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REGULATION OF PROTON-TRANSLOCATING V-ATPases

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Summary

Vacuolar-type ATPases (V-ATPases) are proton-translocating enzymes that occur in the endomembranes of all eukaryotes and in the plasma membranes of many eukaryotes. They are multisubunit, heteromeric proteins composed of two structural domains, a peripheral, catalytic V_1 domain and a membrane-spanning V_o domain. Both the multitude of locations and the heteromultimeric structure make it likely that the expression and the activity of V-ATPases are regulated in various ways. Regulation of gene expression encompasses control of transcription as well as control at the post-transcriptional level. Regulation of enzyme activity encompasses many diverse mechanisms such as disassembly/reassembly of V_1 and V_o domains, oxidation of SH groups, control by activator and inhibitor proteins or by small signalling molecules, and sorting of the holoenzyme or its subunits to target membranes.

Key words: H^+-translocating vacuolar-type ATPase, H^+ V-ATPase, proton pump, regulation.

V-ATPases are multisubunit proton pumps accomplishing a broad range of biological functions

The term ‘cellular homeostasis’ summarizes the constant composition of cellular components. Thus, characteristic values of cell [K^+] are high, cell [Na^+] and [Mg^{2+}] are low and cell [Ca^{2+}] are extremely low. These characteristic values are maintained even in cells, such as those of lepidopteran midgut, through which K^+ in amounts that are equal to the total cell content pass every few minutes (Harvey *et al.* 1983) or in the cells of heteropteran Malpighian tubules through which water, in amounts that are equal to the total cell volume, passes every few seconds (Maddrell, 1969). In particular, the concentration of ATP is held constant through a variety of controls that respond mainly to increases in ADP and intracellular phosphate (P_i) concentration as cellular work is performed. Thus, the activity of ATP synthases is regulated so that the rate of ATP synthesis balances that of its utilization (Fillingame, 1997). Here, we discuss the regulation of H^+ V-ATPases as they respond to metabolic perturbations in compartments bounded by endomembranes and plasma membranes of cells ranging from fungi to insect intestine and vertebrate kidney.

V-ATPases partially resemble F-ATPases, with which they evidently share common ancestors (Nelson, 1992), in their complex overall structure and in their subunit composition. Like F-ATPases, V-ATPases are composed of two structural domains (Fig. 1). The peripheral V_1 domain, with a molecular mass of approximately 500 kDa, is the catalytic sector, whereas the membrane-spanning V_o domain, with a molecular mass of approximately 100–250 kDa, is the proton-conducting sector (Nelson, 1992).

In eukaryotic cells, F-ATPases occur only in mitochondria and chloroplasts, whereas V-ATPases are found in membranes of several organelles as well as in animal plasma membranes (see

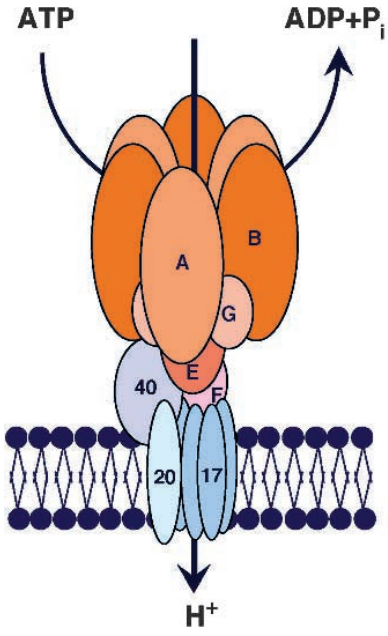

Fig. 1. Putative subunit structure of V-ATPases drawn according to the insect enzyme. Numbers indicate relative molecular masses of subunits. Letters indicate subunits, according to the common nomenclature for V-ATPases.

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Harvey and Nelson, 1992). Examples of organelles that contain V-ATPases are plant and fungal vacuoles, clathrin-coated vesicles, the Golgi complex, lysosomes, synaptic vesicles and chromaffin granules. Examples of plasma membranes that contain V-ATPases are kidney proximal and distal tubule cells, the mitochondria-rich cells of the epididymis, macrophages, osteoclasts, frog skin mitochondria-rich cells and insect epithelial cells. Eukaryotic V-ATPases are electrogenic proton pumps and therefore energize membranes initially by generating a transmembrane voltage. If this voltage is dissipated by the parallel flux of other ions, the *trans*-compartments are rendered acidic, basic or neutral, depending upon the nature of channels or porters and upon the counterion that accompanies the proton (Harvey, 1992). It is this multitude of locations in the cell and the multitude of biological functions which make it likely that the structure and function of eukaryotic V-ATPases are regulated in diverse ways, depending on the physiological demand. In this review, we will focus on the various mechanisms of regulation. Although a unifying picture has not yet emerged, it appears that there is a multitude of these mechanisms controlling the gene expression and enzyme activity of this important class of ion-transporting ATPases (Fig. 2).

