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The multigene family of the tobacco hornworm V-ATPase: novel subunits a, C, D, H, and putative isoforms[1]

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Abstract

The plasma membrane V-ATPase from *Manduca sexta* (Lepidoptera, Sphingidae) larval midgut is composed of at least 12 subunits, eight of which have already been identified molecularly [Wieczorek et al., J. Bioenerg. Biomembr. 31 (1999) 67–74]. Here we report primary sequences of subunits C, D, H and a, which previously had not been identified in insects. Expression of recombinant proteins, immunostaining and protein sequencing demonstrated that the corresponding proteins are subunits of the *Manduca* V-ATPase. Genomic Southern blot analysis indicated the existence of multiple genes encoding subunits G, a, c, d and e. Moreover, multiple transcripts were detected in Northern blots from midgut poly(A) RNA for subunits B, G, c and d. Thus, these polypeptides appear to exist as multiple isoforms that could be expressed either in different tissues or at distinct locations within a cell. By contrast subunits A, C, D, E, F and H appear to be encoded by single transcripts and therefore should be present in any *Manduca* V-ATPase, independent of its subcellular or cell specific origin.

Keywords: Vacuolar H^+-translocating ATPase; V-ATPase; Midgut; Multigene family; Isoform; *Manduca sexta*

1. Introduction

Vacuolar H^+ ATPases (V-ATPases) constitute a highly conserved and ubiquitous family of proton pumps whose primary function is the establishment of a proton motive force across membranes by utilization of the free energy from ATP hydrolysis. According to their localization in the endomembrane system, where they are found in all eukaryotic cells, or in plasma membranes of specialized cells, many biological functions are accomplished, ranging from the energization of epithelial transport processes to receptor mediated endocytosis (for review see [1]). V-ATPases are highly complex, heteromultimeric proteins consisting of a peripheral V_1 complex that catalyzes the hydrolysis of ATP and a membrane bound V_o complex that forms the proton conducting pore. During the last decade 12 subunits have been identified and it is still not clear whether the discovery of novel constituents has neared completion (for

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[1] The nucleotide sequences reported in this paper have been submitted to the GenBank/EMBL data bank with accession numbers AJ249388, AJ249389, AJ249390 and AJ251992.

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review see [2]). The isolated V_1 complex, which exhibits Ca^{2+} but not Mg^{2+} dependent ATPase activity [3], appears to consist of the eight different subunits A–H. The head subunits, A and B, are arranged in an A_3B_3 hexamer and presumably form the ATP binding pocket [4]. The remaining subunits form a stalk that connects the hexamer to the V_o portion. The V_o complex, which in higher eukaryotes is composed of subunits ac_6de, is capable of transporting protons only in association with the V_1 complex [5]. Since dissociation of the holoenzyme into V_1 and V_o complexes is an in vivo regulatory mechanism for the control of V-ATPase activity [6], both the disappearance of Mg^{2+} dependent ATPase activity of the detached V_1 complex and the loss of proton conductance of the remaining V_o complex are intriguing properties of these subcomplexes. Dissociation of the holoenzyme and the existence of an intact cytosolic V_1-ATPase were first reported for the V-ATPase from *Manduca sexta* [3,7]. This finding, together with the first demonstration of animal plasma membrane energization by the proton motive force [8] and the detection of novel subunits as constituents of V-ATPases [9–11], has made the insect enzyme a model ATPase. The *Manduca* V-ATPase is expressed in large amounts in the apical plasma membrane of larval midgut goblet cells, where it is the sole energizer of all secondary active transport processes across the midgut epithelium, such as the extrusion of potassium or the uptake of amino acids [12]. The high level of proton pumps present in goblet cell apical membranes and their dissociation into cytoplasmic V_1 and membrane bound V_o subcomplexes during starvation make the larval midgut an excellent source for purification of the V_1V_o holoenzyme as well as of its subcomplexes ([3,13], Huss et al., in preparation). The large amount and high purity of the isolated V_1 complex made possible the first structural analysis of the insect V_1-ATPase by X-ray scattering and electron microscopy [4,14]. To crystallize the V-ATPase and to determine its atomic structure, it is necessary to know not only the precise subunit composition but also the amino acid sequence of each single subunit. During the past decade we have isolated cDNAs encoding subunits A, B, E, F, G, c, d and e and identified the corresponding proteins in the purified V-ATPase [9–11,15–19]. Here we report the first cloning of cDNAs encoding subunits C, D, H and a from insects and demonstrate that they are constituent parts of the *Manduca* V-ATPase. Some subunits of V-ATPases from other organisms appear to have many isoforms whereas other subunits appear to have none [6]. Since the character of isoforms may affect the structure and localization of the *Manduca* V-ATPase, we performed the first systematic screening for potential V-ATPase isoforms by Southern and Northern blot analysis.

2. Materials and methods

2.1. Insects

Larvae of *M. sexta* (Lepidoptera, Sphingidae) were reared under long-day conditions (16 h of light) at 27°C using a modified synthetic diet [20].

2.2. Cloning of V-ATPase subunits

To determine the primary structure of the V_o subunit a, we performed RT-PCR on larval midgut mRNA, using a set of four degenerate primers corresponding to conserved regions of a cDNA clone encoding the *Drosophila* vha100 protein (provided by Dr. J.A.T. Dow, University of Glasgow). The forward primers were 5′-TTC(AC)GI(AT)(GC)IGA(AG)GA(AG)ATG-3′ and 5′-TTCGA(AG)(AC)GIATG(CT)TITGG-3′ and the reverse primers were 5′-GTGGTA(AG)ATIGC(CT)TTCAT-3′ and 5′-ICCIA(AG)GTCICC(AG)AACAT-3′. This approach led to the amplification of a specific 770 bp fragment that encodes part of subunit a. The remaining parts of the cDNA were obtained by anchored PCR using the 3′-/5′-RACE Kit from Roche Diagnostics, Germany. 5′-RACE was performed on a poly(A) tailed cDNA using an oligo(dT) anchor as forward primer and the reverse primer 5′-GAACTTGTTGTTGCGGTTGT-3′ which was specific for the partial subunit a cDNA sequence obtained by RT-PCR. The resulting 666 bp fragment was cloned and sequenced. It contained the 5′ sequence of subunit a and shared an overlapping region of 51 bp with the PCR fragment described above. 3′-RACE was performed with the oligonucleotide 5′-AGATCAAGGCGATCTACCAC-3′ and an anchor primer provided by the manufacturer. PCR yielded a product

of 2538 bp that included a match with 337 nucleotides of the first PCR fragment obtained by nested PCR.

