The Journal of Experimental Biology 205, 1059–1067 (2002)
Printed in Great Britain © The Company of Biologists Limited 2002
JEB4072

Expression of *Manduca sexta* V-ATPase genes *mvB*, *mvG* and *mvd* is regulated by ecdysteroids

Stephan Reineke, Helmut Wieczorek and Hans Merzendorfer*
Department of Biology/Chemistry, Division of Animal Physiology, University of Osnabrück, 49069 Osnabrück, Germany
*Author for correspondence (e-mail: merzendorfer@biologie.uni-osnabrueck.de)

Accepted 7 February 2002

Summary

V-ATPases are complex proteins consisting of a peripheral, ATP-hydrolysing V_1 complex and a membrane-bound H^+-translocating V_o complex. The plasma membrane V-ATPase from the tobacco hornworm (*Manduca sexta*) midgut is made up of eight different V_1 and four different V_o subunits. During starvation and moulting, V-ATPase activity decreases as a result of the dissociation of the V_1 complex from the V_o complex. To determine whether subunit biosynthesis is reduced during periods of enzyme inactivity, we measured the transcript levels and transcriptional activities of V-ATPase genes. Northern blots revealed the downregulation of almost all V-ATPase transcripts during starvation. During moulting, transcript levels of the three V-ATPase genes examined, *mvB*, *mvG* and *mvd*, also decreased, and this decrease was negatively correlated with the titre of 20-hydroxyecdysone (20-HE) and positively correlated with the titre of juvenile hormone (JH). To test the biological significance of these correlations, we injected both hormones into feeding larvae and measured transcript levels several hours later. A short-term increase and a long-term decrease in levels of mRNA were observed after 20-HE injection, whereas JH injection had no significant effect. Immunohistochemical studies of the midgut epithelium revealed that 20-HE injection led to changes in goblet cell morphology and in the subcellular distribution of the V_1 complex comparable with the situation during the moult and during starvation. Reporter gene assays in *Sf*21 cells using *mvB*, *mvG* and *mvd* promoters to initiate transcription of firefly luciferase led, after incubation of the cells with 20-HE, to results comparable with those obtained in the injection experiments. These findings suggest that putative ecdysone-responsive elements are present in all three promoters. Taken together, our results suggest that the expression of V-ATPase genes is controlled in a coordinated manner by ecdysteroids.

Key words: vacuolar H^+-translocating ATPase, V-ATPase, *Manduca sexta*, promoter, transcription, ecdysteroid.

Introduction

Vacuolar ATPases (V-ATPases) are multisubunit enzymes that consist of a peripheral, ATP-hydrolysing V_1 complex and a membrane-bound H^+-translocating V_o complex (for a review, see Forgac, 2000). These proton-pumping enzymes are found ubiquitously in endomembranes of eukaryotic cells. Furthermore, they are also found in the plasma membranes of a variety of eukaryotic cells (Wieczorek et al., 1999).

The plasma membrane V-ATPase from the midgut of the tobacco hornworm *Manduca sexta* (Lepidoptera, Sphingidae) is made up of eight different V_1 and four different V_o subunits with presumed stoichiometries of $A_3B_3CDEFG_3H$ and ac_6de, respectively (Merzendorfer et al., 2000). In the larval midgut, the V-ATPase is present at high levels in the apical membrane of goblet cells, where it exclusively energizes all secondary active transport processes across the epithelium. As a result of the absence of functional anion channels, a transmembrane voltage in excess of 250 mV is generated, and this drives the electrogenic exchange of H^+ for K^+ (Wieczorek et al., 1991). The combined action of the V-ATPase and the $K^+/2H^+$ antiporter leads to net K^+ secretion and thus to a transepithelial K^+-motive force that drives the absorption of amino acids *via* K^+-coupled amino acid symporters (Castagna et al., 1998). Because of the stoichiometry of the antiporter, the gut lumen is alkalized to a pH of more than 11, the most alkaline value produced by any biological system (Azuma et al., 1995).

Regulation of V-ATPases may encompass many diverse mechanisms such as oxidation of -SH groups or control *via* activator or inhibitor proteins (Merzendorfer et al., 1997a). During the larval/larval moult and periods of starvation, the insect plasma membrane V-ATPase is downregulated by the reversible dissociation of the enzyme into its V_1 and V_o complexes (Sumner et al., 1995; Gräf et al., 1996). As a result of this disassembly, cytoplasmic levels of the V_1 complex increase, a fact that incidentally allowed its efficient purification and the first structural studies of the V_1 complex (Svergun et al., 1998; Grüber et al., 2000). For economical

reasons, it appears plausible that, during periods when V-ATPase activity is shut down, biosynthesis of V-ATPase subunits is downregulated concomitantly. We have therefore measured the levels of transcripts and the transcriptional activities of several V-ATPase genes. Here, we show that transcript levels of V-ATPase subunits decrease gradually during starvation and the larval/larval moult. Moreover, we provide evidence that the control of transcript levels for V-ATPase genes is mediated by ecdysteroids, a class of steroid hormone known to control larval development.

