Sense and antisense RNA for the membrane associated 40 kDa subunit M40 of the insect V-ATPase

Hans Merzendorfer, William R. Harvey[1], Helmut Wieczorek*

Zoologisches Institut der Universität, Luisenstr. 14, D-80333 München, Germany

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Abstract For the first time a cDNA encoding the membrane associated subunit M40 of an invertebrate V-ATPase has been isolated and sequenced, based on a cDNA library from larval midgut of the tobacco hornworm, *Manduca sexta*. Immunoblotting with monospecific antibodies raised against the recombinant M40 polypeptide demonstrated that it is a subunit of the insect plasma membrane V-ATPase. Since M40 subunits had been identified only in endosomal V-ATPases till now, this result indicates that they are constitutive members of all, endomembrane and plasma membrane V-ATPases.

A phagemid clone representing a polyadenylated antisense transcript was also isolated and sequenced. Using RT-PCR, endogenous antisense RNA was detected in poly(A) RNA isolated from the larval midgut. Since Southern blots indicated a single gene locus, both the antisense RNA as well as the sense mRNA encoding subunit M40 seem to originate from the same gene.

Key words: H^+ translocating vacuolar-type ATPase; V-ATPase; cDNA cloning; Antisense RNA; Insect midgut

1. Introduction

Proton pumping V-ATPases are multisubunit proteins that are composed of a peripheral, catalytic V_1 complex and a membrane bound, H^+ translocating V_0 complex. They were originally characterized as a component of endomembranes, but they also play an important role in plasma membrane ion transport (see [1]). In the larval midgut of the model insect, *Manduca sexta* (Lepidoptera, Sphingidae), they occur in the apical plasma membrane of epithelial goblet cells where, in contrast to their default acidifying function in organelles, they energize the alkalinization of the gut lumen up to a pH of more than 11 [2]. The plasma membrane V-ATPase in the tobacco hornworm midgut is highly regulated; for example, during the moult or when deprived of food, the peripheral V_1 complex reversibly detaches from the membrane [3–5].

To gain more insight into this mechanism of regulation, more information about the constituent subunits of the V-ATPase is needed. Although five subunits of the insect V_1 complex have already been cloned and sequenced (see [6]), much less is known about the V_0 complex. Up to now only the 17 kDa proteolipid has been cloned and sequenced [7]. Endomembrane V-ATPases contain two further V_o subunits in the range of 100 kDa [8,9] and of 40 kDa [10–13]. Although there is no sign of a 100 kDa subunit in the insect plasma membrane V-ATPase, a candidate for a 40 kDa subunit appears to exist; V-ATPase purified from midgut goblet cell apical membranes as well as from Malpighian brush border membranes of the tobacco hornworm contains a polypeptide of ca. 40 kDa which is not a V_1 subunit [14,15] and which remains in the membrane during moult [3].

Using degenerate primers to conserved regions of bovine and yeast cDNA, we have cloned and sequenced the first cDNA encoding an invertebrate 40 kDa V_0 subunit. Moreover, we have provided the first evidence that this M40 V-ATPase subunit occurs not only in endomembranes, but also in plasma membranes. Finally, we present data suggesting that posttranscriptional regulation of the M40 subunit mRNA is mediated by the expression of antisense RNA.

2. Materials and methods

2.1. Isolation and sequencing of 40 kDa subunit cDNA

A 137 bp fragment of a λZAP II cDNA library from the midgut of *M. sexta* [16] was amplified by direct PCR [17] using nested degenerate primers (Fig. 2) which were synthesized by Pharmacia and corresponded to conserved regions of the bovine and yeast cDNA sequences [10,11]. After establishing optimal annealing conditions for the nested amplification step (55°C, 1 min), a labeled PCR product [18] was generated by repeating the reaction in the presence of digoxigenin-11-dUTP (Boehringer) and subsequently purified by electrophoresis in 2% FMC Sea Plaque agarose. Hybridization screening of the λZAP II cDNA library with this fragment led to the isolation of six independent phage clones. The phagemid sections of two clones containing 40 kDa protein related cDNA's were rescued by in vivo excision and subsequent *E. coli* XL1-Blue infection [19]. The derived Bluescript® SK (−) plasmid clones ($BSK1V_040$ and $BSK3V_040$) were purified by using a plasmid miniprep kit (Qiagen). Since the clone $BSK3V_040$ turned out to be truncated in the 5′ region of the coding sequence, an additional PCR based screening was performed on the initially isolated phage clones, using T3 and a reverse orientated 5′ primer raised against the $BSK3V_040$ cDNA. This procedure led to the isolation of the full length clone $BSK2V_040$ encoding the *M. sexta* membrane associated V_0 subunit M40.