To obtain sequence information for subunit H, we performed direct PCR [21] on a Lambda ZAP II cDNA library from the larval midgut of *M. sexta* [15] using a set of degenerate primers complementary to conserved regions of the corresponding yeast and bovine subunits [22,23]. The forward primer was 5′-CACAA(AG)(AT)(GC)IGA(AG)AA(AG)TTCTGG-3′, the reverse primer was 5′-(CT)TG(CT)TTICCIA(AG)GTA(CT)TCCCA-3′. This reaction amplified a specific cDNA fragment of 300 bp. Reaction cycles were repeated in the presence of digoxigenin-11-dUTP leading to a labeled DNA probe which was used for hybridization screening of the *M. sexta* Lambda ZAP II library. With this approach a complementary antisense cDNA for subunit H was cloned. It was 1.6 kb in length and polyadenylated at the 3′ end similar to the antisense transcript of subunit d, which we had identified previously [19]. To obtain the corresponding sense cDNA, the library was rescreened with a 500 bp RNA probe derived by in vitro transcription from the antisense clone. This procedure led to the isolation of two identical sense cDNA clones that contained the open reading frame for subunit H.

Subunit C was cloned by RT-PCR with degenerate primers by an approach that was similar to one previously described [19]. Four degenerate oligonucleotides against conserved regions of known sequences of subunit C were employed: 5′-ATG(AT)(GC)IGA(AG)TACTGGAT-3′, 5′-C(GT)IA(AG)IGC(CT)TTIACGTG-3′, 5′-AC(CT)(AC)GITT(CT)CA(AG)TGGGATATG-3′ and 5′-AAGTTIAC(CT)TTIA(AG)CCAGCG-3′. The resulting 550 bp PCR product was cloned into pGEM-T easy (Promega) and confirmed by nucleotide sequencing. To synthesize a digoxigenin labeled RNA probe, in vitro transcription was performed with SP6 polymerase in the presence of digoxigenin-11-UTP. Hybridization screening followed by in vivo excision resulted in a pBluescript SK(−) plasmid containing a cDNA of 1826 bp that encoded subunit C of the V-ATPase.

Protein sequencing of the putative V_1 subunit D resulted in the amino acid sequence KGRLAGAQKGHGLL which exhibited significant similarity to the corresponding yeast and bovine proteins [24]. Hence, this peptide sequence was used to design a degenerate forward primer 5′-GG(ACT)GCICA(AG)AA(AG)GG(ACT)CATGG-3′. Direct PCR using the *M. sexta* Lambda ZAP II cDNA library and a reverse primer corresponding to the T7 promoter of the phasmid resulted in the amplification of a specific 1.5 kb fragment which was cloned and sequenced. The deduced amino acid sequence confirmed that it encoded subunit D. To clone the complete cDNA, hybridization screening was performed with a DNA probe that had been generated by PCR in the presence of digoxigenin labeled 11-dUTP using the primers 5′-TGGGTGAAGTGATGAAGGAG-3′ and 5′-AAGGTCTTCGTCTCCTTCGT-3′, and the partial cDNA for subunit D as a template. In vivo excision led to the isolation of a cDNA clone which was homologous to corresponding cDNA sequences of subunit D from other organisms.

2.3. Expression of V-ATPase subunits

Cytoplasmic expression of the 20 kDa N-terminus of subunit H as a fusion product with the maltose binding protein was performed as reported previously [17], using the pMal-c2 system of New England Biolabs. To obtain the expression vector, a PCR fragment of 550 bp was amplified with the forward primer 5′-TACTCAGGATCCATGGCTAACATCGGTGATGA-3′, which contained a 5′-nonsense sequence and a *Bam*HI site (underlined), and the reverse primer 5′-TACTCAAAGCTT*TTA*GAGTTGATCCTTCAGCCAAGA-3′, which contained a 5′-nonsense sequence, a *Hin*dIII site (underlined) and a stop codon (italics). The PCR product was ligated into the *Bam*HI and *Hin*dIII sites of the predigested pMal-c2. Polyclonal antibodies against the recombinant fusion protein were raised in guinea pigs by Charles River, Germany. Subunit C and the cytosolic N-terminus of subunit a were expressed in *Escherichia coli* cells using the pET expression system of Novagen. The complete coding region of the cDNA for subunit C was amplified with the forward primer 5′-GAAACATATGTCGGAGTACTGG-3′ containing a *Nde*I site (underlined) and the reverse primer 5′-TGGGGGATATCTAAGCCGCC-3′ containing an *Eco*RV site (underlined). The cleaved product was ligated into the *Nde*I and the blunted *Bam*HI sites of pET-16b. The recombinant

plasmid was transformed into *E. coli* BL21 cells and expression was induced with 0.4 mM IPTG. Purification of the recombinant protein, which contained an N-terminal His tag, was performed via Ni^{2+} columns (Novagen) according to the manufacturer's protocol, except that the elution step was done with a linear gradient from 0 M imidazole, 0 M NaCl, 20 mM Tris–HCl (pH 7.9) to 1 M imidazole, 0.5 M NaCl, 20 mM Tris–HCl (pH 7.9). Recombinant subunit C eluted at approximately 0.3 M imidazole/0.1 M NaCl and was essentially free of contaminating proteins.

The N-terminal region of subunit a was expressed using a similar approach. For the construction of the expression vector, the corresponding cDNA was amplified with the primer pair 5′-TACTCACATATGGGGTCTTTGTTCAGGAGT-3′ and 5′-TACTCACCCGGG*TTA*CTCGCGATAGGTGGCGACAC-3′, each of which contained a 5′-nonsense sequence, as well as a *Nde*I and *Sma*I site (underlined). The latter primer additionally carried a stop codon (italics). The PCR product was cloned into the *Nde*I and the blunted *Bam*HI site of pET-16b. Expression of the recombinant N-terminus of subunit a was performed as described above.

2.4. Other methods

Poly(A) RNA from the midgut of fifth instar larvae was prepared as described previously [19]. First strand cDNA for RT-PCR was synthesized from poly(A) RNA of larval midgut using the the First Strand cDNA synthesis kit for RT-PCR (AMV) from Roche Diagnostics, Germany. For optimal design of degenerate primers the codon usage of *M. sexta* was taken into consideration [25]. RNA probes were synthesized according to the manufacturer's protocol by in vitro transcription using the DIG-RNA labeling kit from Roche Diagnostics, Germany. Before transcription the template plasmids were linearized with appropriate restriction enzymes. DNA probes were generated by PCR in the presence of digoxigenin-11-dUTP [26]. PCR, hybridization screening, in vivo excision, Southern and Northern blotting were performed as described previously [19]. Purification of the V_1-ATPase from midgut cytosolic extracts and V_1V_o-ATPase from goblet cell apical membranes was performed according to published procedures [3,13]. Protein extraction from the gel, cyanobromide cleavage and N-terminal sequencing was done according to Schmidt et al. [27]. Protein determination by amido black, SDS–PAGE, Western blotting and immunostaining were performed as described previously [3,8,28].