Materials and methods

Construction of a genomic library

To isolate upstream regions of V-ATPase genes, a genomic library was constructed from *Manduca sexta* (L.) larval midgut. Genomic DNA (100 μg) was purified according to Sambrook et al. (1989), partially cleaved with *Mbo*I (0.025 units μg^{-1}, 1 h at 37 °C) and extracted with phenol/chloroform. The 5′ ends were filled in with dATP and dGTP in the presence of Klenow polymerase. The DNA fragments were separated on a continuous sucrose gradient (10 % to 40 % sucrose, 10 mmol l^{-1} Tris-HCl, 10 mmol l^{-1} NaCl, 1 mmol l^{-1} Na-EDTA, pH 8.0) by centrifugation at 20 °C for 22 h at 22 000 ***g***. Fractions containing DNA fragments of 15–23 kb were dialyzed against a buffer consisting of 10 mmol l^{-1} Tris-HCl (pH 8.0) and 1 mmol l^{-1} Na-EDTA. After volume reduction to 500 μl by 2-butanol extraction, DNA was precipitated with 2 mol l^{-1} ammonium acetate and ethanol. The pellet was resuspended in 10 μl of water. The DNA fragments were ligated, according to the manufacturer's protocol, into λ-Fix II vector arms (Stratagene) which had been predigested with *Xho*I and filled with dCTP and dTTP. Packaging of 0.7 μg of λ-DNA was performed with the Gigapack II packaging extracts from Stratagene following the recommended protocol. The titre of the primary library was 3×10^6 plaque forming units (pfu), and the phasmids contained genomic inserts of 12–20 kb. After amplification of the library according to Sambrook et al. (1989), the titre was determined to be 5×10^5 pfu μl^{-1}.

Cloning of the 5′ regions of mvB, mvG *and* mvd

To isolate the gene encoding V-ATPase subunit B (*mvB*), the *Manduca* gDNA library was screened by a plaque-hybridization procedure (Sambrook et al., 1989). A digoxigenin-labelled DNA hybridization probe was generated by polymerase chain reaction (PCR) using the cDNA primers 5′-ATGGCAAAAACCCTATCCGC-3′ (positions 57–76) and 5′-CATGATCATCCAGCACAGAC-3′ (positions 678–659) and, as a template, the cDNA clone encoding V-ATPase subunit B (Novak et al., 1992). Hybridization and stringency wash steps were carried out at 68 °C. Repeated isolation of positives plaques led to two independent phage clones, λ-mvB_1 and λ-mvB_2. After Southern blotting of the phage DNA, which had been isolated from λ-mvB_2 according to Sambrook et al. (1989) and cleaved using different restriction enzymes, a 4.1 kb *Nco*I fragment containing *mvB* upstream sequences was identified. This DNA fragment was cloned into pBluescript KS(–), which had been modified before by blunt-end ligation of an *Nco*I-linker (Stratagene) into the *Sma*I site of the multiple cloning site. Cloning of the upstream regions of *mvG* and *mvd* was performed using a similar approach.

The primer sets employed for probe synthesis were 5′-CTAAGATCGATGCTGAGACC-3′/5′-TGTCATGTGAC-AAAGTGGCGCT-3′ (cDNA positions 256–275/523–502 of subunit G) (Lepier et al., 1996) and 5′-TTACTTGAA-CTTGGTGCAAT-3′/5′-TTCAATGTACCTACATACAG-3′ (cDNA positions 168–188/1644–1624 of subunit d) (Merzendorfer et al., 1997b), respectively. Hybridization screening at 68 °C led to the isolation of several independent phage clones for both genes (λ-mvG/d_x). Following restriction pattern analysis, Southern blotting and hybridization with appropriate probes, a λ-mvG_1-derived *Cla*I-fragment of 4 kb and a λ-mvd_1-derived *Pst*I/*Sac*I-fragment of 1.7 kb were identified as regions carrying corresponding upstream gene sequences. Both fragments were subcloned in pBluescript KS(–) and sequenced.

Reporter gene assays

The promoter activities of the *mvB*, *mvG* and *mvd* 5′ upstream regions were determined with the Dual Luciferase Reporter Assay (Promega) using the pRL-CMV vector as an internal standard for transfection efficiency. To construct the reporter gene plasmids, PCR fragments comprising a region of 973 bp upstream of the start codons of *mvB*, *mvG* and *mvd* were ligated into the *Sac*I and *Hin*dIII cloning sites of pGL2-basic vector. Amplification was performed in the presence of the pBluescript KS(–) plasmids containing genomic fragments of *mvB*, *mvG* and *mvd*. Primer pairs containing 6 bp nonsense nucleotides at their 5′ ends followed by either a *Sac*I site in the case of the forward primers or a *Hin*dIII site in the case of the reverse primers were as follows: 5′-TACTCAGAGCTC-AAATTTGGCATAGGCATGGC-3′/5′-TACTCAAAGCTA-TAGGGTTTTTGCATT-3′ for *mvB*; 5′-TACTCAGAGCTCT-GCGAATCTTCCGTCAC-3′/5′-TACTCAAAGCTTCATGT-GTCTGACTCGCCATT-3′ for mvG; and 5′-TACTCAA-GCTCAGCATATCTCGTTTTTTCGA-3′/5′-TACTCAAGA-TCTAAATATGCAGCCCTTT-3′ for *mvd*. After ligation of the *Sac*I/*Hin*dIII-digested PCR fragments, the resulting *mvB*/*G*/*d*-pGL2-basic constructs were checked by sequencing. The corresponding reporter gene plasmid and the pRL-CMV control plasmid (2 μg of each) were co-transfected into *Sf*21 cells using 5 μl of Cellfectin (Life Technologies) and an incubation period of 12 h at 27 °C. Further steps of the assay were performed according to the manufacturer's manual. Luminescence signals were measured in a Lumat LB 9507 luminometer (EG&G Berthold, Germany) and specified as relative light units normalized to the *Renilla* luciferase expression of pRL-CMV.

