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The Multifunctional *Drosophila melanogaster* V-ATPase Is Encoded by a Multigene Family

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In animals, V-ATPases are believed to play roles in the plasma membrane, as well as endomembrane. To understand these different functions, it is necessary to adopt a genetic approach in a physiologically tractable model organism. For this purpose, *Drosophila melanogaster* is ideal, because of the powerful genetics associated with the organism and because of the unusually informative epithelial phenotype provided by the Malpighian tubule. Recently, the first animal "knockouts" of a V-ATPase were described in *Drosophila*. The resulting phenotypes have general utility for our understanding of V-ATPase function and suggest a screen for novel subunits and associated proteins. Genome project resources have accelerated our knowledge of the V-ATPase gene family size and the new *Drosophila* genes *vhaSFD, vha100-1, vha100-2, vha100-3, vha16-2, vha16-3, vha16-4, vhaPPA1, vhaPPA2, vhaM9.7.1,* and *vhaM9.7.2* are described. The *Drosophila* V-ATPase model is thus well-suited to both forward and reverse genetic analysis of this complex multifunctional enzyme.

KEY WORDS: *Drosophila melanogaster*; V-ATPase; vacuolar proton pump; genome project.

F-ATPases VERSUS V-ATPases

Ion transport has always attracted a defined succession of workers from multiple disciplines. After a transport is characterized physiologically, the polypeptides are characterized biochemically, the relevant genes are cloned, and a structure–function analysis performed by site-directed mutagenesis combined with heterologous expression in a simple system. At this stage, hard-core structural biologists move into the field and seek to crystallize and determine the three-dimensional structure of the proteins.

Although structurally closely related, the F- and V-ATPases are at different stages in the scientific succession. The F-ATPases, or ATP synthases of bacteria, mitochondria, and chloroplasts are now in the realm of structural biology. By contrast, V-ATPases, discovered only relatively recently, are at a much earlier stage in the succession. This is probably fortunate, because they are probably much the more interesting, and their complexity suggests the adoption of additional approaches.

V-ATPases ARE MULTIFUNCTIONAL

The V-ATPases are ubiquitous among eukaryotes (Finbow and Harrison, 1997; Nelson and Taiz, 1989). Originally identified in plant and yeast vacuoles, they are known to acidify a range of intracellular organelles (Mellman, 1992). In addition to their primary role, there is evidence that they may also serve as cytoskeletal anchors to assist in membrane sorting and trafficking.

In animals, however, they play a unique additional role. They are found in plasma membranes of specialized epithelial cells (Baron, 1995; Gluck, 1992; Gluck *et al.*, 1996; Harvey and Wieczorek, 1997; Wieczorek *et al.*, 1991). In such cells, rather than maintain the pH of a tiny vesicle, they perform bulk transport roles. Accordingly, they can achieve remarkable densities in the plasma membrane, forming semicrystalline arrays

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of portasomes, tightly packed in a 10-nm repeating pattern (Harvey *et al.*, 1981). This extraordinarily high density of ATPase implies a rapid rate of ATP turnover, and an appropriately high rate of cell metabolism. Indeed, specialized V-ATPase cells are frequently described as mitochondria-rich in both vertebrate and invertebrate epithelia (Brown and Breton, 1996).

When an organism spends such a large fraction of its gross metabolic product on a single transport process [(in caterpillars, this has been estimated to be as high as 10% of all ATP production (Dow, 1984; Dow and Harvey, 1988)], the question of control becomes critical. For example, once the V-ATPase is implicated in such epithelial systems as the kidney (Brown *et al.*, 1992), it becomes a candidate for control by a number of hormones. This places the V-ATPase on a different level of sophistication (and arguably interest!) from the more prosaic F-ATPases.

Our group considered that the most interesting problems in V-ATPases were those that were unique to the family and that could not be solved by extension from the more advanced work on F-ATPases. In particular, we wanted to study the differences between plasma membrane and endomembrane V-ATPases and the significance of cell-specific transcript selection. We also wanted to study the mechanisms by which this potent transport process is regulated in real tissues. The nature of these questions has governed both our approach and our model organism.