3. Results and discussion

3.1. Deduced primary structures of the V-ATPase subunits C, D, H and a

cDNA clones encoding the V-ATPase subunits C, D, H, and a of *M. sexta* were isolated using several different approaches.

V_o subunit a was obtained by RT-PCR using degenerate primers and rapid amplification of cDNA ends. The cDNA was 3586 bp in length and contained an open reading frame of 841 amino acids (nucleotide positions 148–2673) encoding a polypeptide with a deduced molecular mass of 95 868 Da and a calculated isoelectric point at pH 6.39. The deduced amino acid sequence was 65.7% identical and

→

Fig. 1. Alignment of predicted *M. sexta* (ms) amino acid sequences with homologous yeast (sc) and bovine (bt) proteins. Using the computer program Clustal, the amino acid sequences of the *Manduca* V-ATPase subunits a (GenBank accession number AJ249390), C (GenBank accession number AJ249388), D (GenBank accession number AJ251992), and H (GenBank accession number AJ249389) were aligned with corresponding yeast and bovine proteins [22–24,29,30,32,33]. Amino acids that are identical in all three sequences are marked with small asterisks; those that are similar are indicated with dots. Hyphens represent gaps introduced for optimal alignment. Potential *N*-glycosylation sites of *Manduca* subunit a are marked with bold asterisks. The predicted leucine zipper of *Manduca* subunit D is underlined. The amino acids obtained from N-terminal sequencing of a 30 kDa protein strictly copurifying with the *Manduca* V_1- and V_1V_o-ATPase are written in italics and are framed together with the corresponding yeast and bovine sequences. Identical amino acids of *Manduca* subunit a sequences obtained from midgut (ms) and from Malpighian tubule (mt) cDNA are highlighted with gray shading.

