoded by these insect cDNAs are the functional analogues of the *VMA3* gene product. This can be studied in a mutant strain of *S. cerevisiae* in which the *VMA3* gene has been inactivated by insertion of the *LEU2* gene (Nelson and Nelson, 1990b). This strain (*LEU::vatc*) has no measurable V-ATPase activity but full activity can be restored by expressing the *VMA3* gene (Nelson and Nelson, 1990a,b; Umemoto et al., 1990) or a *Nephrops* cDNA (Holzenburg et al., 1994; Harrison et al., 1993). The restoration of V-ATPase activity can be quickly monitored by examining growth at pH 7.5. Cells with an inactive V-ATPase are unable to grow at this pH but can grow at pH 5.5 (Nelson and Nelson, 1990a,b).

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Drosophila cDNA     MSSEVSSDNPIYGPFFGVMGAASAIIFSAL
Manduca cDNA          MAENPIYGPFFGVMGAASAIIFSAL
Manduca 16 kDa          ENPIYG?FFGVMGAASAIIFSAL
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Fig. 3. Sequence of the *Manduca* 16 kDa gap junction polypeptide. The 16 kDa polypeptide shown in Fig. 2 was purified for sequencing as previously described (Finbow et al., 1992). N-terminal amino acid sequences deduced from the *Drosophila* (Meagher et al., 1990) and *Manduca* (Dow et al., 1992) cDNAs are shown for comparison.

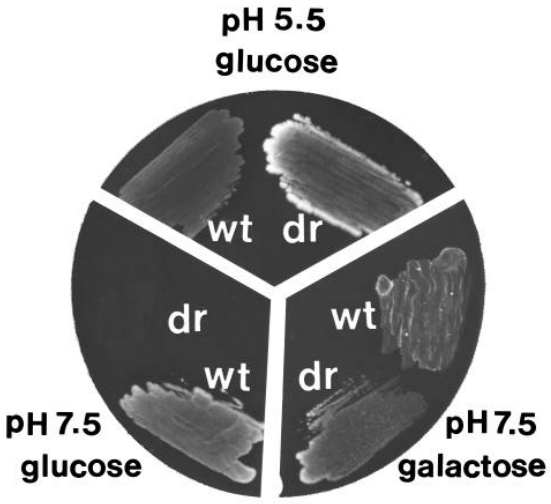


Fig. 4. Rescue of growth at pH 7.5 by the PCR-generated cDNA. W303-1B transformants (dr) grow at pH 5.5 but not pH 7.5 in the presence of glucose. They do grow at pH 7.5 in the presence of galactose, however. YPH500 (wt) grows under all three conditions. Streaked cells were incubated for 10 days at pH 7.5 (W303-1B transformants), 5 days at pH 7.5 (YPH500), and 4 days at pH 5.5 (both).

Full length *Drosophila* cDNA (pDm16k; Meagher et al., 1990) or a cDNA containing the coding region only, was incorporated into the pYes 2 yeast shuttle vector and transfected into *LEU::vatc* cells. Expression of inserted cDNA was under the control of the *GAL1* promoter, and therefore the polypeptide encoded by the cDNA, is only expressed when the cells are grown in the presence of galactose as a carbon source. Transfected cells and YPH500 (*VMA3*) cells were grown in YPD medium at pH 5.5 with glucose, or in YPD at pH 7.5 with galactose or glucose.

YPH500 cells grew, as expected, under all three conditions (Fig. 4). Cells transfected with the *Drosophila* cDNAs failed to grow at pH 7.5 in glucose but did grow in the presence of galactose where the incorporated cDNA would be expressed (results for the *Drosophila* cDNA containing only the coding region are shown). The rate of growth at the elevated pH was slower than that of the YPH500 cells. These results suggest that the 16 kDa polypeptide encoded by the *Drosophila* cDNA can functionally substitute for the proteolipid subunit c component of the *S. cerevisiae* V-ATPase. Analysis of ATPase activity in vacuolar preparations (results not shown) showed the ATPase activity attributable to the V-ATPase (i.e. amount inhibited by *N,N′*-dicyclohexamide carbodiimde) was 20% of the wild-type rate, suggesting the *Drosophila* polypeptide could only partially substitute for the 16 kDa proteolipid encoded by the *VMA3* gene.