Determination of transcriptional start sites

RNA hybridization probes complementary to the 5′ upstream regions of *mvB*, *mvG* and *mvd* were synthesized by

in vitro transcription, as described previously (Merzendorfer et al., 2000). The 973 bp upstream fragments of the *mvB/G/d*-pGL2-basic constructs were excised and ligated into pBluescript KS(+) using the restriction enzymes *Sac*I and *Hin*dIII for the *mvB* and the *mvG* constructs, respectively, and *Sac*I and *Bam*HI for the *mvd* construct. The resulting plasmids (1.5 μg of each) were linearized with *Sac*I at their 3′ cloning sites, purified on an agarose gel and used as template DNA for *in vitro* transcription, which was performed with T3 polymerase at 38 °C for 40 min in the presence of 3 MBq of [α-^{32}P]CTP (Amersham Pharmacia Biotech; 30 TBq mmol l^{-1}). Subsequently, the template DNA was degraded by DNase I (Roche Diagnostics) treatment. Nucleotides that had not been incorporated were separated from the labelled transcripts by centrifugation through Sephadex G25 spin columns (Sambrook et al., 1989). RNA integrity was checked by agarose gel electrophoresis and Radient Red (BioRad) staining.

Radioactive transcripts (9×10^5 cts min^{-1}) and 6 μg of the target mRNA isolated from the midgut of fifth-instar larvae were coprecipitated with sodium acetate and ethanol and resuspended in hybridization buffer consisting of 40 mmol l^{-1} Pipes (pH 6.4), 400 mmol l^{-1} NaCl, 1 mmol l^{-1} EDTA and 80 % formamide. Hybridization was carried out overnight at 50 °C. Single-stranded RNA was degraded by treatment with RNase A and T1 at 30 °C for 30 min. After inactivation of the RNase by proteinase K treatment, double-stranded RNA hybrids were extracted with phenol/chloroform/isoamyl alcohol and coprecipitated with 5 μg of yeast tRNA. The denatured RNA probes were separated on a 6 % polyacrylamide gel containing 7 mol l^{-1} urea. Autoradiography was performed by exposing the gel to X-ray film (Kodak X-omat AR) for 14 days at –70 °C using the BioMax TranScreen HE intensifying screen system (Kodak).

Immunohistochemistry

Larval midguts were dissected, and the gut contents were removed. After excision of the longitudinal muscles, the tissue was stretched, cut into small pieces of approximately 5 mm^2 and fixed for 90 min at room temperature in PLP fixative [0.1 mol l^{-1} sodium *m*-periodate, 75 mmol l^{-1} L-lysine, 2 % (w/v) paraformaldehyde in 0.1 mol l^{-1} Sørensen phosphate buffer, pH 7.4]. Tissue embedding, cryosectioning and immunostaining were performed as described previously (Klein et al., 1991). To label the V_1 complex, cryosections were treated with the monoclonal antibody 221-9 to subunit A of *Manduca sexta* V-ATPase (Klein et al., 1991). Visualization of the primary antibody was performed with Cy3-conjugated anti-mouse F(ab′)$_2$ fragments (Sigma). To test for nonspecific binding of the secondary antibody, control reactions were carried out without primary antibodies. The sections were covered with Mowiol (Aventis, Germany) and viewed with an Olympus IX70 fluorescence microscope. To visualize Cy3 emission, the SWG filter set (Olympus) and monochromatic excitation at 535 nm were used.

Other methods

Manduca sexta was reared under long-day conditions (16 h:8 h L:D photoperiod) at 27 °C using a synthetic diet for the larvae, modified according to Bell and Joachim (1974). Total RNA and mRNA were prepared from the larval midgut of different developmental stages using Qiagen RNA purification kits according to the manufacturer's protocol. Northern blots were performed as described previously (Merzendorfer et al., 1997b), except for the use of CPD-Star (Roche Diagnostics) as a chemiluminescence substrate. RNA levels were quantified densitometrically using the Fluor-S Multi-Imager and Quantity One software (Biorad). Chemiluminescence signals on X-ray film (Kodak) were scanned, and the intensities were measured in units of optical density×mm^2. Sequencing was performed on both DNA strands using the Sequenase 2.0 Kit (Amersham Pharmacia Biotech) and following published protocols. Several nucleotide sequences were obtained from the custom sequencing service of MWG-Biotech. Hormone treatment of caterpillars was carried out by injection of 20-hydroxyecdysone [20-HE; 40 μl, 5 μg μl^{-1} phosphate-buffered saline (PBS) containing 10 % (v/v) 2-propanol] or juvenile hormone III [JH; 40 μl, 0.5 μg μl^{-1} PBS containing 10 % (v/v) methanol] into the dorsal vessel. Control animals were injected with 40 μl of the solvent.