SUBUNIT a

```
bt  ----MGELFRSEEMTLAQLFLQSEAAYCCVSELGELGKVQFRDLNPDVNVFQRKFVNEVR 56
ms  ----MGSLFRSEEMTLCQLFLQSEAAYACVSELGELGLVQFRDLNPDVNAFQRKFVNEVR 56
sc  MAEKEEAIFRSAEMALVQFYIPQEISRDSAYTLGQLGLVQFRDLNSKVRAFQRTFVNEIR 60
          :*** **:* *::: .* :  ..  **:** *******..*..***.****:*

bt  RCEEMDRKLRFVEKEIRKANIPIMDTG---------ENPEVPFPRDMIDLEANFEKIENE 107
ms  RCDEMERKLRYLEKEIRRDGIPMLEIPG--------ECPEAPQPREMIDLEATFEKLENE 108
sc  RLDNVERQYRYFYSLLKKHDIKLYEGDTDKYLDGSGELYVPPSGSVIDDYVRNASYLEER 120
    * ::::*: *:. . ::: .* : :          *    *    : *   . . :*:.

bt  LKEINTNQEALKRNFLELTELKFILRKTQQFFD--EMADPDLLEESSSLLEPSEMGRG-T 164
ms  LREVNQNAEALKRNYLELTELKHILRKTQVFFD--EMADPSREEEQVTLLGEEGLMAGGQ 166
sc  LIQMEDATDQIEVQKNDLEQYRFILQSGDEFFLKGDNTDSTSYMDEDMIDANGENIAAAI 180
    * :::   : :: :  :* : :.**:. : **   : :*.    :.  :          .

bt  PLRLGFVAGVINRERIPTFERMLWRVCRGNVFLRQAEIENPLEDPVTGDYVHKSVFIIFF 224
ms  ALKLGFVAGVILRERIPAFERMLWRACRGNVFLRQAEIDTPLEDPSSSDQVYKSVFIIFF 226
sc  GASVNYVTGVIARDKVATLEQILWRVLRGNLFFKTVEIEQPVYDVKTREYKHKNAFIVFS 240
       :.:*:*** *:::.::*::***. ***:*:: .**: *: *  : :  :*..**:*

bt  QGDQLKNRVKKICEGFRASLYPCPETPQERKEMASGVNTRIDDLQMVLNQTEDHRQRVLQ 284
ms  QGDQLKTRVKKICEGFRATLYPCPESPADRREMAMGVMTRIEDLNTVLGQTQDHRHRVLV 286
sc  HGDLIIKRIRKIAESLDANLYDVDSSNEGRSQQLAKVNKNLSDLYTVLKTTSTTLESELY 300
    :** : .*::**.*.: *.**   .:   * :   * ..:.**  **  *.   .  *

bt  AAAKNIRVWFIKVRKMKAIYHTLNLCNIDVTQKCLIAEVWCPVTDLDSIQFALRRGTEHS 344
ms  AAAKNIKNWFVKVRKIKAIYHTLNLFNLDVTQKCLIAECWVPALDLETIQLALRRGTERS 346
sc  AIAKELDSWFQDVTREKAIFEILNKSNYDTNRKILIAEGWIPRDELATLQARLGEMIARL 360
    * **::  ** .* : ***:. **  * *..:* **** * *  :* ::*  * .   :

bt  GSTVPSILNRMQTNQTPPTYNKTNKFTYGFQNIVDAYGIGTYREINPAPYTIITFPFLFA 404
ms  GSSVPPILNRMETLEDPPTYNRNNKFTQAFQNLIYAYGVATYREVNPAPYTIITFPFLFA 406
sc  GIDVPSIIQVLDTNHTPPTFHRTNKFTAGFQSICDCYGIAQYREINAGLPTIVTFPFMFA 420
    *  **.*:: ::* . ***:::.**** .**.:  .**:. ***:*..  **:****:**

bt  VMFGDLGHGILMTLFAVWMVLKESRILSQKNENEMFSTIFSGRYIILLMGVFSIYTGLIY 464
ms  VMFGDLGHGALMAAFGFWMCYKEKPLQAKKIDSEIWNIFFGGRYIILLMGLFSMYTGLIY 466
sc  IMFGDMGHGFLMTLAALSLVLNEKKINKMK-RGEIFDMAFTGRYIILLMGVFSMYTGFLY 479
    :****:*** **:  .. :  :*. :   *  .*::.  * *********:**:***::*
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                                        *
bt  NDCFSKSLNIFGSSWSVRPMFDIYNWTEETLRGNPVLQLNPAVTGVFGGPYPFGIDPIWN 524
ms  NDIFSKSLNIFGSSWRQN-------YNASTLTENKLLQLNPDSPDYLQYPYPFGIDPVWQ 519
sc  NDIFSKTMTIFKSGWKWP----------------DHWKKGESITATSVGTYPIGLDWAWH 523
    ** ***:.** *.*                    : .   .     .**:*:*  *:

bt  IATN-KLTFLNSFKMKMSVILGIIHMLFGVSLSLFNHTYFKKPLNIYFGFIPEIIFMTSL 583
ms  LAEANKIIFMNAYKMKISIIIGVFHMLFGVCLSLWNHLVFKRRISVYVEFIPQLLPLTLL 579
mt                                                  IVLEFIPQIVPLCLL
sc  GTEN-ALLFSNSYKMKLSILMGFIHMTYSYFFSLANHLYFNSMIDIIGNFIPGLLFMQGI 582
     :   : * *::***:*:::*.:** :.  :** ** **:  :.:   *** ::*:  :

bt  FGYLVILIFYKWTAYN--------------AKTSEKAPSLLIHFINMFLFSYGD----SG 625
ms  FFYMVLLMFIKWTSYGPTPGAFGSQDPAIVKTSAYCAPSILITFINMMLFKTDANTRPQC 639
mt  FLDMVFMMFFKWVAVSTK-----SED---LAYSQGCAFSVLILFINMMLFSKNE-PEAGC
sc  FGYLSVCIVYKWAVDWVK--------------DGKPAPGLLNMLINMFLSPG------TI 622
    * *: : :. **:                    .  **.:*  :***:*

bt  NSMLYSGQKGIQCFLVVVALLCVPWMLLFKPLVLRRQYLRR----------KHLGTLNF 674
ms  DDTMYAGQLQLQKFFVIVALLCVPVMLFGKFYFIMKEQKQR----------AROGHQPV 688
mt  KEFMFDSQGQIQRVFVLVALCCIPVMLIGKPLYLLVTVSKKKDHAKPEQSNGSVNQGIEMQ
sc  DDELYPHQAKVQVFLLLMALVCIPWLLLVKPLHFKFTHKKK----------SHEPLPST 671
    :. :*. *  :* *::::**:*:* :*: ** :     ::          :
          *
bt  GGIRVGNGPTEEDAEIIQHDQLSTHSEDAEEPTEDEVFDFGDTMVHQAIHTIEYCLGCIS 734
ms  EGAAENG-------------TAG---GAPVPSSGHHDDDITEVFIHQGIKTIEYVLGSVS 732
mt  EQTEVDG-------------QSK---PEAKASHDHEDEPFSEVMIHQAIHTIEYVLSTIS
sc  EADASSE---DLEAQQLISAMDADDAEEEEVGSGSHGEDFGDIMIHQVIHTIEFCLNCVS 728
     .   .                .         :  .  *: : ::** *****: *.:*

bt  NTASYLRLWALSLAHAQLSEVLWTMVIHIGLKVKSLAGGLALFFIFAAFATLTVAILLIM 794
ms  HTASYLRLWALSLAHAQLAEVAWNMLLRKGLMSTDFQGGIFLYIVFAGWAAISVSILVLM 792
mt  HTASYLRLWALSLAHAELSEVLWNMVLTFGLKDHDYIGAIKLYVAFCFWALFTLAILVMM
sc  HTASYLRLWALSLAHAQLSSVLWTMTIQIAFGFRGFVGVFMTVALFAMWFALTCAVLVLM 788
    :*****************:.* *.* :: .:   .: * :    :** : ::: ::*::*

bt  EGLSAFLHALRLHWVEFQNKFYSGTGFKFLPFSFEHIREGKFDD-------- 838
ms  EGLSAFLHTLRLHWVEFQSKFYAGEGLSLPTGSRFEVILDSAGPGRGVN--- 841
mt  EGLSAFLHTLRLHWVEFMSKFYVGLGYIFQPFC-FKTILEQEENKDD
sc  EGTSAMLHSLRLHWVESMSKFFVGEGLPYEPFAFEYKDMEVAVASASSSASS 840
    ** **:**:******  .**: * *:   . :
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SUBUNIT C

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bt  -------MTEFWLISAPG---EKTCQQTWEKLHAATSKNNNLAVTSKFNIPDLKVGTLDV 50
ms  -------MSEYWLISAPG---DKTCQQTWEALNQATKANN-LSLNYKFPIPDLKVGTLDQ 49
sc  MATALYTANDFILISLPQNAQPVTAPGSKTDSWFNETLIGGRAFVSDFKIPEFKIGSLDT 60
          .:: *** *      *.  :         .  .  :.  .* **::*:*:**

bt  LVGLSDELAKLDAFVEGVVKKVAQYMADVLEDSKDKVQENLLANGVDLVTYITRFQWDMA 110
ms  LVGLSDDLGKLDTFVEGVTRKVAQYLGEVLEDQRDKLHENLTANNDDLPHYLTRFQWDMA 109
sc  LIVESEELSKVDNQIGASIGKIIEILQGLNETSTN-AYRTLPINNMPVPEYLENFQWQTR 119
    *:  *::*.*:*  : .   *: : :  : * . :   ..*  *.  :  *: .***:

bt  KYPIKQSLKNISEIIAKGVTQIDNDLKSRASAYNNLKGNLQNLERKNAGSLLTRSLAEIV 170
ms  KYPIKQSLRNIADIISKQVGQIDADLKVKSSAYNALKGNLQNLEKKQTGSLLTRNLADLV 169
sc  KFKLDKSIKDLITLISNESSQLDADVRATYANYNSAKTNLAAAERKKTGDLSVRSLHDIV 179
    *: :.:*::::  :*::   *:* *::   : **  * **   *:*::.*.* .*.* ::*

bt  KKDDFVLDSEYLVTLLVVVPKLNHNDWIKQYETLAEMVVPRSSNVLSEDQDSYLCNVTLF 230
ms  KKEHFILDSEYLTTLLVIVPKSMFNDWNANYEKITDMIVPRSTQLIHQDGDYGLFTVTLF 229
sc  KPEDFVLNSEHLTTVLVAVPKSLKSDFEKSYETLSKNVVPASASVIAEDAEYVLFNVHLF 239
    * :.*:*:**:*.*:** ***   .*:  .**.::. :** *:.:: :* :  * .* **
```

```
bt  RKAVDDFRHKARENKFIVRDFQYNEEEMKADKEEMNRLSTDKKKQFGPLVRWLKVNFSEA 290
ms  KKVVDEFKLHARERKFVVREFAYNEADLVAGKNEITKLLTDKKKQFGPLVRWLKVNFSEC 289
sc  KKNVQEFTTAAREKKFIPREFNYSEELIDQLKKEHDSAASLEQSLRVQLVRLAKTAYVDV 299
    :* *::*   ***.**: *:* *.*  :   *:*     : ::.    ***  *. : :

bt  FIAWIHVKALRVFVESVLRYGLPVNFQAMLLQPNKKTLKKLREVLHELYKHLDSSAAAII 350
ms  FCAWIHVKALRVFVESVLRYGLPVNFQAALLVPSRRSARRLRDTLHALYAHLDHSAHHHA 349
sc  FINWFHIKALRVYVESVLRYGLPPHFNIKIIAVPPKNLSKCKSELIDAFGFLGGNAFMKD 359
    *  *:*:*****:********** :*:  ::    :.  : :. *   : .*. .*

bt  DAP-------MDIPGLNLSQQEYYPYVYYKIDCNLLEFK- 382
ms  NAQQD----SVELAGLGFGQSEYYPYVFYKINIDMIEKAA 385
sc  KKGKINKQDTSLHQYASLVDTEYEPFVMYIINL------- 392
    .              .: : ** *:* * *:
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SUBUNIT D