Structure and location of the *Drosophila* gene *Vha16*

The high identity of ductin of gap junctions with the 16 kDa proteolipid of the V-ATPase suggests they may be one and the same. However, Southern blot analyses have suggested that there are

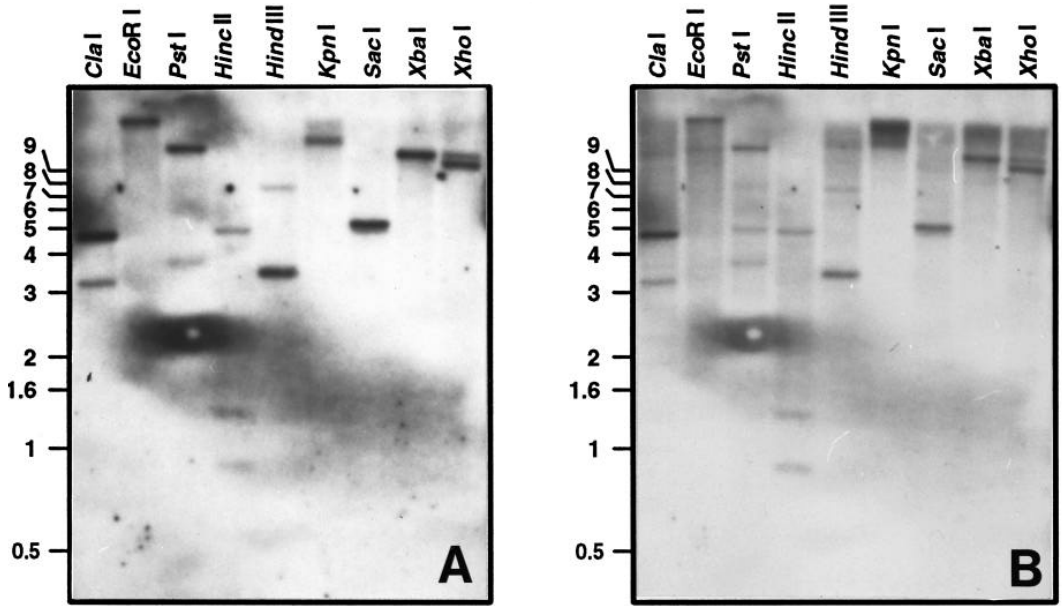


Fig. 5. Southern blot analysis of *Drosophila* genomic DNA. (A) high stringency; (B) low stringency. Sizes are given in kb.

multiple genetic loci encoding ductin and proteolipid-related polypeptides in mammals. It is therefore possible that there are two or more genes that separately code for ductin of gap junctions and the 16 kDa proteolipid of the V-ATPase in *Drosophila*. Alternatively, there may be a single gene whose transcript undergoes differential splicing. A molecular genetic analysis was therefore undertaken to investigate these possibilities.

Drosophila genomic DNA, cleaved with various restriction enzymes, was blotted and probed with pDm16k. Single bands are seen in a number of lanes after hybridisation and washing at high stringency (Fig. 5A), suggesting a single genetic locus. This is consistent with in situ hybridisation to polytene chromosome squashes, which identifies a single locus (42B1-2) on the right arm of chromosome 2 (Fig. 6B). Northern blotting of adult poly(A)$^+$ RNA generates a single band corresponding to a transcript(s) of approximately 3.1 kb (Fig. 6A).