Sequencing of the plasmids was performed by the dideoxynucleotide chain termination method [20] using Sequenase 2.0 [21] from USB and different sets of custom synthesized 20-mer oligonucleotides for a primer walk which was started with oligonucleotides matching with the T7 and T3 promoters. Sequences were determined on both strands of the plasmids.

2.2. Southern and Northern blot analysis

For Southern hybridization 10 μg aliquots of genomic DNA [14] were digested to completion using six different restriction enzymes, electrophoresed through a 0.8% agarose gel in Tris-acetate/EDTA buffer (pH 8.0) and blotted onto a Hybond N nylon membrane (Amersham) according to Sambrook et al. [22]. To obtain a better signal to noise ratio [23] a digoxigenin labeled ssRNA probe was synthesized by in vitro transcription, using a DIG RNA labeling kit (Boehringer), T7 polymerase and, as a template, $BSK3V_040$; the latter had been linearized by *Not*I restriction to prevent polymerization of vector DNA. Hybridization was performed overnight at 50°C in 50%

*Corresponding author. Fax: (+49) 89-5902325.
E-mail: wieczo@zi.biologie.uni-muenchen.de

[1]*Permanent address:* Whitney Laboratory, University of Florida, St. Augustine, FL 32086, USA.

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formamide, 5× SSC buffer, 0.02% SDS, 0.1% *N*-lauroylsarcosine and 2% (w/v) blocking reagent (Boehringer) at a probe concentration of approximately 100 ng/ml. Stringency washing was performed at 68°C in low salt buffer (0.1× SSC, 0.1% SDS). Following the Boehringer Mannheim digoxigenin protocol, the hybridized probes were detected colorimetrically with NBT and BCIP.

For Northern hybridization, 4 μg aliquots of poly(A) RNA, purified from the midgut of 5th instar larvae (Quickprep Micro Kit, Pharmacia), were loaded onto a 1% agarose/formaldehyde gel. Sample preparation, gel electrophoresis and Northern transfer were performed according to [22], except for the modification of supplementary 2% formaldehyde present in the agarose gel, the running buffer and the gel loading buffer. After UV crosslinking the membranes were hybridized with a digoxigenin labeled ssRNA probe (ca 100 ng/ml), generated as described above but using either *Not*I precut $BSK2V_040$ or *Bgl*II precut $BSK1V_040$ as templates for polymerization. Hybridization and stringency washing were performed at 68°C under the same buffer conditions as for the Southern blot.

2.3. Anchored PCR for specific amplification of antisense RNA

For cloning of cDNA derived from the 3′ region of either the M40 sense or antisense RNA, anchored PCR [24] was carried out using a 5′/3′ RACE kit (Boehringer Mannheim). First strand cDNA synthesis, initialized with the oligo d(T)-anchor primer, was performed according to the manufacturer's protocol using 2 μg of larval midgut poly(A) RNA. After heat inactivation of the AMV reverse transcriptase (5 min at 95°C), 1 μl of the reaction mixture was used for amplification of the cDNA. The reaction was performed with the anchor primer of the RACE kit and either the oligonucleotide 5′-GCAAGCAGATTGGTGGTACTACAGCTGATG-3′ (positions 663–692 of the sense cDNA), specific for the sense orientation, or the complementary oligonucleotide 5′-CATCAGCTGTAGTACCACCAATCTGCTTGC-3′ (positions 1375–1404 of the antisense cDNA), specific for the antisense orientation. The buffer used for PCR consisted of 50 mM KCl, 10 mM Tris/HCl (pH 8.3), 1.5 mM $MgCl_2$, 0.01% gelatin and 0.2 mM each of dATP, dGTP, dTTP and dGTP. The reaction was started by the addition of 1.25 U of TAQ polymerase (Perkin Elmer) after heating the reaction mixture for 2 min at 95°C. Forty cycles were carried out, each cycle performed by the succession of 30 s denaturation at 95°C, 40 s annealing at 57°C and 40 s elongation at 73°C. PCR products were separated by electrophoresis in an 1% agarose gel and stained with ethidium bromide. After gel extraction the fragments were cloned into the pUC18 plasmid using the SureClone ligation kit from Pharmacia and a standard $CaCl_2$ protocol to transform competent *E. coli* XL1-Blue cells (Stratagene). The recombinant plasmids were further characterized by nucleotide sequencing as described above.