```
bt  MSGKDRIEIFPSRMAQTIMKARLKGAQTGRNLLKKKSDALTLRFRQILKKIIETKMLMGE 60
ms  MSGKDRLAIFPSRGAQMLMKRGRLAGAQKGHGLLKKKADALQVRFRLILSKIIETKTLMGE 60
sc  MSG-NREQVFPTRMTLGLMKTKLKGANQGYSLLKRKSEALTKRFRDITKRIDDAKQKMGR 59
    ***..*  :**:* :  :** :* **: * .***:*::**  *** * .:* ::*  **.

bt  VMREAAFSLAEAKFTAGDFS-TTVIQNVNKAQVKIRAKKDNVAGVTLPVFEHYHE-GTDS 118
ms  VMKEAAFSLAEAKFTTGDFN-QVVLQNVTKAQIKIRSKKDNVAGVTLPIFESYQD-GSDT 118
sc  VMQTAAFSLAEVSYATGENIGYQVQESVSTARFKVRARQENVSGVYLSQFESYIDPEIND 119
    **: *******.:::*:     * :.*..*:.*:*::::**:** *. ** * :   :

bt  YELTGLARGGEQLAKLKRNYAKAVELLVELASLQTSFVTLDEAIKITNRRVNRIEHVIIP 178
ms  YELAGLARGGQQLAKLKKNFQSAVKLLVELASLQTSFVTLDEVIKITNRRVNAIEHVIIP 178
sc  FRLTGLGRGGQQVQRAKEIYSRAVETLVELASLQTAFIILDEVIKVTNRRVNAIEHVIIP 179
    :.*:**.***:*: : *. :  **: ********:*: ***.**:****** *******
```

```
bt  RIERTLAYIITELDERE-REEFYRLKKIQEKKKILKEKSDKDLEQRR---------AAGEV 229
ms  RLERTLAYIISELDELEREEFYRLKKIQDKKKIIKDKAEAKKAALR---------AAGQD 229
sc  RTENTIAYINSELDELDREEFYRLKKVQEKKQNETAKLDAEMKLKRDRAEQDASEVAADE 239
    * *.*:*** :**** :********:*:**:  . * : .    *         .*.:

bt  IEPANLLAEEKDEDLLFE 247
ms  LRDSANLLDEGDEDLLF- 246
sc  EPQGETLVADQEDDVIF- 256
       .  *  : ::*::*
```

SUBUNIT H

```
bt  -----------MTKMDIRGAVDAAVPTNIIAAKAAEVRANKVNWQSYLQGQMISSEDCEF 49
ms  MANIGDEKVSQLIPTLGDDKIDMIAATSVLQIRASEIRQTQINWQSYLQGQMITQRDHDF 60
sc  -MGATKILMDSTHFNEIRSIIRSRSVAWDALARSEELSEIDASTAKALESILVKKNIGDG 59
                 . :     :     :: *:  . .  . *:. ::... :

bt  IQRFEMKRS------PEEKQEMLQTEGSQRAKTPINLMTHISKEQTVQYILTLVDDTLQE 103
ms  IVNLDQRG--------Q--KDLPDKNPDACADVFLNLLTHISKDHTIQYILVLIDDILSE 110
sc  LSSSNNAHSGFKVNGKTLIPLIHLLSTSDNEDCKKSVQNLIAELLSSDKYGDDTVKFFQE 119
    :   :          :  . .  .  .: . *:: :  . :.*

bt  NHQRVSIFFDYAKRSKNTAWSYFLPMLNRQDLFTVH-----MTARIIAKLAAWGKELMEG 158
ms  DKSRVKIFRETKYSG--NIWQPFLNLLNRQDEFVQH-----MTARIIAKLACWHPQLMDK 163
sc  DPKQLEQLFDVSLKG--DFQTVLISGFNVVSLLVQNGLHNVKLVEKLLKNNNLINILQNI 177
    : .::. : :    .       ::  :*  . :. :       .. : *     * :

bt  SDLNYYFNWIKTQLSSQSSQYVQCVAGCLQLMLRVNEYRFAWVEADG--VNCIMGVLSNK 216
ms  SDLHFYLSWLKDQLKMNNNDYIQSVARCLQMMLRVDEYRFAFLSVDG--ISTLLSILASR 221
sc  EQMDTCYVCIRLLQELAVIPEYRDVIWLHEKKFMPTLFKILQRATDSQLATRIVATNSNH 237
    .::.     ::   .       : *    :  :    :::     .*.   . ::.  :.:

bt  CGFQLQYQMIFSVWLLAFSPQMCEHLRR--YNIIPVLSDILQESVKEKVTRIILAAFRNF 274
ms  VNFQVQYQLVFCLWVLTFNPLLAEKMNK--FNAIPILADILSDSVKEKVTRIVLAVFRNL 279
sc  LGIQLQYHSLLLIWLLTFNPVFANELVQKYLSDFLDLLKLVKITIKEKVSRLCISIILQC 297
    .:*:**: :: :*:*:*.* :.:.: :   . :  * .::. ::****:*: :: : :
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bt  LEKSVERETRQEYALAMIQCKVLKQLENLEQQKYDDEDISEDIKFLLEKLGESVQDLSSF 334
ms  IEKPQDQQVAKEHCIAMVQCKVLKQLSILEQKRSDDEDIMNDVEFLNERLQASVQDLSSF 339
sc  CSTRVKQHKKVIKQLLLLGN-ALPTVQSLSERKYSDEELRQDISNLKEILENEYQELTSF 356
     ..  .:.      : ::   .*  :. *.::: .**:: :*:. * * *  . *:*:**