DEDUCING THE FUNCTION OF V-ATPase GENES DEMANDS REVERSE GENETICS

Framing such questions of V-ATPase function requires an appropriate, organotypical context. Ideally one desires to create an organism in which specific changes are made to the expression patterns of particular genes in particular tissues of an otherwise normal animal. Implicitly, this demands reverse genetic and transgenic approaches.

CHOICE OF MODEL ORGANISM

The application of such technologies to V-ATPases is neither new nor unfamiliar to the V-ATPase community. The discovery of new subunits and associated mutations has been hugely expedited by the discovery of the yeast conditional mutant system (Anraku *et al.*, 1992; Kane, 1992; Noumi *et al.*, 1991). Yeast lacking functional V-ATPase can survive at pH 5.5, but not at pH 7.5. Replica-plating of mutagenized yeast thus provides probably the best way to identify V-ATPase subunits. It also provides an ideal test bed for complementation analysis and thus for structure–function analysis. Unfortunately, yeast is a unicellular organism and so its relevance to our main questions is limited.

It is also possible to perform genetics in fungal models such as *Neurospora* and this too has proved useful (Bowman *et al.*, 1988; Ferea and Bowman, 1996). However, the lack of differentiated tissues limits the applicability of the model.

A reverse genetic approach is also possible in plants. Indeed, rather early on, antisense was used to demonstrate that V-ATPase was necessary for healthy plant growth (Gogarten *et al.*, 1992). In such systems, it is possible to address questions of transcript selection and of response to environmental cues. For example, it is known that there are multiple genes encoding the proteolipid subunit c in plants (Sze *et al.*, 1992), that there are further genes encoding the related subunits c′ and c″ in yeast (Hirata *et al.*, 1997), and that expression levels of the c subunit change in response to salt stress and consequent switch to crassulacean acid metabolism (Low *et al.*, 1996).

However, the questions we wish to address are specific to animals. Here, there is a defined set of genetic models with the transgenic and knockout capabilities associated with reverse genetics. These also tend to be organisms for which genome projects are underway, with further advantages discussed below. Such organisms include mouse, zebra fish, *Drosophila melanogaster*, and *Caenorhabditis elegans*. At first sight, the mouse might be considered the ideal such genetic model, because its body plan resembles that of humans most closely. However, the costs of rearing mice are very high, and the genetics relatively inflexible. While it is relatively straightforward to generate precise genetic knockouts by homologous recombination, the phenotypes of such mutations are likely to be embryonic lethal mutations with a lethal phase too early to be informative. The technology for performing conditional genetic manipulations (e.g., to knock out a gene at a particular stage in the life cycle) is only just becoming available in mouse.

Of the remaining models, we have chosen *Drosophila* as providing probably the best balance of genetic power and relevance to the broad spectrum of animals, including humans.

DROSOPHILA MELANOGASTER AS A GENETIC MODEL

Drosophila has one of the best characterised genetic maps of a higher organism (Rubin, 1988). Since the turn of the century, genetic loci have been mapped by recombination and mutant lines have been preserved for posterity. With the discovery of the salivary giant (polytene) chromosomes, it became possible to reconcile the genetic and cytological maps, so that genes could be attributed to particular physical bands. The advent of molecular biology allowed DNA and deduced protein sequences to be attributed to many of these loci and the formal genome projects are now setting these islands of previously characterized DNA into a context of ever more complete multimegabase contigs of genomic sequence (Ashburner and Drysdale, 1994; Bellen and Smith, 1995; Rubin, 1996, 1998).

Reverse genetics, as we have discussed, also requires knockout, transformation, and ectopic expression technologies. In *Drosophila*, these are provided by engineered transposons, particularly, the P-element. DNA with flanking P-element repeats is efficiently taken up into the genome if embryos are microinjected with DNA, together with a source of P-element transposase (Rubin and Spradling, 1982). As engineered P-elements do not themselves contain a source of transposase, they can be mobilized within the genome by crossing in, then out, a genetically marked source of transposase. As the insertion sites of the P-element are essentially random, this provides a route to inactivating any *Drosophila* gene by insertional mutagenesis (Ballinger and Benzer, 1989; Cooley *et al.*, 1988; Kaiser and Goodwin, 1990; Spradling *et al.*, 1995). It is also possible to generate multiple new alleles from an insertional mutant, by selecting for the rare classes of excision event where either the P-element excises incompletely, or it takes with it an adjoining segment of DNA, leaving behind a small deficiency (deletion).