2.4. Other methods

The 40 kDa protein was expressed as a fusion protein using the pMal c2 system from New England Biolabs. The complete coding sequence was amplified by PCR using a 32-mer upstream primer extended by an in-frame *Eco*RI site together with a 6-base nonsense sequence and an 35-mer downstream primer extended by an in-frame stop codon together with a *Sal*I site and the 6-base nonsense sequence. The *Eco*RI and *Sal*I digested DNA was ligated into the pMal c2 expression vector, and the resulting plasmid p40MBP-c2 was transformed into competent, protease-deficient UT5600 *E. coli* cells. After expression and purification, the fusion protein was cleaved overnight at room temperature using factor Xa protease. Polyclonal antibodies against the cleaved fusion protein were generated from guinea pigs by Charles River, Germany.

For amplification of a partial sequence of the M40 gene, direct PCR [17] was performed as described above using the primers 5′-AATGTTGAAATTCGATGACG-3′ and 5′-TTTTTTTTTATCTCTTTGGG-3′ (positions 1741–1760 and 2066–2085, respectively, of the antisense cDNA). 3000 pfu of a phage clone isolated from a genomic *M. sexta* λFix II library were used as a template. By hybridization with a M40 cDNA specific probe this clone had been shown before to contain sequences of the corresponding gene. Cloning and sequencing of the PCR product was performed as already mentioned.

Larvae of *M. sexta* were reared under long day conditions (16 h of light) at 27°C using a synthetic diet (modified according to Bell and Joachim [25]). A membrane fraction enriched with midgut goblet cell apical membranes and V-ATPase purified from goblet cell apical membranes were prepared from 5th instar larvae following published protocols [26,27]. Isolation of Malpighian tubule brush border membranes and stripping of V_1 complexes was performed by treatment with 0.8 M KI [15] in a buffer consisting of 0.3 M mannitol, 17 mM Tris-HCl (pH 7.5), 5 mM EGTA and 9.4 mM mercaptoethanol. SDS polyacrylamide gel electrophoresis, Western blotting on nitrocellulose membranes (B85), immunostaining and protein determination with Amido black were performed as described previously [14,26–28].

3. Results and discussion

3.1. Primary structure of the 40 kDa subunit

Screening of a *M. sexta* cDNA library with a digoxigenin-labeled double stranded probe resulted in the detection of two individual clones, both of which were sequenced by primer walk. The cDNA insert of $BSK2V_040$ was 2082 bp in length and contained the complete open reading frame coding for a putative polypeptide of 348 amino acids (Figs. 1 and 2). The protein had a deduced molecular mass of 39 594 Da and a pK_i of 4.73, was hydrophilic and exhibited no striking transmembrane regions. The *M. sexta* protein showed a high amino acid similarity with 36–41 kDa V_0 subunits sequenced previously [10–13]. The highest similarity was found for the bovine sequence (89% similarity/79% identity), followed by *Dictyostelium* (77%/61%), *Neurospora* (71%/52%) and yeast (68%/47%). The overall similarity between these five species was 51%, the overall identity 32% (Fig. 2). Similarity with other 40 kDa subunits was observed not only with regard to the primary structure in general, but also included the highly conserved regions, the overall hydropathy, the predicted secondary structure and three conserved cysteines at amino acid positions 36, 125 and 332.

3.2. Expression, Western blot analysis and stripping experiments

To confirm that the M40 cDNA encodes a subunit of the insect V-ATPase, we expressed a recombinant fusion protein and used it to generate antibodies. Western blots with V-ATPase that was purified from midgut goblet cell apical membranes or from Malpighian tubule brush border membranes and blots with a midgut preparation that was enriched with goblet cell apical membranes together proved that the resulting polyclonal antibodies were highly specific for a 40 kDa protein present in all of these preparations (Fig. 3). Anti-MBP antibodies, affinity purified from the antiserum, did not cross-react with any protein from the different V-ATPase preparations (not shown). These results demonstrate that the cloned 40 kDa protein is part of the *M. sexta* V-ATPase.