A genomic DNA clone representing the insert of pDm16k was isolated from an EMBL3 library (see Materials and Methods). Sequencing of subclones generated a 7.2 kb contig (Fig. 7A). It contains three exons flanked on the 5′ side by a region that includes three TATAAAA motifs; 60 bp upstream of the most 5′ motif is the sequence CCAAT. Intron/exon boundaries are all related to donor and acceptor consensus sequences, and the exons combine to produce a sequence identical to that of the 1.1 kb cDNA. The second exon contains the initiation codon of the deduced polypeptide.

The above results suggest a single gene and, in adults at least, a single transcriptional unit. Genomic DNA probed at low stringency reveals some additional bands, however (Fig. 5B). These may represent distantly related members of a gene family.

In keeping with typical *Drosophila* nomenclature, the gene has been called *Vha16* (vacuolar H$^+$-ATPase 16 kDa subunit). The EMBL accession number of the genomic DNA sequence is X77936 DMCSDUC.

‘Site-selected’ transposon tagging of *Vha16*

As described above, null mutants of the *VMA3* gene are available in *S. cerevisiae* permitting analysis of V-ATPase function. In *Drosophila*, site-selected P-element mutagenesis provides a means of obtaining lines with P-element transposons inserted in or near to target genes as a step in the generation of null mutants (Ballinger and Benzer, 1989; Kaiser and Goodwin, 1990). The method utilises the polymerase chain reaction (PCR) to amplify DNA lying between a specific region of the target gene (defined by a gene-specific PCR primer) and a newly inserted transposon in its immediate vicinity (defined by a transposon-specific PCR primer). New insertions are detected initially within a large population of flies, and are then followed (as specific amplification products) while the population is subdivided. Detection at the molecular

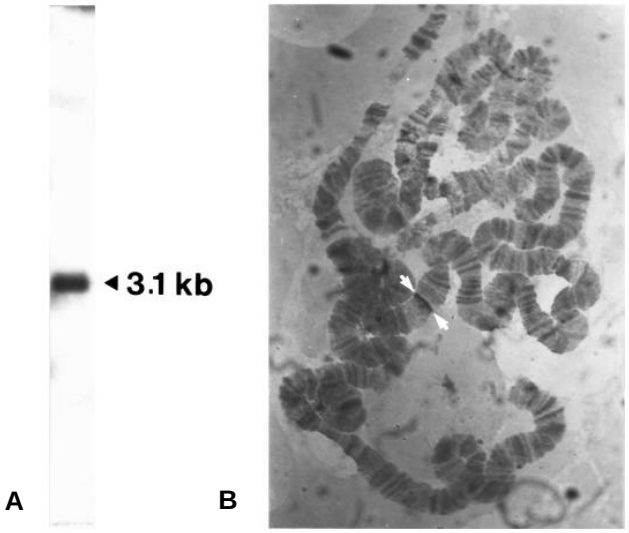
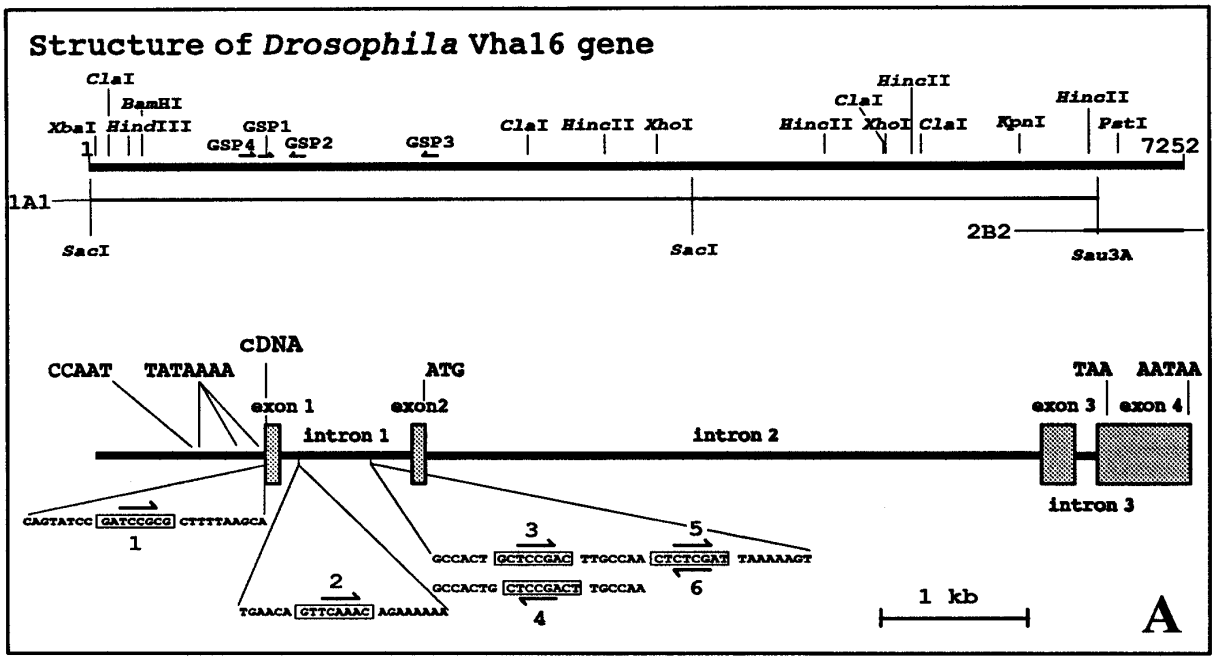