The ability to intervene in specific cell types in a differentiated organism is provided, in principle, by placing a genetic construct of choice under the control of the relevant promoter and injecting embryos with the resulting construct. However, this requires that clean promoters for the region in question must previously have been characterized. In *Drosophila*, however, a generic technology side-steps this requirement. The GAL4–UAS binary enhancer trap technique allows gene expression to be driven by the yeast UAS promoter, which is activated in *trans* by a GAL4 enhancer detector. This provides the toolbox both to modulate expression levels and to introduce transgenes in a very precise manner (Brand and Perrimon, 1993).

DROSOPHILA PHENOTYPE FOR REVERSE GENETICS

Reverse genetics also requires a phenotype relevant to the gene under study. In *D. melanogaster*, this is provided by the Malpighian (renal) tubule. Although the smallest such insect epithelium yet studied, its transport physiology is relatively well defined (Fig. 1). Like most insect epithelia (Wieczorek and Harvey, 1995), this tissue is energized by an apical plasma membrane V-ATPase (Davies *et al.*, 1996; Dow, 1994; Dow *et al.*, 1994b, 1998). In this case, we know that the V-ATPase is confined to a particular cell type, the mitochondria-rich, metabolically active principal cell (Davies *et al.*, 1996; Sozen *et al.*, 1997). This is strongly reminiscent of the position in many other animal epithelia, where the plasma membrane V-ATPase is confined to a specialized cell type (Brown and Breton, 1996). This tissue thus provides an ideal

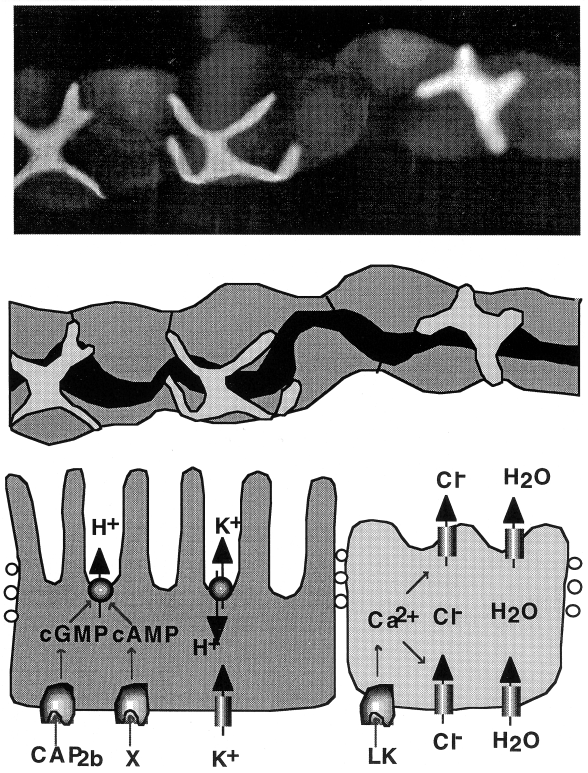

Fig. 1. Overview of the transport physiology of the Malpighian tubule. After O'Donnell *et al.*, 1996.

phenotype for reverse genetic analysis of the multifunctional V-ATPase.