V-ATPases are complex, heteromultimeric proteins

The number of subunits that make up the peripheral V_1 sector of eukaryotic V-ATPases differs in various tissues. In chromaffin granules, the V_1 domain consists of at least five different subunits (Nelson, 1992): subunit A (approximately 70 kDa), subunit B (approximately 57 kDa), subunit C (approximately 44 kDa), subunit D (approximately 30 kDa) and subunit E (approximately 26 kDa). The subunit stoichiometry of the V_1 domain in clathrin-coated vesicles was reported to be A_3B_3CDE (Arai *et al.* 1988). A 14 kDa protein, subunit F, was originally cloned from *Manduca sexta* (Gräf *et al.* 1994) and was subsequently found in yeast (Graham *et al.* 1994; Nelson *et al.* 1994) and in mammals (Peng *et al.* 1996). A 13 kDa protein, subunit G, was first detected in yeast (Supeková *et al.* 1995) and subsequently identified as part of the insect V_1 domain (Lepier *et al.* 1996); therefore, it may also be a ubiquitous V-ATPase subunit, especially since it also occurs in bovine chromaffin granules (Supeková *et al.* 1996). Subunits A and B are not only structurally homologous to the respective catalytic/regulatory F-ATPase subunits β and α, but also functionally similar (Nelson, 1992). Subunits C, D and E may be involved in contacting the proton-conducting subunit

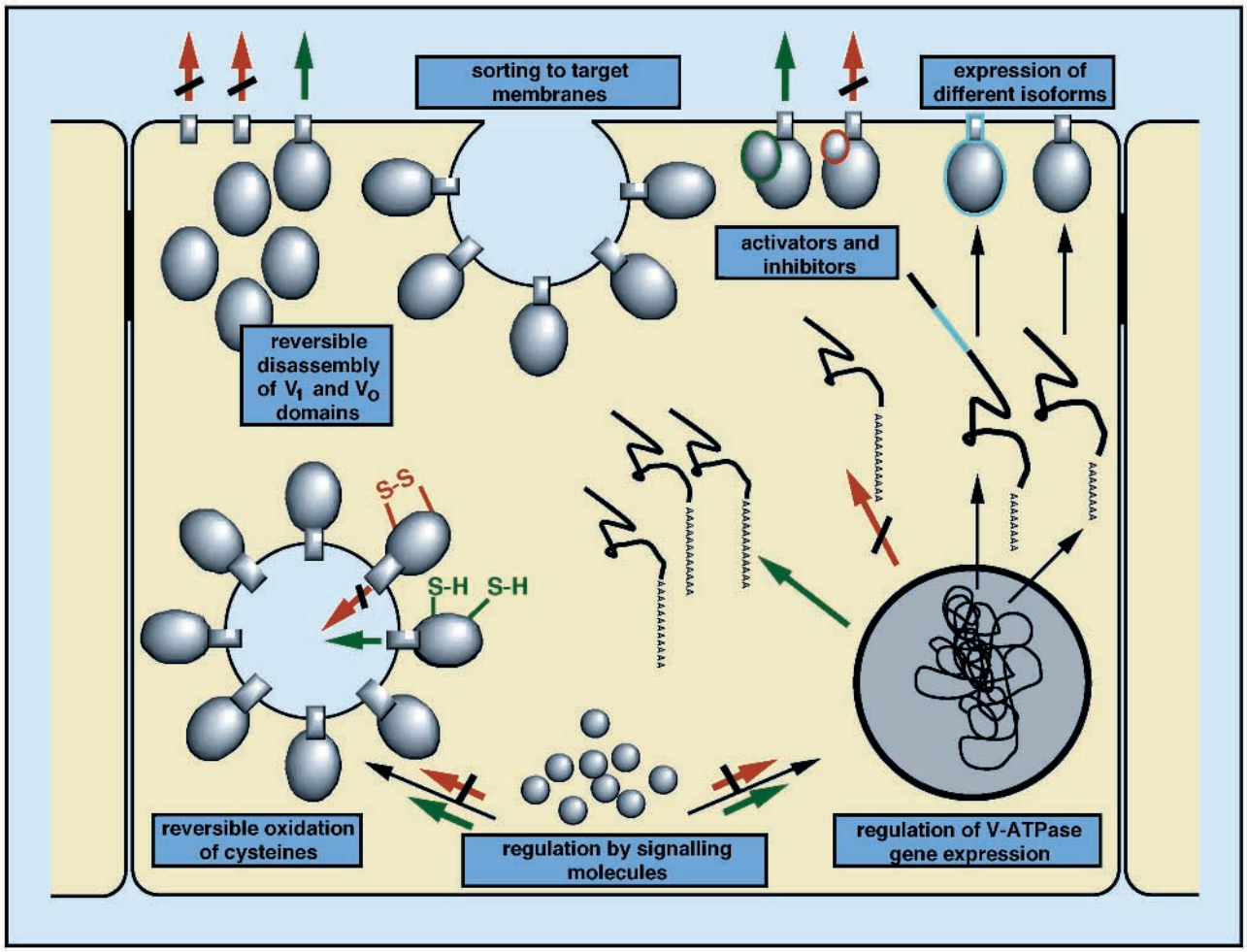

Fig. 2. Summary of regulatory processes mediating the control of either V-ATPase expression or V-ATPase activity.

c of the V_o domain (Adachi *et al.* 1990), and subunit F may be a good candidate for connecting the V_1 domain with the V_o domain, since it exhibits some affinity for the V_1 domain (Gräf *et al.* 1994, 1996), but it also appears to be involved in the assembly and stability of the V_o domain (Graham *et al.* 1994). Some controversy still exists regarding subunit G, which is clearly a peripheral V_1 subunit in the insect enyzme (Lepier *et al.* 1996), but appears to be part of the V_o domain in yeast (Supeková *et al.* 1995).