Results

V-ATPase transcripts are downregulated upon starvation and during the larval/larval moult

During moulting and periods of starvation, midgut transepithelial voltage, an indicator of active K^+ transport, falls to zero as a result of the inactivation of the V-ATPase by reversible disassembly into its soluble V_1 complex and its membrane-bound V_o complex (Sumner et al., 1995; Gräf et al., 1996). For reasons of energetic economy, it would make sense also to stop the biosynthesis of V-ATPase subunits during these periods.

To investigate whether levels of V-ATPase subunit transcripts depend on food intake, we performed a series of northern blots. Total RNA was isolated from 16 h starved and from feeding larvae, dotted onto nylon membranes and hybridized with ssRNA probes for V-ATPase transcripts. Hybridization signals were quantified densitometrically and normalized to the amounts of ribosomal protein S7 mRNA (Jiang et al., 1996). Starvation of fifth-instar larvae (days 2–3) resulted in decreased transcript levels for all the V-ATPase subunits except for subunit D (Fig. 1). Our findings suggest that, upon starvation, both the activity of the V-ATPase and the biosynthesis of most V-ATPase subunits are downregulated.

To address the question of whether levels of V-ATPase transcripts are also downregulated during the moult, we determined the time courses for transcript levels of subunits B, G and d, each encoding a subunit from a different part of the holoenzyme complex: B from the V_1 head, G from the V_1 stalk and d from the membrane-bound V_o portion (Wieczorek et al., 2000). Total RNA was isolated from the midguts of larvae at different fourth- to fifth-instar moulting stages, classified

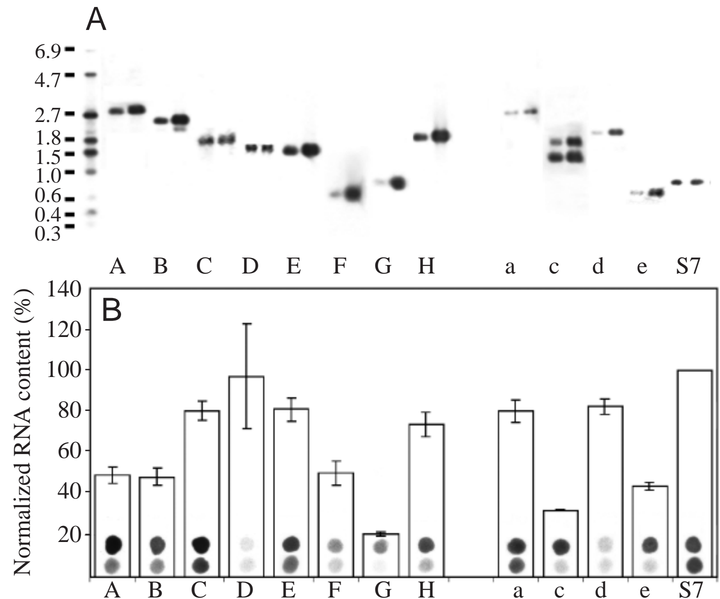

Fig. 1. Transcript levels for V-ATPase subunits from *Manduca sexta* during starvation. (A) Pooled total RNA from the midgut of three fifth-instar larvae (2 μg each) was separated by gel electrophoresis, transferred onto nylon membranes and hybridized with digoxigenin-labelled ssRNA probes specific for the indicated V-ATPase subunits. Labelled transcripts from starved (left) or feeding (right) larvae were visualized by chemiluminescence and autoradiography. The far left column shows molecular mass standards (in kb). (B) Pooled total RNA was dotted onto nitrocellulose and hybridized, and chemiluminescence signals were quantified densitometrically. Values (± S.E.M., N=3) are given as a percentage of signal intensities obtained from feeding larvae and are normalized to the transcript levels of ribosomal protein S7 (Jiang et al., 1996). Representative dots have been inserted in the corresponding columns with RNA from feeding larvae in the upper row and RNA from starved larvae in the lower row. A–H, subunits of the V_1 complex; a–e, subunits of the V_o complex.