To test whether the 40 kDa protein is the M40 subunit of the V_0 complex, we stripped peripheral proteins including the V_1 subunits from Malpighian tubule brush border membranes by treatment with a high concentration of chaotropic iodide (Fig. 4). In contrast to a selection of known, peripheral V_1 subunits (A, E and G), which all were removed efficiently from the membrane, most of the 40 kDa subunit remained in the membrane fraction. Thus, the 40 kDa subunit was stably associated with the membrane under conditions where V_1 subunits clearly fell off. The 40 kDa protein appeared to be only faintly stained by Coomassie Blue and to be covered by another peripheral protein of the same electrophoretic mobility, since its location in the membrane could be derived only from immunostaining of Western blots, but not from protein staining after SDS-PAGE. Taken together, these results show

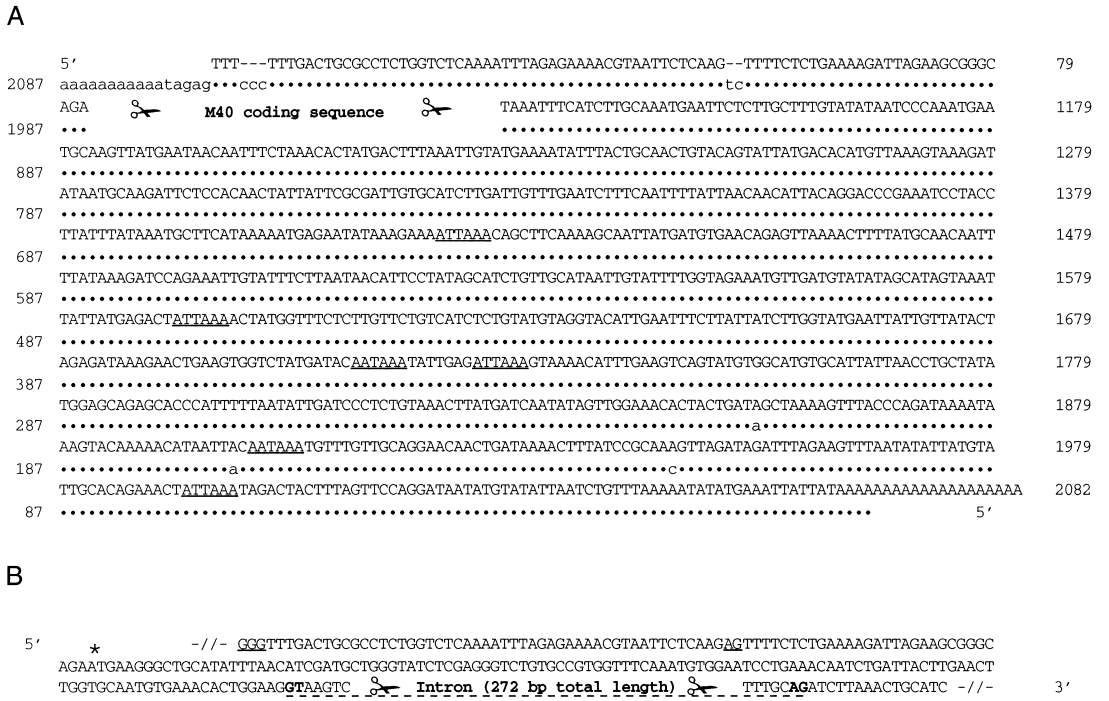

Fig. 1. (A) Partial nucleotide sequences of sense and antisense cDNA for the M40 V-ATPase subunit of *M. sexta*. The cDNA sequence of the sense strand (uppercase letters) is written from 5′ to 3′, that of the antisense strand (lowercase letters) from 3′ to 5′. The complete nucleotide sequences including the open reading frame have been submitted to the GenBank/EMBL Data Bank and have been given the accession number X98825. In the M40 coding region (not shown here), sense and antisense cDNAs are completely complementary; dots indicate nucleotides of the antisense strand that are complementary to the cDNA in the non-coding region. Putative polyadenylation sites are underlined. (B) Partial nucleotide sequence of the M40 gene spanning an intron in the 5′ region (omitted for the most part) that is marked with a dotted line. 5′ donor and 3′ acceptor sites are in bold letters. Deviations from the sense cDNA are underlined. The asterisk marks the start codon of the M40 coding region.

that we have cloned and sequenced the first invertebrate cDNA encoding the membrane associated 40 kDa V-ATPase subunit M40. All M40 subunits hitherto described have been from cells in which V-ATPases are expressed in endomembranes, but not in the plasma membrane [10–13]. The presence of the M40 subunit in the plasma membrane V-ATPase of insect cells supports the idea that this subunit is a constitutive part of V-ATPases in general.