bt  DEYSSELKSGRLEWSPVHKSEKFWRENPARLNEKNYELLKILTKLLEVSD---------D 385
ms  DQYATEVKSGRLEWSPVHKSAKFWRENAARLNERGQELLRTLVHLLEKSH---------D 390
sc  DEYVAELDSKLLCWSPPHVDNGFWSDNIDEFKKDNYKIFRQLIELLQAKVRNGDVNAKQE 416
    *:* :*:.* * *** * .  ** :*  .::: . :::: * .**: .           :

bt  PQVLAVAAHDVGEYVRHYPRGKRVIEQLGGKQLVMNHMHHEDQQVRYNALLAVQKLMVHN 445
ms  PVVLAVACYDVGEYVRHYPRGKHIIEQLGGKQRVMYLLSHDDPNVRYEALLAVQKLMVHN 450
sc  KIIIQVALNDITHVVELLPESIDVLDKTGGKADIMELLNHSDSRVKYEALKATQAIIGYT 476
     :: **  *: . *.  *..  :::: ***  :*  : *.* .*:*:** *.* :: :.

bt  WEYLGKQLQSEQPQTAAARS----- 465
ms  WEYLGKQLEKEQIDKQAGTVVGAKA 475
sc  FK---------------------- 478
    ::
```

73.9% similar to the bovine a1 isoform [29]; it was also 40.3% identical and 50.6% similar to the yeast vph1 protein [30] (Fig. 1). Prosite search revealed two putative *N*-glycosylation sites at amino acid positions 486 and 694. The first one is at a position similar to that in the bovine a1 isoform. This site is presumably localized in an extracellular loop between transmembrane helices 3 and 4 that has been predicted for the yeast vph1p by cysteine scanning mutagenesis [31]. Since lectin staining had also revealed that the putative *Manduca* homolog may be a glycoprotein [11], this site could serve for glycosylation. By contrast, the second site is located in a region between transmembrane helices 6 and 7, which is presumed to be on the cytoplasmic face, and therefore it is unlikely to be glycosylated.

Subunit C was obtained by screening the *Manduca* cDNA library with a corresponding probe derived from nested PCR with degenerate primers. A plasmid clone was obtained which contained 1826 bp of a cDNA in an open reading frame encoding 385 amino acids (nucleotide positions 66–1223). Calculation of the protein's molecular mass and its isoelectric point revealed values of 44 124 Da and pH 8.31, respectively. The deduced *Manduca* protein was 65.1% identical and 75.6% similar to the bovine subunit C [32]; it was also 37.3% identical and 50% similar to the yeast subunit C ([33], Fig. 1).

Subunit D was cloned by reverse genetics yielding a cDNA that was 1648 bp in length with an open reading frame of 246 amino acids at nucleotide positions 50–791. The deduced protein exhibited a molecular mass of 27 504 Da and an isoelectric point at pH 10.04. Its deduced amino acid sequence was 52.7 and 75.1% identical, as well as 64.1% and 82.9% similar to the yeast and bovine subunits [24], respectively (Fig. 1). Prosite search indicated a potential leucine zipper at amino acid positions 223–244 which is not present in any of the corresponding polypeptides described so far, but may mediate specific protein interaction in insects.

Subunit H was obtained by hybridization screening using the *Manduca* cDNA library. The isolated cDNA contained 1922 bp with an open reading frame at nucleotide positions 33–1460. The deduced protein consisted of 475 amino acids with a molecular mass of 55 022 Da and an isoelectric point at pH 6.30. The amino acid identity of the *Manduca* protein to the yeast and bovine proteins [22,23] was 27.3% and 40.9% whereas the similarity was 55.9% and 68.1%, respectively (Fig. 1).

3.2. Identification of individual subunits in preparations of the V_1V_o complex

Cloning of a cDNA provides evidence that the corresponding mRNA is expressed in the tissue from which the cDNA library was generated. How-

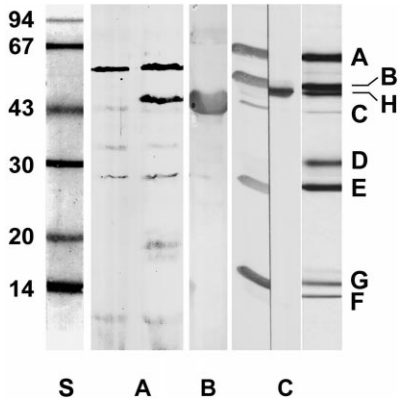

Fig. 2. Immunological identification of *Manduca* V-ATPase subunits. Lane S: standard proteins and their molecular masses indicated in kDa. Lane A: The first 390 amino acids of the cDNA encoding *Manduca* subunit a were expressed in *E. coli* BL21 cells. Subsequently, 10 μl of the crude bacterial extract was subjected to SDS–PAGE and blotted onto nitrocellulose. In contrast to material from cells not induced with IPTG (left lane), immunoblotting with polyclonal antibodies staining the bovine subunit a [36] labeled an additional protein band of approximately 45 kDa (right lane). Unspecific labeling observed for some bacterial proteins was due to the polyclonal character of the bovine antiserum. Lane B: The putative subunit C of the *Manduca* V-ATPase was expressed as a His-tagged version in *E. coli* BL21 cells, purified by affinity chromatography on a Ni^{2+} column, separated by SDS–PAGE and transferred to nitrocellulose. The purified 40 kDa protein was immunoreactive to the anti-V_1 complex antiserum directed to the purified *Manduca* V_1 complex. Lane C: V_1-ATPase was prepared as reported previously [3], separated by SDS–PAGE and blotted on nitrocellulose. One lane was cut in half and subjected to immunostaining. The first lane was stained with the antiserum to the V_1 complex that shows only weak reactivity with subunit H (left lane). The second lane was stained with monospecific antibodies raised against the 20 kDa N-terminus of subunit H. Only a single protein of 55 kDa migrating closely below subunit B was immunodetected in the same preparation by this procedure (middle lane). V_1-ATPase was prepared as reported previously [3], separated by SDS–PAGE and stained with Coomassie (right lane).

ever, no information is provided as to whether or not the mRNA is translated. To determine whether or not the isolated cDNA sequences encode polypeptides that are V-ATPase constituents, recombinant expression was carried out on all of the cloned subunits, except for subunit D, which was identified by reverse genetics.