The major V_o subunit in every V-ATPase is the dicyclohexylcarbodiimide-binding, proton-conducting 16–17 kDa proteolipid, subunit c. The proteolipid is remarkably conserved among V-ATPases of different sources and also shows a rather high similarity to the 8 kDa F_0 subunit c of F-ATPases; it has therefore been suggested that it evolved from a smaller ancestor by gene duplication and subsequent fusion (Nelson, 1992). In accordance with this assumption, the proteolipid appears to occur in six copies per holoenzyme (Arai *et al.* 1988), whereas subunit c of F-ATPases is believed to occur in 12 copies per holoenzyme. The V_o domain contains, among others, two further subunits with molecular masses of approximately 100 and 40 kDa, as found in clathrin-coated vesicles where they occur as single copies (Arai *et al.* 1988). The 40 kDa polypeptide seems to be common to all V-ATPases, whereas the100 kDa polypeptide is present in some but not all preparations.

Regulation of V-ATPase gene expression

Transcriptional regulation

In many cells, V-ATPases are located only in membranes of acidic intracellular compartments such as lysosomes or vacuoles, where they appear to be expressed continuously because their main function is the generation of an acidic organelle interior. Thus, in these cells, V-ATPases function as housekeeping enzymes, with no evident need for particular control of their expression. This was recently demonstrated for the V- ATPase of *Neurospora crassa* (Wechser and Bowman, 1995). The promoters of three V-ATPase genes – *vma-1*, *vma-2* and *vma-3*, encoding subunits A, B and c, respectively – were examined regarding structure, common promoter elements and similarities to other housekeeping genes. Although the upstream regions were largely dissimilar in sequence, the *Neurospora vma* genes shared several common features resembling mammalian housekeeping genes. They exhibited multiple transcription initiation sites and a G+C-rich region just upstream of the initiation sites. In addition, they lacked TATA and CAAT box elements. Close to the transcription initiation sites, two sequence elements were identified in all *Neurospora vma* genes studied so far: a putative Sp1 site, which is also found frequently in mammalian housekeeping genes, and a sequence that is identical or similar to CCAACCTC. The latter has been described previously for several other housekeeping genes of *Neurospora*.

In many cases, cell-specific demands require particular control of V-ATPase expression that leads to increased or decreased amounts of protein. During monocyte to macrophage differentiation, the amount of V-ATPase is increased because of the development of an extensive lysosomal system which enables the macrophage cells to digest pathogens and to present antigen. For both native monocytes and related THP-1 cells, which were used as tetra decanoyl phorbol acetate (TPA)-inducible model cells for monocyte to macrophage differentiation, transcriptional regulation could account for the increase in V-ATPase content (Lee *et al.* 1995). In particular, one subunit, the B2 isoform, was amplified transcriptionally as shown by nuclear run-off experiments. As in the *Neurospora vma* genes, the promoter of the B2 isoform gene had a high G+C content and also lacked TATA or CAAT box elements. However, in contrast to the *Neurospora* gene, primer extension and ribonuclease protection analysis revealed only a single major transcriptional initiation site which is not typical for promoters without TATA boxes. Moreover, two Sp1 recognition sites, multiple sites for transcription factor AP2 binding and two GA/CT stretches were found. Analysis of several promoter–luciferase constructs led to a minimal fragment of 179 base pairs (bp) containing multiple AP2 sites. These AP2 sites evidently account for the induction of phorbol esters and for cyclic AMP responses which seemed to be involved in increased expression of the B2 isoform. The 5′ flanking region of the human B1 isoform gene showed little similarity in structure to that of the B2 isoform, but this promoter also lacked TATA box elements. In the only other published mammalian promoter sequence of V-ATPase genes, the 5′ flanking region of the human proteolipid gene showed a high G+C content, putative Sp1 recognition sites and no TATA or CAAT boxes (Gillespie *et al.* 1991), as in the examples described above.

In the midgut of larval *Manduca sexta*, the tobacco hornworm, high densities of V-ATPase are found in the apical membranes of goblet cells, whereas immunocytochemistry could not locate corresponding amounts in endomembranes or in the plasma membranes of the other cell type, the columnar cell (Klein, 1992; Wieczorek, 1992). To determine how V-ATPase expression is regulated differentially in membranes and cells, we have isolated and partially sequenced the gene encoding subunit B of the *Manduca* V-ATPase (H. Merzendorfer and H. Wieczorek, unpublished results). Although no transcriptional initiation site has yet been mapped, the overall structure of the *Manduca* promoter seemed to be different in several respects from the V-ATPase promoters just described (Fig. 3). Within 973 base pairs sequenced upstream to the initiation codon, multiple putative TATA boxes, two of them corresponding to the consenus sequence TATAWAW, three putative CCAAT boxes and a single GC box were found. The average G+C content was 35 %, with only few short regions of less than 40 bp exhibiting a G+C content as high as 66 %. In a section of 248 bp (from −157 to −405), an A+T-rich region with an A+T content of 70–93 %, averaged over 30 bp windows, was found. The TATAAA sequence at position −724 and the CCAAT box at position −912 are located at a distance relative to the initiation codon which is presumably