3.3. *M40 antisense RNA*

The cDNA clone BSK1V$_0$40 did not show any similarity with an mRNA encoding subunit M40. Rather its sequence revealed nearly complete identity with the antisense transcript. The M40 antisense cDNA was 2087 bp in length, polyadenylated at the 3′ terminus and constituted a precise complement to the coding region of the M40 sense cDNA (Fig. 1A). Due to the lack of an appropriate open reading frame, the antisense transcript obviously does not encode a protein. The 3′ terminus of the M40 antisense cDNA was found to be extended by 16 nucleotides, 11 of them comprising the poly(A) tail. Conversely, the antisense 5′ terminus was truncated by 16 nucleotides, lacking most of the complementary thymidines of the sense strand poly(A) tail. Besides the 3′ overhangs, only eight non-matching nucleotides were observed over the complete sequence; these deviations were exclusively localized in the periphery of the untranslated regions. Since the cDNA library used for screening had been constructed by directional cloning in λZap II [16] and since, in addition, the cloning sites of BSK1V$_0$40 were found to be intact, we conclude that the M40 antisense cDNA is a genuine representative of a corresponding endogenous, cytoplasmic antisense transcript.

In order to detect independently antisense RNA in poly(A) RNA isolated from the midgut of 5th instar larvae, we performed Northern blots under high stringency conditions. Using the sense strand specific probe for hybridization, two major and one minor messages with lengths of approximately 2.2, 1.8, and 1.5 kb, respectively were detected (Fig. 5). This result indicated the presence of three different M40 mRNA species in tobacco hornworm midgut cells. They might be generated by alternative polyadenylation, since multiple polyadenylation signals were observed in the 3′ UTR of the M40 cDNA (Fig. 1A). Unexpectedly, no message was detected using an antisense strand specific probe for hybridization. Since it was not clear which amounts of endogenous antisense RNA could be expected in the midgut, we performed RT-PCR which is a more sensitive method than Northern blotting. After reverse transcription of poly(A) RNA, we amplified by 3′ RACE specifically sense or antisense M40 transcripts. For both, sense and antisense messages, we obtained fragments of the expected size (1.44 and 0.75 kb, respectively, see Fig. 6). In line with the results from Northern blotting, the sense product was much more abundant then the antisense product. The establishment of both nucleotide sequences verified their identity with the initially isolated sense and antisense M40 cDNA clones (Fig. 6, result shown only for the antisense sequence). Some slight sequence differences at the 3′ termini were most likely due to non-perfect annealing of the oligo