DROSOPHILA MELANOGASTER V-ATPase FAMILY

One of the powerful advantages of selecting an experimental model for which there is a genome project is that gene cloning by homology ceases to be necessary. V-ATPase subunits are highly conserved and are reliably identified in novel sequence by sequence similarity searches. We have thus been able to identify genes encoding all of the presently known V-ATPase subunits, either from genomic sequence, or from expressed sequence tags (ESTs) corresponding to the 5′ ends of random cDNAs from several cDNA libraries. Although our group has characterized several genes by traditional means (Davies *et al.*, 1996; Guo *et al.*, 1996a, b, c), the availability of ESTs has transformed the operation, as cDNAs encoding V-ATPase subunits can usually be identified unambiguously by deduced sequence homology. Further genes have been identified in *Drosophila* genomic sequences; in this case, the chromosomal localizations are also revealed. The power of the genome projects is attested by the data shown in Table I, which represent (to our knowledge) the first description and naming of the *D. melanogaster* genes: *vhaSFD*, *vha100-1*, *vha100-2*, *vha100-3*, *vha16-2*, *vha16-3*, *vha16-4*, *vhaPPA1*, *vhaPPA2*, *vhaM9.7-1*, and *vhaM9.7-2*.

In general, cyberscreening for DNA will not always work. Searches may not recognize genes that are not conserved at their 5′ ends and the cDNAs may never be cloned if the mRNA transcripts are originally of low abundance. However, the relative abundance of V-ATPase message means that there are multiple hits on subunits corresponding to each of the known V-ATPase subunits (Table I). With time, the completion of the genome project will allow us to identify genes encoding even very rarely transcribed subunits.

From this table, several observations can already be made. Some subunits appear to be represented by a single gene, and this is borne out, for example, in the case of *vha55*, by genomic Southern analysis. However, multiple transcripts of the same gene may then be observed (Davies *et al.*, 1996). Where there are several genes encoding a single subunit, there seems to be a highly expressed ubiquitous subunit (which we have tried to number first in the series), and genes with head-enriched and body-enriched transcripts. Examples in the table are provided by *vha67*, *vha100*, and *vha16*. The head–body division is significant; heads are enriched for (indeed, include little other than) nervous tissue and thus V-ATPases play an endomembrane role. By contrast, bodies contain effectively all the V-ATPase energized epithelia of the organism. Our present model is thus that particular genes tend to be coexpressed to make distinct classes of V-ATPase holoenzyme.

For those subunits encoded by multigene families, there is sometimes a gene whose expression cannot be detected, even by reverse transcriptase–polymerase chain reaction (RT–PCR). Pseudogenes are rare in *Drosophila* (Petrov and Hartl, 1997), so the data suggest that the latter class of genes may have very specific and narrow expression.

As the genes are all encoding subunits of the same holoenzyme, one might expect to observe similarities both in their transcripts, expression levels, and genomic organisation. For example, there is consistency in the length of the 5′ and 3′ UTRs between subunits, although the 5′ UTRs in the genome project ESTs are significantly longer than those reported previously in both *Drosophila* and other organisms. The latter probably reflects the quality of the cDNA libraries selected by the Berkeley *Drosophila* Genome Project (BDGP). These comparisons, and those of translational start and polyadenylation consensus (Table I) take on added interest because they are of subunits of a multisubunit enzyme complex with a defined stoichiometry.

It is surprising to note the relative numbers of EST “hits” on each subunit. One might *a priori* expect a rough stoichiometry between mRNAs of different subunits, perhaps with a slight excess of heavier subunits, as these take longer to translate. Consistent with this, the multiple copy subunits A, B, and c have 13, 16, and 39 hits, compared with 20 for vha100. However, some other subunits are also very highly represented, for example, subunit C at 13, SFD at 14, E at 14, and AC39 at 27. By contrast, some other subunits that one might expect to see as single copy in the holoenzyme are extremely rarely found in the EST library, for example, M9.7 (two times), PPA1 (three times) and F (one time). While an EST-based strategy for mapping expression levels is a relatively blunt tool, these data raise some interesting questions about the relationship between mRNA levels and protein stoichiometry.