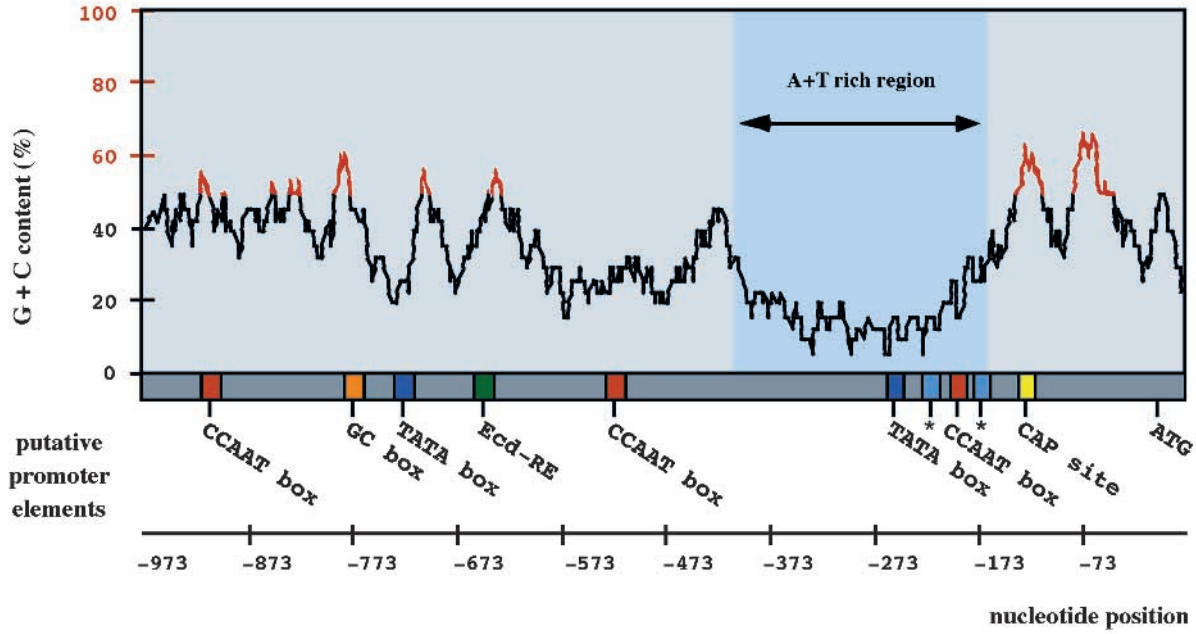

Fig. 3. Base composition of the 5′ upstream region of the gene encoding *Manduca sexta* subunit B. The G+C content is shown averaged over a window of 30 base pairs. Coloured boxes indicate putative promoter elements at the nucleotide positions shown below. The A+T-rich region is highlighted in blue. Asterisks mark H sequences similar to TATA consensus motifs.

not relevant for the transcriptional initiation process. This interpretation is supported by the finding that the upper size for subunit B mRNA detected in a northern blot was only approximately 350 bp longer than the corresponding cDNA clone encoding subunit B (R. Gräf and H. Wieczorek, unpublished results). Thus, assuming even a very short poly(A) tail and considering the absence of splice sites in the proximal promoter region, the 5′ untranslated region would be restricted to a maximum of approximately 400 bp. On the basis of the usual distance and location of TATA and CAAT boxes in eukaryotic promoters, the arrangement of two CCAAT boxes at nucleotide positions –521 and –200 seemed not to be correlated to the TATAAA element at position –249. This indicates that the more proximal TATA motif at position –171, which shares less similarity with the TATA box consensus sequence, may be involved in transcriptional initiation. This site would be in good agreement with the location of a putative CAP site at position –139, since the centre-to-centre spacing between both elements is approximately 30 bp, a distance often found in promoters of higher eukaryotes (Bucher, 1990).

In addition to the recognition sites frequently found in the upstream regions of eukaryote protein coding genes, some further elements sharing sequence similarity to non-common motifs could be relevant for cell- or development-specific gene regulation. Among other promoter elements, a sequence motif at position –658, corresponding to the consensus sequence KNTCANTNNMM of *Drosophila melanogaster* ecdysterone response elements (Luo *et al.* 1991), could contribute to cell-specific regulation of V-ATPase expression during larval development in *Manduca sexta*.

The overall promoter structure of the gene encoding subunit B of the *Manduca* V-ATPase shares features common with upstream regions known from inducible or tissue-specific genes. This conclusion is also based on a comparison of this promoter sequence with all sequences in the eukaryotic promoter database (GenBank, BLASTN program), which resulted in a significant 52 % identity to the *Xenopus laevis* adult beta-globin gene upstream region over a window of 133 bp. However, despite all indications that it is not a housekeeping gene and based just on the presence of TATA and CCAAT elements, the subunit B promoter of the tobacco hornworm resembles the promoter of the carrot V-ATPase A subunit gene, which also contains TATA and CCAAT boxes but is presumed to be a housekeeping gene (Struve *et al.* 1990).

Differential targeting or regulation of V-ATPases could be accomplished by compartment- or cell-specific subunit isoforms, deriving from different genes. In some cases, isoforms indeed originate from different genes, as already reported for the human subunit isoforms B1 and B2 and as further indicated by genomic Southern blots for the bovine subunit B (Puopolo *et al.* 1992) and the cotton subunit c (Hasenfratz *et al.* 1995), or by polymerase chain reaction (PCR) analysis across exon–intron boundaries for subunit A of *Psilotum nudum* and *Equisetum arvense* (Starke *et al.* 1991). Moreover, in yeast, two genes have been isolated for subunit c, the proteolipid (Umemoto *et al.* 1991) and the 100 kDa subunit, which will be discussed later in the present paper (Manolson *et al.* 1994). However, until now, no studies have been reported which could elucidate either genetic mechanisms or particular signals for differential expression of subunit isoforms by multiple V-ATPase genes.