Subunit a is a major part of the V_o complex and is presumed to have nine transmembrane helices [31]. The N-terminal part of the protein, which covers approximately 400 amino acids, is believed to be exposed to the cytoplasm where it may be involved in stabilizing the V_1V_o complex, probably by the formation of a peripheral stator as deduced from electron microscopic data [34,35]. To determine whether or not the cloned cDNA encodes the 100 kDa protein that is observed in SDS–PAGE of the V_o complex and the V_1V_o-ATPase (Huss et al., in preparation), the N-terminal part from amino acid positions 1–390 was expressed in *E. coli* BL21 cells. As shown in Fig. 2A, SDS–PAGE and immunoblotting revealed immunoreactivity of the recombinant protein to polyclonal antibodies against bovine subunit a [36] which also stain the 100 kDa protein present in the *Manduca* V-ATPase (Huss et al., in preparation). Thus, we conclude that the cDNA encodes the *Manduca* subunit a.

Subunit C is believed to be a peripheral stalk component which may not be essential for enzyme activity in a reconstituted system [37], but is important for V_1 complex assembly [33,38]. In yeast the cytoplasmic V_1 complex appears to lack subunit C, whereas the holoenzyme contains this polypeptide [39]. The native V_1 complex of *M. sexta* also possesses only substoichiometric amounts of subunit C, indicating rather weak interactions of C with other stalk subunits, suggesting that it has a peripheral localization in the V_1 complex [3,7,12]. Expression and purification of the recombinant putative subunit C led to a protein that cross-reacted in immunoblots with polyclonal antibodies to the V_1 complex (Fig. 2), indicating that this cDNA encodes V-ATPase subunit C.

Subunit H seems to have characteristics similar to those reported for subunit C. The polypeptide is essential for activity of the yeast V-ATPase and is believed to have a peripheral localization in the V_1 stalk, since its absence does not lead to an inhibition of V-ATPase assembly, but rather to a destabilization of the holoenzyme [22]. To confirm that the isolated cDNA for subunit H corresponds to a

Table 1
Characteristics of subunits in the multigene family of the tobacco hornworm V-ATPase

V-ATPase subunit (deduced/observed mol. mass in kDa)	Yeast homolog	GenBank accession number	Transcript size (kb)	Gene copy number	Reference
A (68.1/66)	vma1	X64233	3.2	1	[15], this work
B (54.9/56)	vma2	X64354	2.4/2.8	1	[16], this work
C (44.1/43)	vma5	AJ249388	2.0	1	this work
D (27.5/30)	vma8	AJ251992	1.9	1	this work
E (26.1/27)	vma4	X67131	1.8	1	[17], this work
F (13.8/14)	vma7	X67130	0.9	1	[9], this work
G (13.7/16)	vma10	X92805	0.8/0.9	2 or more	[10], this work
H (55.0/55)	vma13	AJ249389	2.0	1	this work
a (95.9/100)	vph1/stv1	AJ249390	3.6	2 or more	this work
c (16.0/17)	vma3/vma11/ vma16	X65051	1.4/1.8	2 or more	[18]
d (39.6/40)	vma6	X98825	2.2/1.8[a]/1.5	1	[19]
e (9.7/20)	vma21	AJ006029	0.9	2 or more	[11], this work

[a]This band was obtained only if a full length ssRNA probe was used for hybridization.

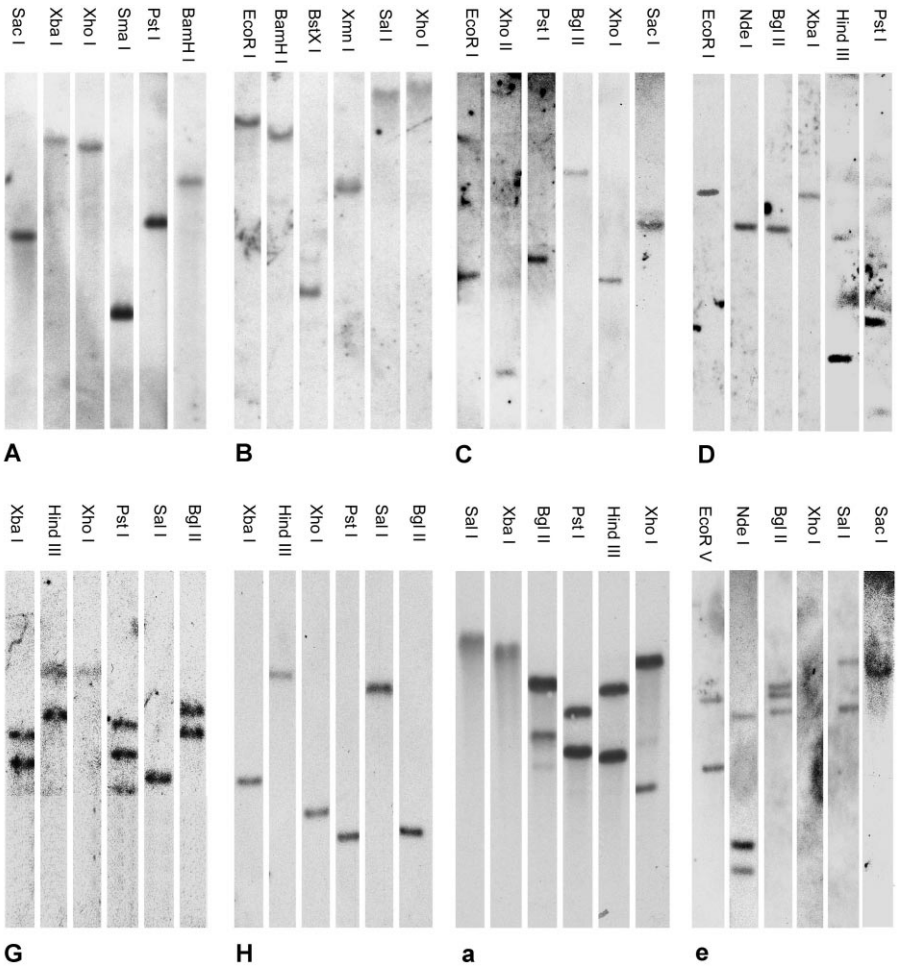

Fig. 3. Genomic Southern blot analysis for different V-ATPase genes. For each lane 5 μg of genomic DNA was digested with 50 units of the indicated restriction enzyme. The purified DNA fragments were separated on an 1% agarose gel, blotted onto a nylon membrane and hybridized with a digoxigenin labeled DNA probe directed to the indicated subunit. For the genes encoding subunits A, B, C, D and H only single bands were labeled with the corresponding hybridization probe, whereas multiple bands were detected for the genes encoding subunits G, a and e.

55 kDa protein that is part of the V-ATPase and migrates in SDS gels just beneath subunit B (Fig. 2C, right lane), the 20 kDa N-terminus was expressed as a fusion protein with maltose binding protein. After purification of the recombinant protein by affinity chromatography on an amylose resin, monospecific antibodies were generated in guinea pig. As shown in the Western blot of Fig. 2C (middle lane), the antibodies specifically stained the 55 kDa protein of the highly purified V_1 complex, clearly demonstrating the correct assignment of the cloned cDNA.

Subunit D appears to be essential for the function and assembly of the V_1 complex and has been proposed to be a structural homolog of the γ subunit of F-ATPases [24] which functions as a transducer between ATP hydrolysis/synthesis in the F_1 head and proton movement in the membrane F_o complex [40]. A 30 kDa protein strictly copurifying with the highly purified V_1-ATPase or V_1V_o holoenzyme was isolated by SDS–PAGE, treated with cyanobromide and subjected to N-terminal sequencing. Fourteen amino acids of one of the resulting fragments were identified. Since the obtained peptide sequence matched the known amino acid sequences encoding subunit D of the yeast and bovine V-ATPases (Fig. 1), the 30 kDa *M. sexta* protein is clearly subunit D. As described above, this amino acid sequence was used to design a degenerate primer that allowed us

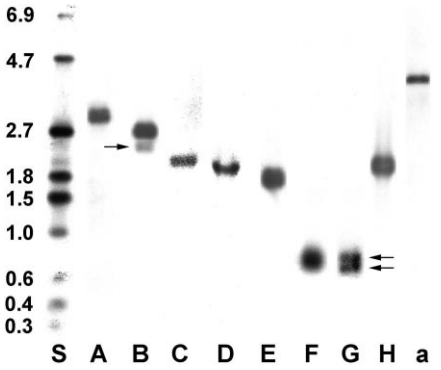