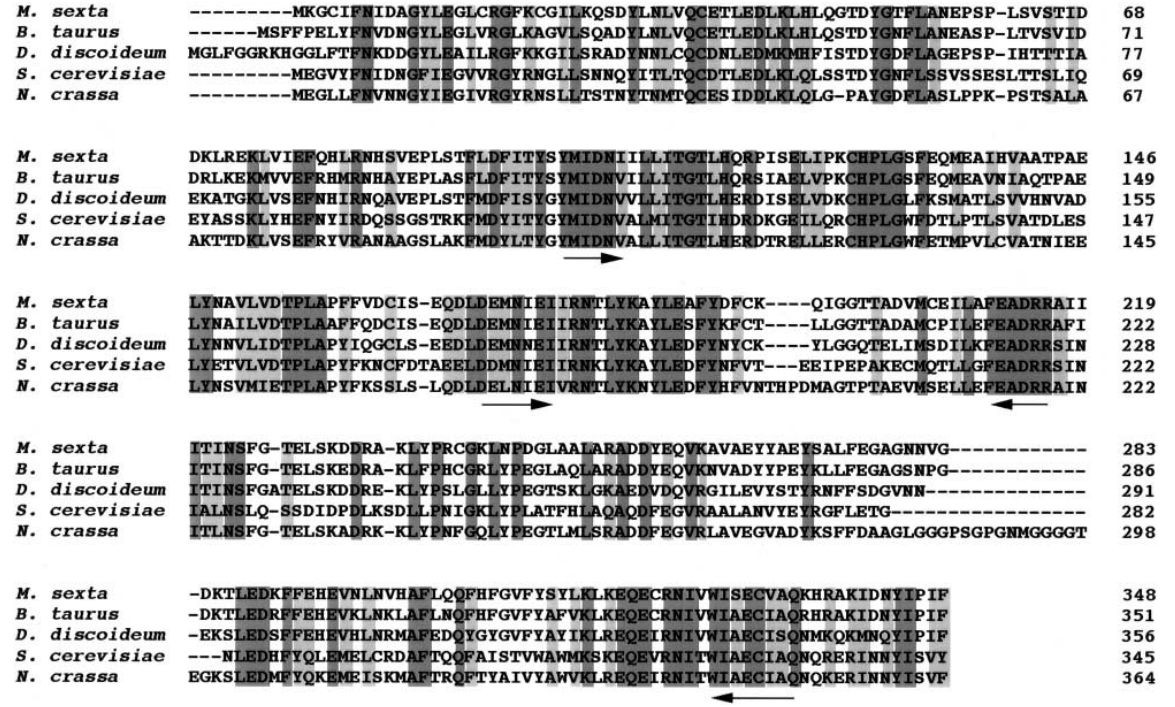

Fig. 2. Multiple amino acid sequence alignment of the *M. sexta* subunit M40 with polypeptides isolated from *Bos taurus* (updated fifth version, GenBank accession number J04204), *Dictyostelium discoideum* [12], *Saccharomyces cerevisiae* [11] and *Neurospora crassa* [13]. Identical amino acids are highlighted with dark, similiar amino acids with light shading. Hyphens indicate gaps introduced for better sequence alignment. A homologous human protein sequence [39] was not considered in this alignment, since it was truncated in the N-terminal region, possibly due to the incorrect determination of the translational start site [13]. Arrows: conserved amino acid sequences which were chosen to design nested primers for direct PCR.

d(T) anchor primer in the A-rich regions close to the poly(A) tails or may have resulted from errors during PCR.

To rule out the possibility of either cDNA contaminations in the poly(A) RNA or the presence of endogenous cDNA we performed several control experiments. Thus treatment of RNA preparations with DNase I or RNase A and reactions performed without AMV-RT to exclude reverse transcriptase activity of the TAQ polymerase all confirmed the absence of cDNA in the template mRNA preparation (data not shown).

To determine whether the detected antisense RNA derives from a different gene than that encoding subunit M40, a Southern blot was performed under high stringency conditions (Fig. 7). Although we digested genomic DNA using six different restriction enzymes without recognition sites within the hybridization target sequence, only one restriction fragment was labeled in each digest indicating a single gene locus for both the sense and the antisense RNA.

The existence of native antisense RNA in bacteria and its mode of action in the regulation of gene expression are well known [29]. For eucaryotes, however, only a few examples of endogenous antisense RNA have been reported. Although there are some hints for a regulatory function (e.g. for the *EB4-PSV* antisense transcript [30]), the respective mechanisms still remain obscure (for review see [31]). To our knowledge this report is the first demonstration of specific antisense RNA for invertebrates as well as for V-ATPases in general. We currently do not know why antisense RNA is expressed in the *M. sexta* midgut. It could be involved in mRNA sorting to specific cell locations; indeed localized mRNA encoding V-ATPase subunits has been reported by us previously [32]. Alternatively it also could be involved in the regulation of sense mRNA stability. As supposed for *Dictyostelium* [30], a simple model would suggest the formation of RNA/RNA hy-