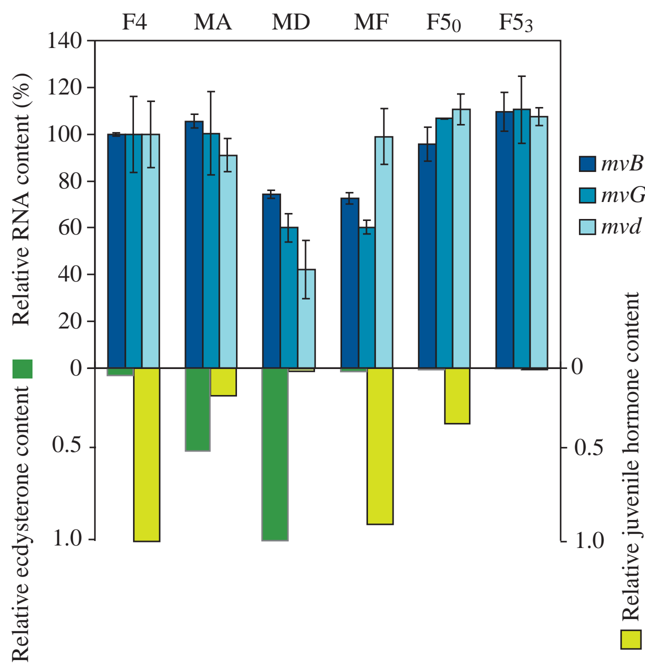

Fig. 2. Transcript levels of genes for V-ATPase subunits B (*mvB*), G (*mvG*) and d (*mvd*) from *Manduca sexta* during the moult. Pooled total RNA from the midgut of three larvae (2 μg each) at the stages indicated was dotted onto nylon membranes and hybridized with digoxigenin-labelled ssRNA probes. Chemiluminescence signals were quantified densitrometrically. In the upper part of the graph, values (± S.E.M., N=3) are given as a percentage of signal intensities obtained from feeding larvae and normalized to the transcript levels of ribosomal protein S7 (Jiang et al., 1996). In the lower part of the graph, published ecdysteroid and juvenile hormone titres are shown at the corresponding moulting stages (Baker et al., 1987; Bollenbacher et al., 1981; Fain and Riddiford, 1975). F4, fourth-instar day 2; MA, moulting stage A; MD, moulting stage D (approximately 20 h after MA); MF, moulting stage F (approximately 20 h after MD); $F5_0$, fifth-instar day 0; $F5_3$, fifth-instar day 3.

according to Baldwin and Hakim (1991). Transcript levels were detected and normalized as described above. The mRNA levels of these subunits decreased during the moult until stage D was reached and increased to control levels when the larvae started to feed again at the early fifth instar (Fig. 2).

Development and metamorphosis of insects are strictly controlled by two major hormone classes; ecdysteroids and juvenile hormones. Increased titres of ecdysteroids such as 20-hydroxyecdysone (20-HE) induce moulting by initiating a regulatory cascade beginning with the activation of primary response genes. In contrast, the function of juvenile hormones (JH) is to prevent metamorphosis and to regulate reproductive maturation in the adult. Interestingly, the changes in transcript levels for V-ATPase subunits B, G and d turned out to be negatively correlated with previously published hormone titres of 20-HE (Baker et al., 1987; Bollenbacher et al., 1981) and positively correlated with those of JH (Fain and Riddiford, 1975; Hiruma et al., 1999). Transcript levels of V-ATPase subunits reached their minima at moulting stage D, when the haemolymph titre of 20-HE exhibits its maximum level. Conversely, V-ATPase transcript levels started to recover in moulting stage F, when JH titres in the haemolymph are highest. Our results suggest that either 20-HE or JH may be responsible for the observed changes in transcript levels of V-ATPase subunits during the moult.

Injection of 20-hydroxyecdysone leads to decreased levels of V-ATPase transcripts and influences midgut morphology

To identify the hormone responsible for the change in RNA levels, we injected either 20-HE or JHIII into the dorsal vessel of fifth-instar larvae (days 2–3). Since haemolymph titres of both hormones are extremely low at this developmental stage (Baker et al., 1987; Bollenbacher et al., 1981; Fain and Riddiford, 1975; Hiruma et al., 1999), injection should mimic the effects of endogenous 20-HE and JH, as has been shown previously (Edgar

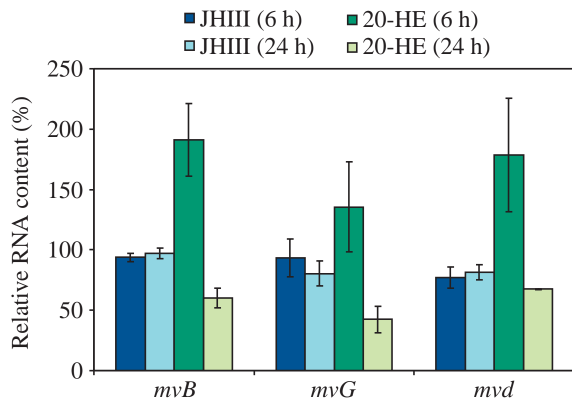

Fig. 3. Effect of 20-hydroxyecdysone and juvenile hormone III on transcript levels of *Manduca sexta* V-ATPase genes *mvB*, *mvG* and *mvd*. 20-Hydroxyecdysone (20-HE) (200 μg; approximately 0.4 mg ml^{-1} haemolymph) or juvenile hormone III (JHIII) (20 μg; approximately 40 μg ml^{-1} haemolymph) was injected into the dorsal vessel of fifth-instar larvae (days 2–3, 2 g body mass). After the indicated period of rearing in the presence of food, total RNA was extracted from the midgut. Pooled total RNA (2 μg) was dotted onto nylon membranes and hybridized with digoxigenin-labelled ssRNA probes. Chemiluminescence signals were quantified densitometrically. Values (± S.E.M., N=3) are given as a percentage of signal intensities obtained from untreated animals and are normalized to the transcript levels of ribosomal protein S7 (Jiang et al., 1996).

et al., 2000; Hewes and Truman, 1994). Caterpillars were exposed for either 6 or 24 h to 200 μg of 20-HE, 20 μg of JHIII or equal volumes of control solution containing the corresponding solvent. They were then dissected, total RNA was isolated from the midgut and transcript levels of subunits B, G and d were quantified as described above. JHIII had, at the most, only a slightly negative effect on transcript levels. In contrast, 20-HE led to a short-term increase and a long-term decrease in transcript levels for all three subunits (Fig. 3).