Table I. Drosophila Melanogaster V-ATPase Gene Family

Subunit	Yeast homolog	Copy number	Genes	Location	ESTs available	Genbank accession	Transcript size (kb)	5′UTR	3′UTR	Lethal mutation?	Start consensus	Poly-A consensus	Reference
V_1 head group													
A	*vma1*	3	*vha67-1*,	34B	13	P48602	2.7	93	644	Yes, embryonic/ early larva	GACC**ATG**C	AATAAA (20 bp)	Guo *et al.*, 1966a y. Guo, unpublished
			vha67-2,	34B									
			vha67-3	34B									
B	*vma2*	1	*vha55/SzA*	87C2-3	16	P48602	2.8, 2.5, 2.0, 1.8	86	1019	Yes, embryonic/ sublethal	CAAG**ATG**A	AATAAA (21bp)	Davies *et al.*, 1996
SFD	*vma13*	1	*vhaSFD*	36B1-2	14	AA439742		132			CAAA**ATG**A		This work
C	*vma5*		*vha44*		13	AF006655		156	506	Yes, second instar	CAAG**ATG**A	AATAAA (28 bp)	Harvie and Bryant, 1996
D	*vma8*	1	*vha36/l(2)k07207*	52A9-11	6	AA696459		91			CAAA**ATG**T		This work
E	*vma4*	1	*vha26*	83B	14	U38951	2.3	77	1307		CAAA**ATG**	AATAAA (26 bp)	Guo *et al.*, 1996c
F	*vma7*	1	*vha14*	52B	1	Q24583	0.65	42	160		CGAA**ATG**G	incomplete 3′	Guo *et al.* 1996b
V_0 transmembrane domain													
100 kDa	*vph1*	3	*vha100-1*,		10	AA440638		102			CACA**ATG**G		This work
			vha100-2,		10	AA978406		206			CAAG**ATG**G		This work
			vha100-3	56F6-13	0	AC004322							This work
AC39	*vma6*		*vhaAC39*		27	AI134340		242			CAAG**ATG**A		This work
c	*vma3*	4	*vha16-1 (ductin)*,	42B1-2	39	P23380		246	516	Yes, embryonic	CAAA**ATG**T	ATTAAA (30 bp)	Meagher *et al.*, 1990
			vha16-2,	68C	0	AC004438							This work
			vha16-3,	68C	0	AC004438							This work
			vha16-4	53D	0	AC004641					AAAT**ATG**G		This work
PPA1	*PPA1*	*2*	*vhaPPA1-1*,	88D-E	3	AA539661		86		Yes	CAAG**ATG**G		This work
			vhaPPA1-2	88D-E	0	AC005721					AAAG**ATG**A		This work
M9.7		2	*vhaM9.2-1*,	64A-B	1	AA735493		52			AATC**ATG**G		This work
			vhaM9.2-2		1	AA540407		81			CAAA**ATG**G		This work

CURRENT WORK

Mutational Analyses of the V-ATPase

In reverse genetics, a function is inferred for a gene based on its mutant phenotype. This is thus an option only for genes characterized in a genetic model.

For one of the subunits, it has been possible to prove that a known and well-characterized existing genetic locus (*SzA*) corresponds to the gene encoding a subunit (*vha55*) (Davies *et al.*, 1996). This means that the phenotypes previously described for a relatively large number of mutant alleles (Gausz *et al.*, 1979) can now be attributed to a mutation in a V-ATPase subunit, making *vha55* the first animal "knockout" of a V-ATPase subunit. There are two striking aspects of the mutant phenotype: a variable lethal phase and a Malpighian tubule phenotype. The lethal phase of homozygous deletions of the entire locus is early larval; the embryo hatches, but the larva fails to thrive and dies after a few hours. By contrast, chemical mutagenesis (traditionally assumed to cause point mutations or relatively local genetic alterations) produces a range of mutant alleles, from sublethal to embryonic lethal (Gausz *et al.*, 1979). How can a point mutation be more severe than deletion of the entire locus?