Fig. 6. (A) Northern blot analysis of adult poly(A)$^+$ RNA; (B) in situ localisation to polytene chromosomes. Northern analysis of poly(A)$^+$ RNA from adult flies shows a single mRNA species of 3.1 kb. In situ hybridisation with the 1.1 kb cDNA shows a single locus on chromosome 2R, 42B1-2 (white arrowheads).

rather than the phenotypic level facilitates fast and efficient screening, and can be carried out on heterozygotes.

Three gene-specific primers were used in conjunction with two P-element primers (see Materials and Methods). The latter were based on regions just internal to the left and right ends of the P-element. In a screen of 3000 mutagenised individuals, each estimated to contain approximately ten new P-element insertions, we detected six individuals with insertions in



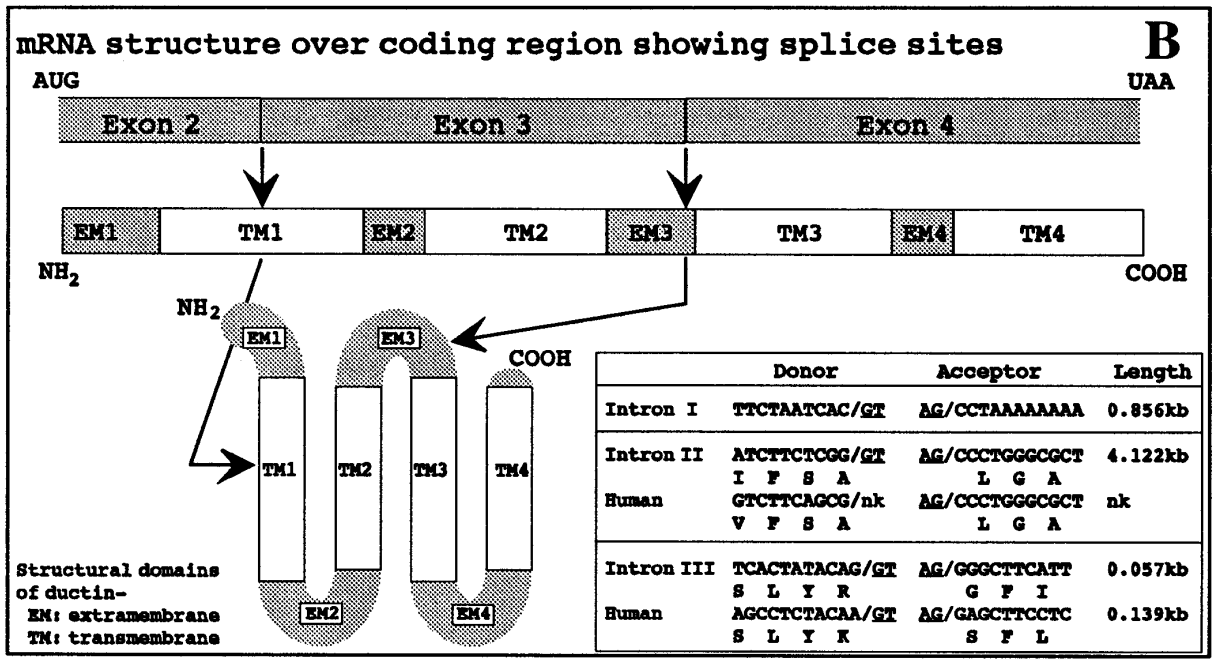


Fig. 7. Structure of the *Vha16* gene and its products. (A) Top, restriction map of the 7.2 kb contig derived from sequencing the genomic DNA clones 1A1 and 2B2. With the exception of the *Sau*3A site, all sites of cleavage by the indicated enzymes are shown. Arrows indicate the approximate positions and orientations of the gene specific primers (GSP) used to detect P-element insertions. The sequenced regions of clones 1A1 and 2B2 are shown as the thicker lines below the restriction map. Bottom, intron/exon structure; putative translation initiation and termination codons; TATA, CAAT and poly(A) addition motifs. The 5′ boundary indicated for exon 1 (coordinate 1085) represents the limit of sequence available from pDm16k. Sequences below the line indicate sites of P-element insertion. P-element insertion generates 8 bp target site duplications. The latter are shown boxed. Arrows indicate orientation of P-elements. (B) Correlation of mRNA structure, protein domain structure, and protein topology in the membrane bilayer. Exon/intron junctions are compared (inset) with known and predicted splice sites of a human gene (Gillespie et al., 1991; Hasebe et al., 1992). The introns lie at identical positions withing the respective open reading frames (nk, not known).