The results of 20-HE injection are in line with the assumption that ecdysteroids are involved in the regulation of levels of V-ATPase transcripts during the moult. To evaluate the effects of 20-HE injection on midgut morphology and the intracellular localization of V_1 complexes, we performed an immunohistochemical study comparing cryosections of posterior midguts from larvae treated with 20-HE for 24 h with those from moulting (fourth larval moult, stage D), starving (16 h, fifth-instar, days 2–3) and control (feeding, fifth-instar, days 2–3) larvae (Fig. 4). In comparison with midguts from feeding larvae, the midgut cells from 20-HE-treated, moulting and starving larvae appeared to be elongated and the goblet cavities appeared to be reduced in diameter.

To visualize the V_1 complex, we used the monoclonal antibody 221-9 to subunit A of the V-ATPase (Klein et al., 1991). In midguts of feeding animals, the signal for V-ATPase subunit A was almost exclusively located in the region of the goblet cell apical membrane. In contrast, 20-HE-treated, moulting and starved larvae exhibited significantly different staining patterns. In all three cases, the antibody to subunit A labelled the cytoplasm of the goblet cells intensively, indicating detached V_1 complexes, as has been demonstrated previously for moulting and starved larvae using biochemical approaches (Sumner et al., 1995; Gräf et al., 1996). These experiments clearly demonstrated that injection of 20-HE leads to changes in general midgut morphology and in the subcellular distribution of V_1 complexes as they are also observed during moulting and starvation.

Upstream regions of V-ATPase genes mvB*,* mvG *and* mvd *differ in general structure but all contain ecdysone response elements*

The injection experiments suggested that the control of

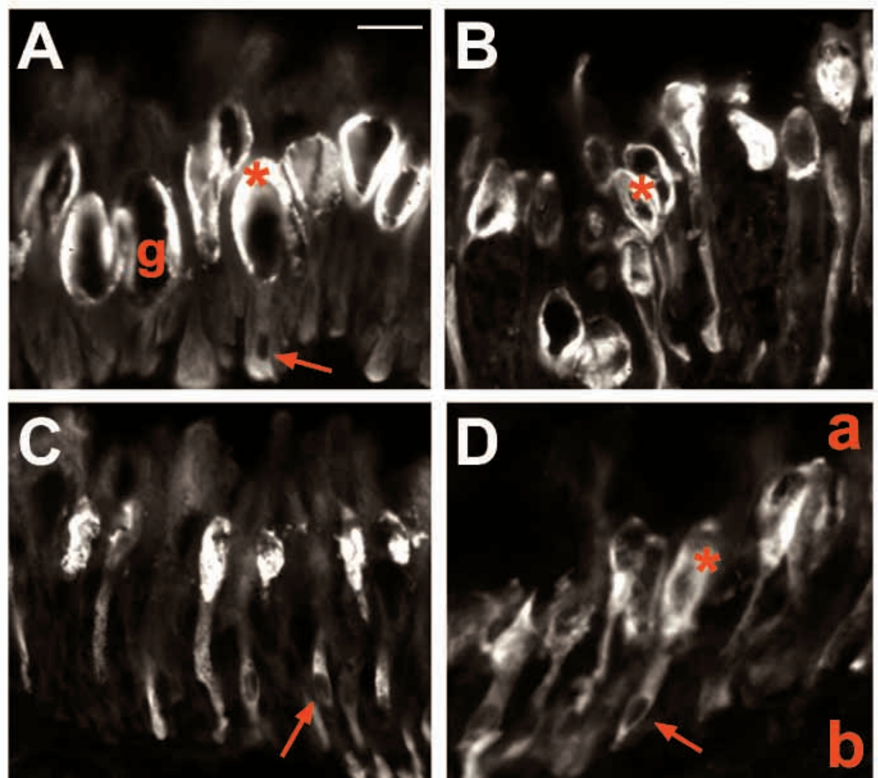

Fig. 4. Immunolabelling of the V_1 complex in midgut goblet cells from *Manduca sexta*. Cryosections from feeding (A; fifth-instar, days 2–3), starving (B; fifth-instar, day 2–3), moulting (C; fourth- to fifth-instar, moulting stage D) and 20-hydroxyecdysone-treated (D; fifth-instar, days 2–3, 24 h after hormone injection) larvae were stained with monoclonal antibody 221-9 to V-ATPase subunit A (Klein et al., 1991). Scale bar, 30 μm; a, apical; b, basal; g, goblet cell cavity; asterisks indicate goblet cell apical membranes; arrows point to goblet cell nuclei.