Our model (Fig. 2) is that this represents a dominant negative effect. When an egg is laid, the mother invests a significant amount of V-ATPase protein in each egg. In the homozygous null progeny, no zygotic protein for one subunit is made and so no new holoenzyme can be assembled. Our model implies that the maternal V-ATPase contribution is just sufficient to permit the embryo to hatch, before dilution and turnover bring the levels of protein down to a critical level. However, in a severe point mutant, normally folding, but defective, protein is made and is incorporated into existing holoenzymes. The effect of this is likely to be devastating. Assuming that the rotational model for catalysis recently graphically demonstrated for F-ATPase (Kato-Yamada *et al.*, 1998; Noji *et al.*, 1997; Yasuda *et al.*, 1997, 1998) is also applicable to V-ATPase, then each of the three *vha55* subunits takes part in turn in the reaction and so must be functional if the holoenzyme itself is to function. It is even possible that severe alleles will demonstrate an effect in heterozygotes; this has been reported for some alleles of *vha55* (Gausz *et al.*, 1979). This dominant negative phenotype is characteristic of proteins that assemble to make a cooperative enzyme complex. There is a more general prediction for V-ATPases; in any organism in which V-ATPase mutants are found, it is possible that they will show a dominant negative phenotype and this effect is likely to be most severe for the multicopy subunits A, B, and c.

The other phenotype associated with the original description of *SzA* is a transparent Malpighian tubule (Gausz *et al.*, 1979). With the benefit of knowing that *SzA* corresponds to *vha55*, we can interpret this phenotype as a defect in urinary acidification (Davies *et al.*, 1996). Insects, like birds, employ a uricotelic excretory system, in which nitrogen is excreted as the insoluble uric acid to conserve water. The urate ion is transported in soluble form into the tubule lumen, where it is acidified in order to precipitate uric acid. In normal embryos, uric acid crystals first become visible shortly before hatching. In the absence of sufficient V-ATPase activity, no uric acid crystals form.

If this explanation is correct, then the phenotype should be general, and displayed by null alleles of other V-ATPase subunits. This is the case; the tubule phenotype is shown by null alleles of *vha67-1* (Fig. 3). This means that there is a plausible epithelial phenotype during the lethal phase of the null mutants. In addition, we can predict that the transparent tubule phenotype will be characteristic of mutations in any V-ATPase subunit (or by extension of any protein that interacts with, targets or anchors V-ATPases) in their plasma membrane role. This phenotype may thus provide a classical, "forward" genetic, screen for proteins that are essential for plasma membrane V-ATPase function. As such a screen is not appropriate in yeast, this

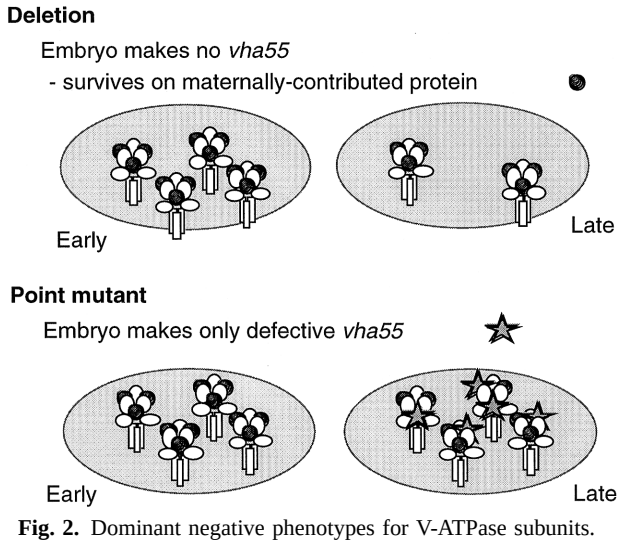

Fig. 2. Dominant negative phenotypes for V-ATPase subunits.

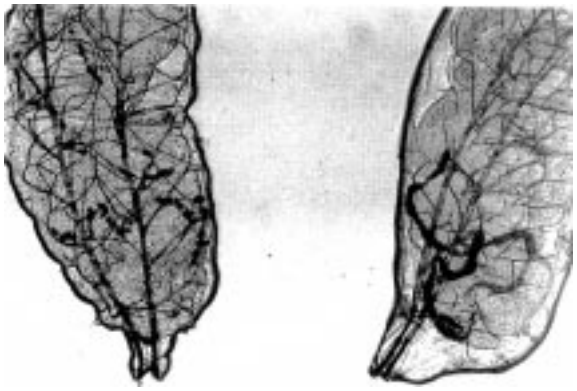

Fig. 3. The homozygous lethal tubule phenotype of *vha* mutants. (Dow *et al.*, 1997).

may prove to be the best way of identifying such proteins.