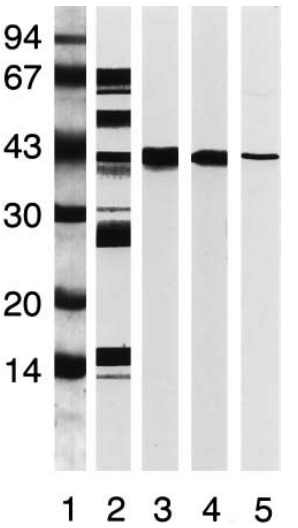

Fig. 3. Western blots of V-ATPases after SDS-polyacrylamide gel electrophoresis. Lane 1: gel stained with Coomassie Blue; standard proteins with molecular masses indicated in kDa. Lane 2: Western blot of V-ATPase purified from midgut goblet cell apical membranes, stained with an antiserum to the V-ATPase holoenzyme [28]. Lanes 3–5: Western blots of different V-ATPase preparations stained with an antiserum raised against the cleaved fusion protein containing the recombinant 40 kDa protein. Membrane fraction enriched in goblet cell apical membranes (lane 3), V-ATPase purified from midgut goblet cell apical membranes (lane 4) and isolated Malpighian tubule brush border membranes (lane 5).

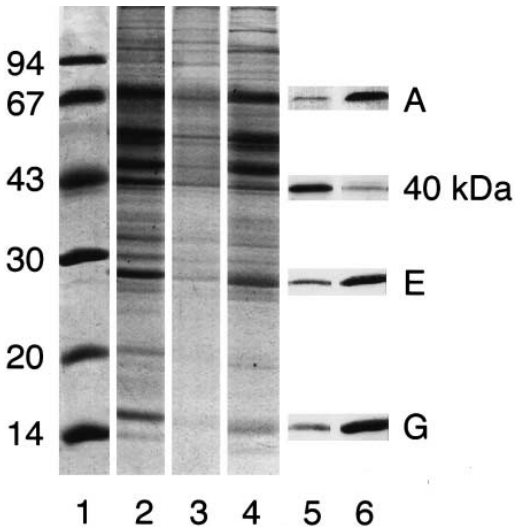

Fig. 4. Stripping of Malpighian tubule brush border membranes with chaotropic iodide. After treatment with 0.8 M KI, proteins were separated by SDS-PAGE and either stained with Coomassie Blue (lanes 1–4) or transferred to a nitrocellulose membrane and stained separately with different antibodies (lanes 5 and 6). Lane 1: standard proteins with molecular masses indicated in kDa; lane 2: Malpighian tubule brush border membranes before stripping. Lanes 3–6: membrane pellet (lanes 3 and 5) and supernatant with soluble proteins (lanes 4 and 6) after KI stripping. The following antibodies were used: a monoclonal antibody (221-9, [3]) to visualize subunit A, the antiserum to the 40 kDa pMal fusion protein to visualize subunit M40, and a polyclonal antiserum to the V-ATPase holoenzyme [28] to visualize subunits E and G.

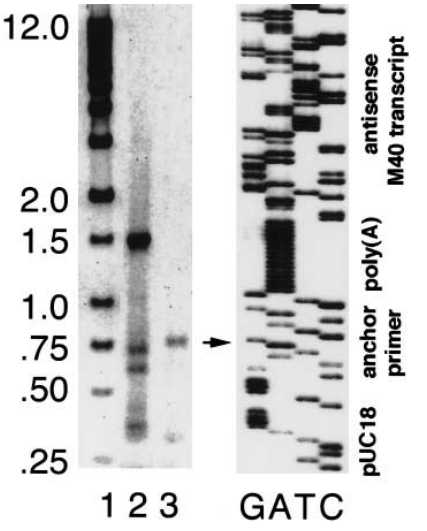

Fig. 6. Detection of endogenous M40 antisense RNA by anchored PCR. Midgut poly(A) RNA was subjected to first strand cDNA synthesis in the presence of an oligo(dT) anchor primer. PCR amplification was performed using an anchor primer and a primer specific for either sense or antisense orientation. PCR products were separated by agarose gel electrophoresis and stained with ethidium bromide. The picture was graphically inverted by a drawing program to increase the contrast. Lane 1: 0.5 μg of standard DNA fragments of the indicated size in kb; lane 2: product of anchored PCR using the sense strand specific primer; lane 3: product of anchored PCR using the antisense strand specific primer. On the right side of the figure, the autoradiogram shows the nucleotide sequence of the 3′-terminal region of the antisense M40 transcript after cloning the PCR product (arrow) into the pUC18 vector.

brids and their rapid degradation catalyzed by dsRNases. Consequently only the one transcript produced in excess should be visible in Northern blots. This deduction is consistent with our observation that the antisense RNA is detectable by highly sensitive RT-PCR but not by Northern hybridization which requires higher amounts of template RNA for detection.