Vha16. This makes *Vha16* something of a 'hot-spot' for insertion (cf. *singed*; Roberston et al., 1988). The exact location of the P-element in each case was determined by direct sequencing of PCR products flanking left and right insertion boundaries (Fig. 7). Five insertions (2-6) occurred within an 800 bp region of intron 1. Insertions 5 and 6 are at exactly the same position, but in opposite orientations. Insertions 3 and 4 are separated by a single nucleotide. Insertion 1 is in the first, non-coding exon.

The second chromosome of each line was balanced. Balanced individuals were then crossed to generate flies homozygous for the P element. Homozygotes were in each case viable and fertile, and presented no obvious phenotypic defects.

DISCUSSION

Our previous studies have shown that ductin (previously called the 16k-18k protein) is a component of the connexons of isolated gap junctions prepared from different sources (crustacean and vertebrate) and appears to have an active role in gap junction-based cell-cell communication (see Finbow and Pitts, 1993, for a review). The results of this present study suggest that a closely related ductin polypeptide is likewise a gap junction component present in the larvae of the lepidopteran *M. sexta*. A recent functional study supports a role for ductin in gap junctional intercellular communication in insects. Antibodies to *Nephrops* ductin block dye coupling between follicular cells and the oocyte/nurse cell syncyctia in *Drosophila* ovarian follicles when injected into the latter cells (Bohrmann, 1993).

The N-terminal region of the ductin polypeptide from *Manduca* gap junctions is identical to the 16 kDa polypeptide encoded by a previously cloned *Manduca* cDNA (Dow et al., 1992). This latter polypeptide has high identity to the polypeptide encoded by the *Drosophila* cDNA used in this study. Expression of the *Drosophila* cDNA, either the 1.1 kb insert of pDm16k or a shortened form containing the coding region only, in *VMA3*-defective (*LEU2::vatc*) *S. cerevisiae* restores growth at pH 7.5. It therefore seems reasonable to conclude that the *Drosophila* cDNA encodes the 16 kDa proteolipid of the V-ATPase. The slow growth rate can be explained by lower amounts of V-ATPase activity. The ductin of *N. norvegicus* also subsititutes for the 16 kDa proteolipid of *S. cerevisiae* but again the chimeric V-ATPase is not as efficient as the wild-type form having a twofold increased K_m for ATP (Harrison et al., 1994).