Regulation of the V-ATPase

Gene Selection

The large number of genes that encode different V-ATPase subunits begs the question of whether expression is tissue-specific. We have some evidence that different genes can be expressed in different tissues. Although the predominant expression pattern of V-ATPase subunits is ubiquitous, with particularly high levels in the labial and antennal palps, the Malpighian tubules, rectum, and uterus (Dow *et al.*, 1997), some P-element insertions appear to report more specific patterns of expression. We are presently trying to study this further by *in situ* hybridization.

Transcriptional

We know that there is far more V-ATPase protein in epithelia in which V-ATPases play a plasma membrane role (Klein, 1992). How is this achieved? The traditional method to assess transcriptional activity is by Northern analysis, but this conflates the rate of transcription with the lifetime of the mRNA; both are potential regulatory mechanisms. *Drosophila* provides a unique opportunity to assess the contribution of transcription rate in isolation. The reporter gene in P-element insertions, *LacZ*, is expressed at levels characteristic of the genomic context in which it is inserted; however, both the *LacZ* mRNA and the β-galactosidase protein (and thus any stability signals they may contain) are invariant. If we thus see high levels of β-galactosidase reporter activity in a tissue, this must be in proportion to the rate at which the reporter gene (and by inference the flanking gene) is expressed. The weight of this finding is increased if several different insertions, or insertions in several subunits, show similar patterns of expression. This is the case. As has been described earlier, insertions in *vha55*, *vha67-2*, and *vha16-1* all show enhanced expression in epithelia where V-ATPases are known to be present at high levels by immunocytochemistry or by pharmacological sensitivity to bafilomycin (Davies *et al.*, 1996; Dow, 1994; Dow *et al.*, 1997, 1998). V-ATPase genes are thus transcribed at a higher rate in tissues where they play a plasma membrane role.

Protein Turnover

The standing crop of V-ATPase holoenzyme could be regulated either by the rate of transcription and translation, or by the rate of turnover of mature protein. In *Drosophila*, we can distinguish the two possibilities genetically, because in heterozygotes for null alleles, the copy number of the gene is halved. In the absence of dosage compensation, this means that transcript levels should also be reduced; if transcription is limiting, then the maximal rate of fluid production by the tubule should be significantly reduced. By contrast, if protein turnover limits the lifetime of protein, the effect should be minimal.

Our preliminary data (Fig. 4) suggests that both mechanisms are acting. Fluid secretion, both resting and stimulated, is significantly lower, but not halved.

Hormonal

Short Term. The hormonal regulation of V-ATPases is of great interest. Despite the small size of the Malpighian tubule, it has been possible to elucidate, in some detail, the hormonal control mechanisms underlying plasma membrane V-ATPase regulation. As in many epithelia, the V-ATPase is expressed at high levels in a specialized cell type, the primary cell, whereas the chloride shunt conductance and water transport is confined to the secondary, or stellate cell (Dow *et al.*, 1997, 1998; O'Donnell *et al.*, 1996, 1998; Sozen *et al.*, 1997). Neuropeptides have been identified which act separately on the V-ATPase and chloride

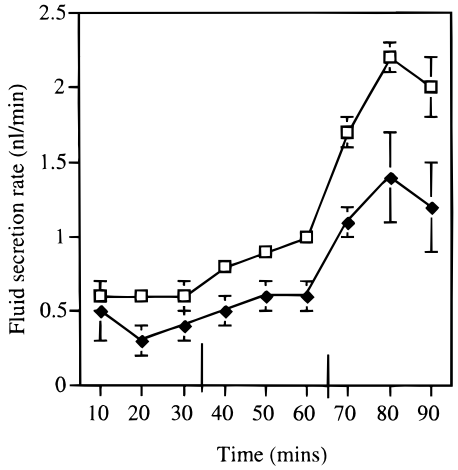

Fig. 4. Effect of hemizygosity for *vha55* on fluid secretion. Fluid secretion rates of wild-type Oregon R (open squares) and *vha55*^{j2eg}/TM3 hemizygotes (black diamonds) were measured according to standard techniques. Cyclic AMP was added to 1 m*M* final at 30 min, and leucokinin I added to 1 μ*M* final at 60 min. Data are presented as mean ± SEM ($N = 6$ tubules) (V. Pollock and J. A. T. Dow, unpublished 1999).