Post-transcriptional regulation

In principle, subunit isoforms could be generated from a single gene by alternative splicing of precursor mRNAs. Indeed, two isoforms of chicken V-ATPase subunit A, which differ in the structure of their catalytic centres, have been generated by this mechanism, although both seem to be expressed ubiquitously (Hernando *et al.* 1995). Furthermore, the two tissue-specific isoforms which have been reported for the bovine 116 kDa polypeptide both originate from the same gene but may differ in their biological half-lives (Peng *et al.* 1994).

The stability of mRNA may represent another control on gene expression. For example, mRNA turnover is affected by the presence of specific elements in the 3′ untranslated region (3′ UTR) of mRNA which promote poly(A) shortening and thus enhanced mRNA degradation (see Decker and Parker, 1994). Alternative usage of different polyadenylation signals could result in the expression of mRNAs that differ in their 3′ UTRs, especially with respect to the presence of special degradation signals. Indeed, differential polyadenylation signals have also been observed for mRNAs encoding V-ATPase subunits (Dow *et al.* 1992; Puopolo *et al.* 1992; Gräf *et al.* 1994). For the *Manduca* subunit B, northern blots revealed two transcripts differing by approximately 400 bp in length (R. Gräf and H. Wieczorek, unpublished results). This difference corresponds precisely to the distance between two putative polyadenylation signals in the 3′ UTR of the corresponding subunit B cDNA. Moreover, Southern blots suggested only a single gene locus for subunit B, additionally supporting the involvement of alternative polyadenylation. Thus, nested PCR, using a *Manduca* cDNA library as the template, forward primers matching downstream sequences of the coding region and reverse primers specific for the T7 promoter of the cloning vector, for both 3′ UTRs or for only the additional 3′ region of the longer transcript, finally demonstrated the presence of two differentially polyadenylated transcripts (H. Merzendorfer and H. Wieczorek, unpublished results). The extra 3′ region of the longer transcript contains two additional AU-rich elements, which may mediate differential control of mRNA stability (Decker and Parker, 1994). Finally, this region may also be involved in differential mRNA sorting. *In situ* hybridization using ssRNA probes specific for the mRNA encoding the V_1 subunit A as well as for the mRNA encoding the V_o proteolipid resulted in an apical localization of the subunit A mRNA, whereas the proteolipid mRNA was found to be uniformly distributed (Jäger *et al.* 1996). This finding suggests spontaneous self-assembly of the V_1 subunits close to the targeting site of the protein. A similar situation has been reported for the biosynthesis of several proteins such as the bicoid protein or the cytoskeleton proteins actin, vimentin and muscle myosin (Wilhelm and Vale, 1993). The sorting signals for the bicoid mRNA were found in the 3′ UTR. Mutagenesis of the 3′ UTR led to the characterization of a 53 bp region which, it was suggested, was essential for directing the early steps of bicoid mRNA localization (MacDonald *et al.* 1993).

A further control mechanism of mRNA turnover could be mediated by the expression of antisense mRNA. Such a mechanism (see Nellen and Lichtenstein, 1993), first proved in prokaryotes and then found in a few eukaryotes, may be based on the formation of RNA/RNA hybrids between sense and antisense mRNA and subsequent degradation *via* dsRNAases. We have recently cloned and sequenced a cDNA encoding the membrane-associated 40 kDa subunit of the *Manduca* V-ATPase (H. Merzendorfer, W. R. Harvey and H. Wieczorek, in preparation), which may play a central role in coupling the V_1 and V_o domains, as suggested in yeast (Bauerle *et al.* 1993). In addition to the sense transcript, we have isolated the corresponding antisense cDNA. To demonstrate independently the presence of antisense mRNA in poly(A) RNA, we have performed reverse transcription-polymerase chain reaction (RT-PCR) analysis resulting in the detection of the endogenous antisense transcript. This antisense mRNA may be involved in the post-transcriptional regulation of the 40 kDa subunit.

Regulation of V-ATPase function

Disassembly and reassembly of V_1 and V_o domains

About half of the V_o domains in the membrane of clathrin-coated vesicles occur freely, without V_1 domains bound to the membrane; these V_o domains do not conduct protons (Zhang *et al.* 1992*a*). Correspondingly, a cytosolic pool of fully assembled, enzymatically inactive V_1 domains of unknown function has been detected in a bovine kidney cell line (Myers and Forgac, 1993*a*). The finding of free V_1 and V_o domains implies, in principle, that a reversible disassembly of V_1 and V_o domains could reflect a general mechanism for the regulation of V-ATPase activity. Dissociation of V_1 subunits from the V_o domain is well known from *in vitro* experiments: for instance, cold-inactivation in the presence of ATP or treatment with chaotropic ions leads to the dissociation of V_1 subunits from the V_1V_o holoenzyme (Moriyama and Nelson, 1989), as do oxidizing conditions (Dschida and Bowman, 1995). Reassembly of V_1 and V_o domains has also been demonstrated *in vitro* (Puopolo and Forgac, 1990).