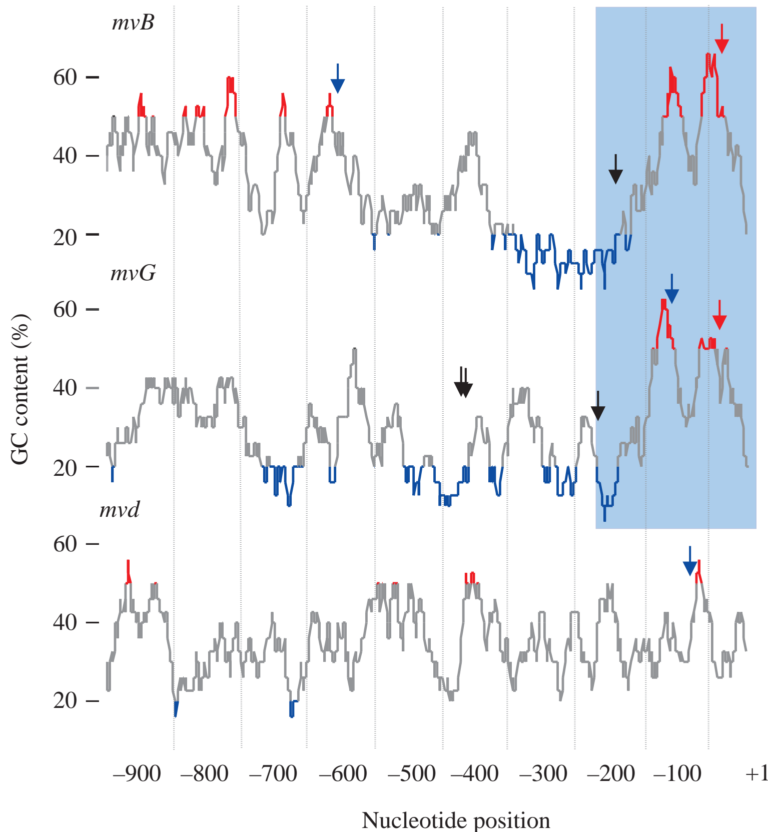

Fig. 5. Overall promoter structures of the *Manduca sexta* V-ATPase genes *mvB*, *mvG* and *mvd*. Hybridization screening of a genomic λ-Fix II library from *M. sexta* led to the isolation of three genes encoding V-ATPase subunits B, G and d. The corresponding 5′ upstream regions were subcloned and sequenced. Fragments of 1 kb are shown, with special emphasis on overall promoter structures in the region close to the translational start site (shaded blue). GC content was determined using a window size of 30 bp; GC contents of more than 50 % are in red, of between 20 % and 50 % are in grey and of less than 20 % are in blue. Black arrows, canonical TATA boxes; red arrows, cAMP-responsive elements (CREs); blue arrows, ecdysterone response elements (EcREs).

transcript levels encoding V-ATPase subunits may be mediated by ecdysteroids. Since steroid hormones are known to be potent regulators of transcriptional activity, we cloned and sequenced the 5′ upstream regions of *mvB*, *mvG* and *mvd*, the *Manduca sexta* genes encoding V-ATPase subunits B, G and d, respectively. Our aim was to compare the promoter structures and to investigate the influence of ecdysteroids on the promoter activities. The nucleotide sequences were compared over a region of approximately 1 kb upstream of the translational start codon (Fig. 5).

Although dissimiliar in sequence, the promoters of *mvB* and *mvG* shared features common with inducible or tissue-specific promoters of vertebrates. They showed canonical TATA boxes and a low GC content of approximately 30 %, with a similiar distribution pattern in the proximal region. In addition, both promoters contained a motif similar to the consensus sequence of the cAMP-responsive element (CRE) (Roesler et al., 1988). In contrast, the promoter of *mvd* lacked apparent TATA boxes and CREs and exhibited a different GC distribution pattern, although the averaged GC content of approximately 35 % was only negligibly higher. Overall, *mvd* appeared to exhibit several characteristics common to housekeeping genes described in other organisms. However, all three promoters contained an ecdysone-responsive element (EcRE) corresponding to the consensus sequence KNTCANTNNMM (Luo et al., 1991).

Thus, the EcREs found in the 5′ regions of *mvB*, *mvG* and *mvd* may act as common trancriptional regulator elements that, upon the release of ecdysteroids, simultaneously control the promoter activities of different V-ATPase genes in a concerted fashion. This interpretation is in line with the observed decline in levels of V-ATPase transcripts during the moult and upon 20-HE injection. To characterize the promoters further, we mapped the transcriptional start sites of the *mvB*, *mvG* and *mvd* 5′ regions by RNase protection assays using ^{32}P-labelled RNA probes covering the corresponding upstream sequences between nucleotide positions –973 and +1. As shown in Fig. 6, analysis of the autoradiograms revealed four transcriptional start sites for *mvB*, one for *mvG* and six for *mvd*, all of which were similar to the CAP consensus sequence KCABHYBY (Bucher, 1990). Thus, the upstream regions of *mvB* and *mvd* exhibit multiple transcriptional start sites, whereas the upstream region of *mvG* contains only a single start site, although there was a second protected fragment at a very distal position but no corresponding CAP site in close proximity.