shunt conductances in the two cell types. Cardio accelleratory peptide 2b (CAP_{2b}), an endogenous *Drosophila* peptide first characterized in *Manduca sexta*, acts through intracellular calcium, only in the principal cell, to stimulate an intrinsic nitric oxide synthase to release nitric oxide (Davies *et al.*, 1995; 1997; Dow *et al.*, 1994a) (Fig. 5). This acts to stimulate a soluble guanylate cyclase, which acts through cGMP-dependent protein kinase ultimately to activate the apical V-ATPase (O'Donnell *et al.*, 1996). The signaling cascade is controlled by a Zaprinast-sensitive phosphodiesterase and an okadaic acid-sensitive protein phosphatase (Dow *et al.*, 1994a). The end effect of cGMP-mediated signaling is to render the apical lumen more electropositive; this can only be achieved by the apical electrogenic ATPase, so we can be confident that this is the final target of the cascade (O'Donnell *et al.*, 1996). A similar cascade exists for cyclic AMP-mediated signaling, although our characterization of this pathway is, at present, less complete.

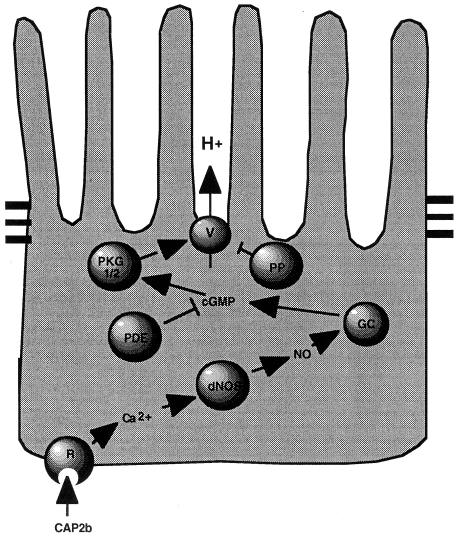

Fig. 5. The principal cell CAP_{2b}/NO/cGMP signaling pathway and its role in controlling apical plasma membrane V-ATPase.

Long Term. There is also evidence for temporal control of expression of some, but not all, subunits. Insects undergo a profound change in their physiology when they molt. In *Manduca sexta*, both ion transport and the responsible V-ATPase activity are lost precipitously before the molt and recover rapidly shortly after the molt is completed (Sumner *et al.*, 1995). This is accompanied by the loss of V_1 headgroups from the plasma membrane into the cytoplasm (Sumner *et al.*, 1995). A similar model has since been identified in other organisms, suggesting that it may represent a general V-ATPase control mechanism. In *Drosophila* during pupation, a period when epithelial transport is also believed to be shut down (Ryerse, 1978), expression levels of some genes, but not others, fall. Genes affected include the V_1 headgroup subunits *vha67-1* and *vha26*, but not *vha14* (Guo *et al.*, 1996c). The hormonal interplay between ecdysone and juvenile hormone that produces molts with specific characters is well understood, especially in *Drosophila*. Clearly, the larval–larval molts and pupal stages provide excellent contexts in which to study genomic regulation of V-ATPase expression.

CONCLUSION

The results described here illustrate the utility of adopting a model in which genetic manipulations can be combined with physiological analysis. Recent work has provided us with the first null alleles of a V-ATPase in an animal and the resulting phenotypes have provided us with general insights about V-ATPase function. Genome projects have provided us with valuable information about the size and complexity of the V-ATPase gene family and the genetic toolbox pro-

vided by the GAL4/UAS binary system provide exquisite tools for the functional analysis of a complex protein in the organotypical context appropriate to a plasma membrane enzyme.

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