The reversible disassembly of V_1 subunits as an *in vivo* control mechanism of V-ATPase activity was first demonstrated in the midgut of moulting *Manduca* larvae. In this tissue, more than 10 % of the tobacco hornworm's total ATP production is used by the H^+-translocating V-ATPase to generate a voltage of greater than 240 mV across the apical membrane of goblet cells; the voltage drives secondary $K^+/2H^+$ antiport and thus net active K^+ secretion (see Wieczorek and Harvey, 1995). The resulting K^+ electrochemical potential energizes all secondary transport processes across the epithelium of the midgut. However, during the moult, when no secondary transport has to be energized because the larva does not feed, one would expect, for reasons of economy, that active K^+ transport might be down-regulated. Indeed, active K^+ transport was found to decrease during the moult as a result of dissociation of the peripheral V_1 subunits from the membrane V_o domain (Sumner *et al.* 1995). The V_1 domain appeared to

dissociate as a complete complex, since the cytosolic concentration of V_1 domains was doubled during the moult (Gräf *et al.* 1996). The reverse process, the reassembly of the V_1V_o holoenzyme, evidently does not need biosynthesis of new V_1 subunits, since inhibition of translation in moulting larvae by cycloheximide does not influence the (re)assembly of V_1 and V_o domains (Jäger and Klein, 1996). V_1 domains detach reversibly from the V_o domains not only during moulting but also during starvation, indicating that this type of regulation of V-ATPase activity is a response to a drop in energy (food) supply (Gräf *et al.* 1996).

Reversible disassembly of V_1 and V_o domains also plays a role in regulation of V-ATPase activity in yeast (Kane, 1995). Using wild-type cells, Kane demonstrated that depriving yeast of glucose, for as little as 5 min, causes the V_1 domains to detach from the V_o domains. Upon restoration of glucose, rapid reassembly of V_1 and V_o domains was observed, without the need for new subunit biosynthesis, as indicated by the inefficiency of cycloheximide treatment. This result shows that, as in the midgut of *Manduca*, free V_1 and V_o domains may be in dynamic equilibrium with the fully assembled V_1V_o holoenzyme. Taken together, these similar findings from two evolutionarily distant organisms such as insects and yeast may indicate that the reversible disassembly of V_1 and V_o domains is a general mechanism for V-ATPase regulation.

Reversible oxidation of SH groups (see also Harvey and Wieczorek, 1997)

The catalytic A-subunits of eukaryotic V-ATPases carry three highly conserved cysteines, homologous to Cys261, Cys284 and Cys538 of yeast (Taiz *et al.* 1994). The first cysteine occurs in the glycine-rich nucleotide-binding loop, the second is located directly downstream from the loop, and the third occurs near the C terminus of subunit A. On the basis of the complete digestion of the *N*-ethylmaleimide (NEM)-labelled subunit A of clathrin-coated vesicles by V8 protease, followed by amino-terminal sequencing of the labelled fragment, Feng and Forgac (1992) showed that the first cysteine is likely to be the residue responsible for the NEM-sensitivity of the V-ATPase. Corroborating this interpretation, Taiz *et al.* (1994) showed for the yeast enzyme that mutant subunits lacking the first cysteine were no longer susceptible to NEM. This first cysteine has also been shown to form an intra-subunit disulphide bond with the C-terminal cysteine (Feng and Forgac, 1994). Although this potential for disulphide bond formation may be the rule for most V-ATPase subunits A, a novel isoform, generated by alternative splicing and lacking the first cysteine, was isolated from chicken osteoclasts (Hernando *et al.* 1995). This isoform may not be able to form a disulphide bond.

More than half of the V-ATPase in native clathrin-coated vesicles exists in a reversibly inactivated disulphide state, implying that there is an equilibrium between reduced and oxidized states *in vivo* (Feng and Forgac, 1992). The oxidation state of sulphhydryl groups and its importance for the regulation of enzyme activity have also been highlighted for V-ATPases in plant cells and in *Neurospora crassa* (Hager and Lanz, 1989; Dschida and Bowman, 1995). For instance, the *Neurospora* V-ATPase is protected by diverse reducing agents, whereas oxidizing agents such as nitrate cause the dissociation of the V_1 domain (Dschida and Bowman, 1995). Also, the cytosolic V_1-ATPase isolated from tobacco hornworm midgut (Gräf *et al.* 1996) retains its Mg^{2+}-dependent activity in the presence of methanol only if β-mercaptoethanol is added to the preparation (M. Huss and H. Wieczorek, unpublished results). In addition to the three conserved cysteines in subunit A, one conserved cysteine in subunit B (Novak *et al.* 1992, and references herein) may be involved in the reversible formation of disulphide bonds. Finally, the three conserved cysteines occurring in the 40 kDa subunit of the membrane V_o domain (Melnik and Bowman, 1996, and references herein) are additional candidates for the regulation of V-ATPase activity; one reasonable speculation is that their redox state might be responsible for the stable link between the V_1 and V_o domains of the holoenzyme.

Although there seems to be ample evidence that reversible disulphide bond formation may be a means of V-ATPase regulation, there is so far no evidence for a physiological situation affecting the equilibrium between the oxidized and the reduced state of the V-ATPase. The cytoplasm is a highly reducing environment and, therefore, one would expect that endomembrane V-ATPases localized there would be in the active, reduced state. However, it is unlikely that the redox environment is the same at the cell surface as it is in the cytoplasm. In all epithelial cells possessing plasma membrane V-ATPases, mitochondria occur in abundance near the V-ATPase-carrying membranes. In certain insect epithelia, such as the Malpighian tubules of the bloodsucking bug *Rhodnius prolixus*, mitochondria even move to the apical plasma membrane when ion transport is induced (Bradley and Satir, 1981). The close proximity of mitochondria and ion-transporting membranes appears to make sense as, by this means, ATP is made readily available. Harvey and Wieczorek (1997) have speculated that mitochondria may also act as redox machines since they remove oxygen and thus assist in producing a highly reducing microenvironment.