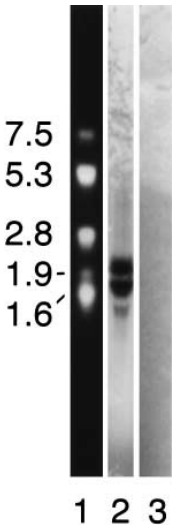

Fig. 5. Northern blot of midgut poly(A) RNA, probed with digoxigenin labeled ssRNA corresponding to the sense and antisense RNA of the 40 kDa protein. Lane 1: 5 μg of standard RNA fragments with the size indicated in kb (the lane was cut off before Northern transfer and stained with ethidium bromide). Lanes 2–3: *M. sexta* poly(A) RNA after Northern transfer and hybridization with the sense (lane 2) and the antisense (lane 3) specific ssRNA probe.

The origin of the antisense transcript remains obscure. According to genomic PCR analysis the M40 gene is organized into at least 3 exons and 2 introns (data not shown). One of them, 272 bp in length, is localized at nucleotide position 202/203 of the M40 cDNA (Fig. 1B). Thus the antisense M40 RNA represents the complement of the spliced sense transcript. Fig. 1B also demonstrates that the sequences of the (−) strand of the 5′ exon and of the corresponding region of the antisense cDNA are identical; the sense transcript, as derived from the sequence of the cDNA clone, differs from both in the lack of some nucleotides (underlined in Fig. 1B). The finding of only one gene locus for subunit M40 argues that the antisense RNA did not derive from transcription of a pseudogene. In such a case one would expect more than one labeled fragment in at least one of the digestion assays. Our assumption is supported furthermore by the total complementarity of the sense and antisense transcripts in the coding region, without nucleotide substitutions which are commonly found in pseudogenes. Antisense RNA could, on the one hand, be produced by transcription from the opposite strand of the M40 gene, as described for human, chicken and *Xenopus bFGF* antisense RNA [33–35]. However, bidirectional transcription of the M40 gene would require the usage of unusual donor and acceptor sites for splicing of the antisense pre-RNA to preserve complementarity. In fact, unusual splicing sites have already been reported [36], but in such a case the precise removal of at least two complementary introns from both M40 pre-RNAs would be necessary. Bidirectional transcription based on unusual splicing was proposed for the

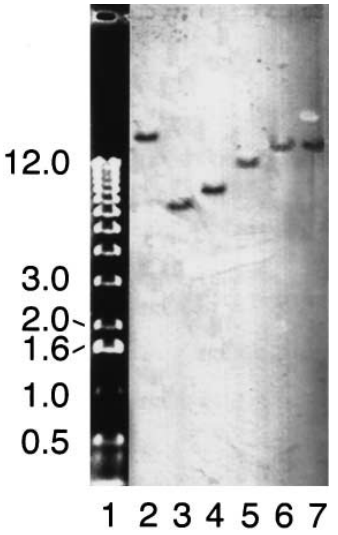

Fig. 7. Southern blot hybridization of genomic DNA with a ssRNA probe derived from M40 cDNA. Lane 1: 0.5 μg of standard DNA fragments of the size indicated in kb (the gel was cut off before Southern transfer and stained with ethidium bromide); lanes 2–7: genomic DNA, after Southern transfer. The DNA had been digested with the following restriction enzymes: *Sal*I (lane 2), *Xba*I (lane 3), *Xho*I (lane 4), *Sac*I (lane 5), *Bam*HI (lane 6) and *Bgl*II (lane 7).

BCMA-antisense RNA that represents, as in our case, the polyadenylated total complement of the spliced BCMA-mRNA [37]. Antisense RNA could, on the other hand, be produced in the cytoplasm, catalyzed by an RNA dependent RNA polymerase. Such a mechanism was suggested for mouse antisense globin RNA which exhibited total complementarity to spliced globin mRNA [38].

To elucidate the mechanism of antisense RNA biogenesis we are currently sequencing the complete gene for the M40 subunit. Moreover, to collect evidence for a regulatory function of the antisense transcript, we are screening several developmental stages of *M. sexta* for differences in the ratio between sense and antisense RNA of the M40 protein; preliminary experiments already yielded evidence for the existence of endogenous dsRNA in *M. sexta* midgut.

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