Fig. 4. Northern blot analysis for different V-ATPase transcripts present in poly(A) RNA isolated from *Manduca* fifth instar larval midgut. For each lane 1 μg of this RNA preparation was denatured and separated on an 1% agarose gel (2% for subunit G) containing 2% formaldehyde. After transfer to a nylon membrane the RNA was hybridized with a digoxigenin labeled RNA probe obtained by in vitro transcription of the antisense strand of the indicated V-ATPase subunit. Labeled fragments were aligned using 0.5 μg of digoxigenin labeled standard RNA fragments with sizes indicated as numbers of kbp (S). Multiple transcripts were detected only for subunit B and G (arrows).

to amplify the corresponding nucleotide sequence for the isolation of a cDNA clone.

3.3. *Southern and Northern blots suggest that isoforms exist*

In higher eukaryotic organisms V-ATPases may be involved in multiple functions not only in different tissues but also within a single cell. Thus, the question arises whether the nature of subunits has an impact on the location and/or function of the enzyme. Subunit composition could be influenced by the expression of different isoforms of one or more subunits which may derive from different genes, alternatively spliced transcripts, or from posttranslational processing. To investigate possible isoforms in the *Manduca* V-ATPase, Southern and Northern blot analysis was performed on all subunits identified so far (Table 1). Using different sets of restriction enzymes which do not cut the genomic DNA in the region selected for probe hybridization, Southern blot analysis was used to investigate gene copy numbers for the various V-ATPase subunits. Single gene loci were identified for subunits A, B, C, D, E, F, H and d whereas multiple genes are likely for subunits G, a, c and e (Fig. 3 and Table 1). Northern blots of mRNA isolated from larval midgut showed two or more transcripts only for subunits B, G, c and d (Fig. 4 and Table 1).

These Southern and Northern blot analyses led to four suggestions. First, subunits A, C, D, E, F and H may have been unequivocally identified as members of the midgut V_1 complex and the V-ATPase holoenzyme derived from goblet cell apical membrane, since no isoforms could be detected for these polypeptides. Second, at least some of the various transcripts encoding subunits c and G may derive from different genes, provided that pseudogenes do not exist. Third, since only one gene locus is detectable for subunits B and d, the multiple transcripts observed in Northern blots may be generated via RNA processing, either by alternative splicing or by differential polyadenylation. Fourth, although different gene loci are likely for subunits a and e, for each subunit there is only one transcript detectable in mRNA derived from midgut tissue, indicating tissue specific isoforms. This deduction was supported for subunit a by the amplification of a PCR fragment from Malpighian tubule mRNA, which is similar but not identical to the coding region of the subunit a cDNA isolated from midgut mRNA (Fig. 1). In comparison with the midgut subunit a, the deduced partial amino acid sequence of the Malpighi isoform is somewhat more similar (~1%) to the bovine isoform a2, which, in contrast to the ubiquitous a1 isoform, is highly expressed only in lung, spleen and kidney [41].

One or more isoforms have been reported for V-ATPase subunits A, B, G, H, a and c, some of which are expressed in specific tissues and some of which seem to have distinct intracellular locations [6,23,42]. In unicellular organisms, such as yeast, isoforms have been reported only for the proteolipid (*vma3*, *vma11*, *vma16*) and for subunit a (*vph1* and *stv1*), suggesting that the functional homologs of higher eukaryotes could also be expressed within one cell. However, at least for subunit a, tissue specific expression has also been observed in multicellular eukaryotes, implying that there are more than two isoforms. Indeed, in higher organisms at least three isoforms appear to exist for this protein [29,41,43]. Most noticeable are genetic studies performed in yeast which demonstrated that the proteolipids vma3p, vma11p and vma16p together may form one proton conducting part within the V_o complex [44]. Moreover, at

least one of the proteolipids of the V-ATPase appears to have an additional function in metazoan tissues, because it is likely to be a major component of gap junctions [45]. Expression of multiple V-ATPase subunits A, B, G, and H may be restricted to multicellular eukaryotes, since in yeast or *Neurospora crassa* no isoforms have been detected [46,47]. In higher eukaryotes these proteins are either expressed ubiquitously in the organism or restricted to specialized cell types, where they could be involved in targeting and regulation processes. Southern and Northern blot analysis for the *M. sexta* V-ATPase gene family suggested that isoforms may be present only for subunits B, G, a, c, d and e. Although all of the ATPase cDNAs that we have isolated come from a *M. sexta* midgut library, we cannot completely exclude the possibility that some of them may represent isoforms which are not expressed in goblet cell apical membranes, but in endosomal membranes instead. This prospect may be true especially for those subunits with multiple transcripts in Northern blots. Consequently, we will try to isolate the corresponding cDNAs of the presumed isoforms, and to generate isoform specific antibodies for their identification.

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