Activators and inhibitors

V-ATPase activity is regulated by the interaction with both activatory and inhibitory proteins. A 35 kDa cytosolic activator from bovine kidney (Zhang *et al.* 1992*b*) and a 6 kDa, evidently membrane-bound, activator from bovine brain (Xie *et al.* 1993) have already been purified. Both proteins were found to be heat-stable and trypsin-sensitive; stimulation of V-ATPase activity was obtained only under acidic conditions. In contrast to the kidney protein, the brain protein stimulated the V-ATPase asymmetrically, evidently exerting its influence from the inside of the vesicles. Since the acidification of organelles appears not to be at thermodynamic equilibrium (Nelson, 1992), an activator could regulate V-ATPase activity and proton pumping kinetically.

An inhibitory 6 kDa protein was isolated from bovine kidney cytosol (Zhang *et al.* 1992*c*). In contrast to the activator proteins, the inhibitor was heat-labile, showed a pH optimum above 7.5 and exhibited a Hill coefficient of approximately 1.5, suggesting a requirement for dimerization. The latter conclusion was supported by the finding that the active fraction from gel filtration appeared at an apparent molecular mass of 12–16 kDa. The mechanisms by which regulatory proteins affect V-ATPase activity remain unresolved, and their regulatory properties *in vivo* are still uncertain.

Sorting to target membranes

Sorting of V-ATPases to a target membrane helps to control the density of V-ATPase and thus the proton transport capacity of the membrane. Three aspects of sorting merit discussion: sorting of V-ATPase-bearing, intracellular vesicles to the plasma membrane, differential sorting of subunit isoforms to specific target membranes, and the recognition of V-ATPases by adaptor proteins in the target membrane.

Sorting of intracellular vesicles to the plasma membrane

The turtle bladder and the mammalian kidney are model organs in which sorting of V-ATPase-containing intracellular vesicles to the plasma membrane has been investigated. In the apical membrane of kidney proximal tubule cells, an H^+-transporting V-ATPase contributes to urinary acidification (Aronson, 1983; Sabolic *et al.* 1985; Brown *et al.* 1988). The V-ATPase is located at the base of the microvilli and appears to be recycled, depending on the tubular pH (Schwartz and Al-Awqati, 1985). In the kidney cortical collecting duct and in the turtle urinary bladder, acidic intracellular vesicles studded with V-ATPase particles are inserted into the apical membrane during acidosis (Gluck *et al.* 1982; Schwartz and Al-Awqati, 1985; Brown *et al.* 1987, 1988). Two morphological types of intercalated cells occur in the cortical collecting duct, proton-secreting cells and bicarbonate-secreting cells (Madsen and Tisher, 1986). Using antibodies to the V-ATPase, Brown *et al.* (1988) found apical, basolateral or intermediate labelling in the intercalated cells, supporting the assumption that different cells are responsible for either proton (apical V-ATPase) or bicarbonate (basolateral V-ATPase) secretion. Although the mechanisms leading to the polarized distribution of V-ATPase have not yet been clarified, it seems that microtubules are involved in the sorting of intracellular vesicles. Using antibodies directed to the V-ATPase, a marked diminution in the amount of plasma membrane V-ATPase was found as a response to colchicine treatment; this diminution was paralleled by a marked accumulation of V-ATPase-bearing intracellular vesicles (Brown *et al.* 1991).

Sorting of subunit isoforms to different target membranes

The most obvious mechanism to direct structurally and functionally distinct enzymes to different membranes is the recruitment of different isoforms containing specific targeting signals for the assembly of the V-ATPase. For instance, two isoforms of the V-ATPase subunit A have been identified in carrot (Gogarten *et al.* 1992). By the use of an antisense mRNA complementary to the mRNA encoding one of the two subunits A, it was possible to inhibit the expression of the tonoplast V-ATPase, whereas the amount of enzyme present in the membranes of Golgi-enriched microsomes was unaffected. Also, the isoforms for the yeast 100 kDa subunits, vph1p and stv1p mentioned above, seem to be involved in differential V-ATPase targeting; vph1p localizes specifically to the membranes of vacuoles, whereas stv1p appears to reside in intracellular membranes elsewhere. Nevertheless, both isoforms were capable of substituting for each other in corresponding deletion mutants, implying that they act as functional homologues (Manolson *et al.* 1994). There may be further examples available in the future, since many differentially expressed isoforms have been identified (see Table 1); at present, however, the mechanism of isoform-mediated targeting to specific membranes remains an enigma.

Adaptor proteins in the target membrane

V-ATPase targeting, mediated by the recognition of adaptor proteins acting as membrane-specific signal molecules, has been suggested for the assembly protein AP-2, which is known to be associated with coated pits and vesicles. The AP-2 complex may function as a specific docking site, since the clathrin-coated vesicle V-ATPase associates with, and is phosphorylated by, the 50 kDa subunit of AP-2 (Myers and Forgac, 1993*b*). Furthermore, Liu *et al.* (1994) have shown that the 50 kDa subunit of AP-2 is required for both activity and *in vitro* reassembly of the V-ATPase.

Regulation by signalling molecules

It appears that V-ATPase activity can be influenced by a multitude of extracellular or intracellular signalling molecules. For instance, activated human neutrophils extrude H^+ equivalents when the cells undergo a large burst of metabolic acid generation while their cytosolic pH is maintained within physiological limits. Determination of intracellular alkalization in the presence of specific V-ATPase inhibitors led to the discovery of a plasma membrane V-ATPase that obviously represents one component of the pH regulatory system in activated neutrophils. Since V-ATPase activity became apparent in resting cells only after treatment with phorbol esters or agonists of protein kinase C (PKC), it was suggested that PKC is involved in activation of the V-ATPase (Nanda *et al.* 1992). Evidence for PKC-dependent activation of a plasma membrane V-ATPase has also been reported for murine peritoneal macrophages (Nordstrom *et al.* 1994). However, it is not clear at present whether PKC also accounts for V-ATPase activation under physiological conditions.