The functional and biochemical analyses therefore suggest that the 16 kDa proteolipid subunit of the V-ATPase and ductin of gap junction are interchangeable and may be one and the same polypeptide at least in *Drosophila* and *Manduca*. The genetic analysis in this study and in an earlier study on *Manduca* (Dow et al., 1992) support this suggestion showing a single genetic locus that, at least in adult *Drosophila*, encodes a single transcriptional unit. In addition, the structure of the *Drosophila Vha16* gene suggests that there is unlikely to be differential splicing of its transcript. Because of the potential for confusion in nomenclature, we suggest that the name ductin be extended to the 16 kDa proteolipid of the V-ATPase as well its present use as a connexon component of gap junctions.

Multiple genetic loci have been found in rat (Nezu et al., 1992) and man (Hasebe et al., 1992). Whether these encode different forms of ductin polypeptides is not known. However, Northern analysis from a variety of mammalian tissues and cell lines shows a single mRNA species, indicating a single ductin polypeptide (Nezu et al., 1992; Prowse and Pitts, personal communication).

Ductin is a tandem repeat of an 8 kDa domain related to the 8 kDa subunit c polypeptide of F_1F_0 ATP synthase (Mandel et al., 1988). This suggests an origin via gene duplication. Intron 3 of the *Drosophila Vha16* gene is positioned such that it divides the open reading frame in two, between the second and third transmembrane segments (Fig. 7B). A recently described human *Vha16* gene (Hasebe et al., 1992), when compared with a corresponding human cDNA (Gillespie et al., 1991), appears to have an intron at an identical position. The human gene also has an intron at the same nucleotide position as intron 2 of *Drosophila Vha16*. The *Vha16* gene would thus appear to have an ancient history.

Although three TATA motifs have been identified in the *Drosophila Vha16* gene, it is unclear which, if any, corresponds to the transcriptional unit in adult. The cDNA insert of pDm16k is only 1.1 kb in length but northern blotting of adult mRNA indicates a 3.1 kb transcript. Since the pDm16k was isolated from a larval cDNA library, this may suggest developmental regulation of *Vha16* transcription.

Low-stringency Southern blot analysis, for both *Drosophila* and *Manduca* (Dow et al., 1992), reveals bands not present at high stringency (Fig. 5B). These could represent more distantly related members of a gene family. In *S. cerevisiae*, for example, there are at least three other genes related to *VMA3* (Apperson et al., 1990; Shih et al., 1990; Umemoto et al., 1991).

S. cerevisiae is providing a suitable organism for analysis of ductin as a V-ATPase component (Noumi et al., 1991; Harrison et al., 1994). *Drosophila* should likewise provide a comparable organism for analysis of ductin as a gap junction component. To this end, we have generated several *Drosophila* lines that have insertions of a transposable element within the *Vha16* gene. Although these insertions appear to be without phenotypic consequences, they provide a route for inactivation of the gene (Kaiser, 1990; Segalat et al., 1992).

This work was supported by grants from the Cancer Research Campaign (M.E.F. and L.M.), the Science and Engineering Research Council (J.B.C.F. and J.K.), the Medical Research Council (K.K. and S.F.G.) and the Agriculture and Food Research Council (N.J.L.). We thank Drs N. Nelson and Y. Anaraku for their kind gifts of strains of *S. cerevisae* containing inactivated *VMA3* genes. We thank Dr J. D. Pitts and Professor J. A. Wyke for their critical comments on the work and manuscript.

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(Received 11 January 1994 - Accepted 5 April 1994)