Activation of human neutrophils by chemoattractants, such as formyl-Met-Leu-Phe (fMLP), also leads to an increase in bafilomycin-sensitive H^+ extrusion. Since V-ATPase activity is inhibited by treatment with pertussis toxin, the mediation of the fMLP effect *via* a heterotrimeric G-protein was suggested. Investigation of the downstream signalling pathways indicated that tyrosine phosphorylation of several polypeptides

Table 1. *Summary of identified and putative V-ATPase isoforms from various organisms*

Organism	Isoform subunits (additional denomination)	Expression pattern (comments)	Reference
Vertebrates			
Human	Subunit A		
	Isoform VA-68	Ubiquitous	1
	Isoform HO68	Plasmamembrane of osteoclasts	2
	Subunit B		
	Isoform B1 (brain type, HO57)	Ubiquitous HO57 is expressed exclusively in osteoclasts	3 4
	Isoform B2 (kidney type)	Kidney and placenta	5
	Subunit c, proteolipid	? (isoforms suggested by different genomic clones)	6
Bovine	Subunit B		
	Isoform B1 (brain type)	Ubiquitous	7
	Isoform B2 (kidney type)	Kidney cortex and medulla	8
	116 kDa polypeptide		
	Isoform type I	Brain	9
	Isoform type II	Ubiquitous (generated by alternative splicing)	
Rat	Subunit c, proteolipid	? (isoforms suggested by Southern blot)	10
Chicken	Subunit A		
	Isoform A1	Ubiquitous	11
	Isoform A2	Ubiquitous (generated by alternative splicing)	
Fungi			
Yeast	Subunit c, proteolipid	? (functional independence suggested)	12
	Isoform VMA-3		
	Isoform VMA-7		
	95 kDa polypeptide		
	Isoform Stv1p	Probably different intracellular locations	13
	Isoform Vph1p		
Plants			
Carrot	Subunit A	One isoform tonoplast-specific (suggested by antisense mRNA transformation)	14
Psilotum	Subunit A	? (two separate genes)	15
Equisetum	Subunit A	? (two separate genes)	15
Barley	Subunit B	? (two cDNA clones)	16
Arabidopsis	Subunit c, proteolipid	? (several distant genes)	17
Tobacco	Subunit A	? (suggested by Southern blot)	18
	Subunit B	? (suggested by Southern blot)	19
Cotton	Subunit B	? (multiple cDNAs)	20
	Subunit c, proteolipid	(two cDNA clones)	21
	CVA 16.2		
	CVA 16.4	Most prevalent in expanding ovules	
Oat	Subunit A	? (suggested by two-dimensional gel electrophoresis and immunoblot)	22
	Subunit B	? (suggested by immuno-precipitation)	23
	Subunit c, proteolipid	? (four cDNA clones, Southern blot)	24
Maize	Subunit c, proteolipid	? (two isoforms, cloned by RT-PCR)	25

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accompanied stimulation by fMLP, whereas PKC was obviously not involved (Nanda and Grinstein, 1995). Interaction of heterotrimeric G-proteins with a plasma membrane V-ATPase has also been demonstrated in rat kidney medulla, but it is not clear whether the G-protein interacts directly with the proton pump or indirectly *via* a second messenger (Brunskill *et al.* 1994).

Finally, several examples deserve brief mention. Aldosterone mediates activation (Khadouri *et al.* 1989) and angiotensin II mediates inhibition (Tojo *et al.* 1994*b*) of V-ATPase activity in the rat cortical collecting duct. Secretin mediates V-ATPase stimulation in pig intrahepatic bile ductules and in pig pancreatic ductules (Villanger *et al.* 1993, 1995), and interleukin-1 increases V-ATPase activity in murine peritoneal macrophages (Brisseau *et al.* 1996). Nitric oxide is involved in inhibition of V-ATPase in murine peritoneal macrophages as well as in rat cortical collecting duct (Swallow *et al.* 1991; Tojo *et al.* 1994*a*). Finally, spontaneous apoptosis of human neutrophils, preceded by intracellular acidification, is delayed by the granulocyte colony-stimulating factor which causes up-regulation of V-ATPase activity (Gottlieb *et al.* 1995). It should be noted that, in all cases mentioned in this paragraph, the site and mode of action of regulatory factors on V-ATPase activity remain unknown.

Conclusions

Less than 20 years ago, Anraku and colleagues discovered the first V-ATPase in the vacuolar membrane of yeast (Kakinuma *et al.* 1981). As a result of exciting discoveries of similar V-ATPases from a variety of species in animals, plants and fungi, our knowledge of the structure and function of V-ATPases has been rapidly expanding. A bewildering array of mechanisms by which V-ATPases might be regulated have already been identified. These ancient enzymes are thought to have been present almost since the dawn of life. Through the ages, they have adapted to a broad array of physiological roles which range from vacuolar acidification and plasma membrane alkalization to the generation of voltages that drive electrophoretic symport and antiport and the movement of ions through channels. The challenge for the coming decade is to assign specific physiological roles to the regulatory mechanisms discovered by membrane biochemistry and molecular biology.

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