Transcriptional activities of mvB*,* mvG *and* mvd *promoters are inhibited by ecdysterone*

To test the effect of 20-HE on the transcriptional activities of V-ATPase genes, we performed reporter gene assays in *Sf*21 insect cells. We ligated 976 bp (positions –973 to +3) of the *mvB*, *mvG* and *mvd* upstream sequences into pGL2-basic, a vector using firefly luciferase as the genetic reporter. Normalization of transfection efficiency was achieved by co-transfection with pRL-CMV, which contains a CMV promoter to provide constitutive expression of *Renilla* luciferase. After co-transfection of *Sf*21 cells with the corresponding reporter gene plasmid, we added 20-HE to the culture medium and incubated the cells for different times. After cell harvest and lysis, we successively measured the luminescence signals derived from firefly and *Renilla* luciferase. All upstream sequences cloned in front of the luciferase coding sequence led to significant transcriptional activites, suggesting that all these regions actually contain promoter sequences that allow binding of basic transcription factors and of RNA polymerase II.

After addition of 2.5 μg ml^{-1} 20-HE to the culture medium, a decrease in promoter activities for *mvB*, *mvG* and *mvd* was observed after 3, 5 and 48 h, suggesting that ecdysteroids

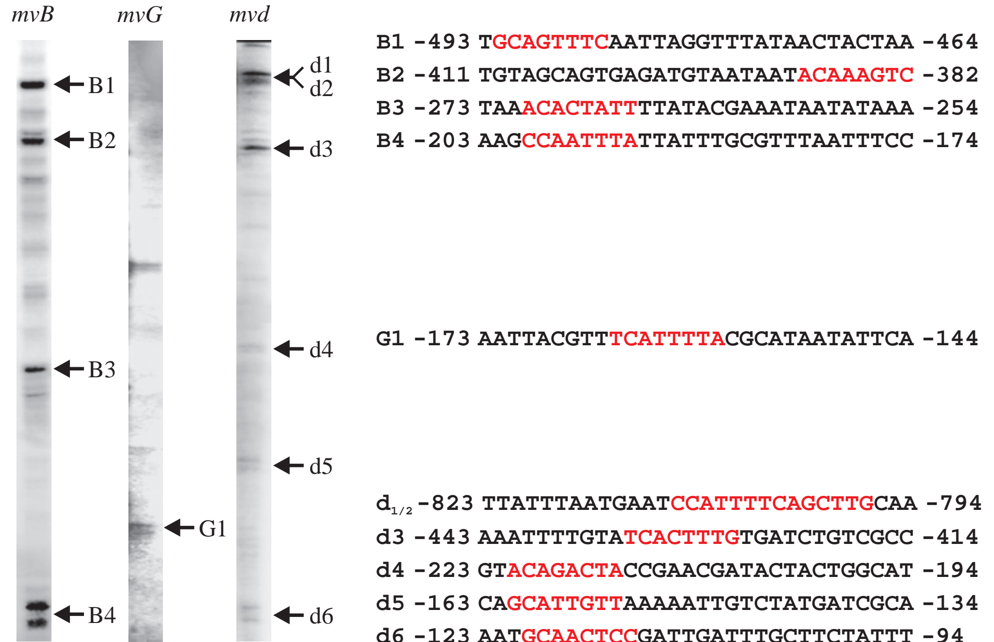

Fig. 6. Determination of transcriptional start sites of the *Manduca sexta* V-ATPase genes *mvB*, *mvG* and *mvd*. The start sites were determined by RNase protection assays using 973 bp each of the 5′ upstream regions as a template for *in vitro* transcription of ^{32}P-labelled ssRNA probes. After hybridization with poly(A) RNA, single-stranded RNA was digested with RNase A and T1. The remaining double-stranded RNA was separated on a denaturing polyacrylamide gel. Visualization of radioactively labelled bands was performed by exposing the gels to X-ray film. Nucleotide positions of identified transcriptional initiation sites that are similar to the consensus sequence KCABHYBY (Bucher, 1990) are shown on the right side of the figure.

inhibit the transcription of V-ATPase genes (Fig. 7). In contrast, short-term treatment for only 1.5 h led to significant activation of the *mvB* and slight activation of the *mvG* promoter (Fig. 7). Except that short-term activation was not detected for the *mvd* promoter, these findings were similar to the results obtained for the 20-HE injection experiments, in which a short-term increase and a long-term decrease in transcript levels were observed.

Discussion

In this study, we examined the transcript levels and transcriptional activities of several V-ATPase genes of *M. sexta* larvae during the moult and during periods of starvation, both states in which V-ATPase activity is shut down by reversible disassembly of the holoenzyme (Sumner et al., 1995). The rapid and enormous growth of larvae from hatching to pupation – they increase their body mass within 3 weeks by a factor of approximately 10^4 – requires that the uptake of nutrients such as amino acids is energized very effectively. For this reason, it is not surprising that 10 % of total larval ATP production is spent on active K^+ transport in the midgut (Dow and Peacock, 1989), and this is driven by the plasma membrane V-ATPase (Wieczorek et al., 2000). However, high rates of ATP consumption entail strict control of enzyme activity and, since V-ATPase is present in high densities in the goblet cell apical membranes (up to approximately 5000 molecules μm^{-2}), also strict control of its biosynthesis. We therefore examined levels of subunit mRNAs during periods when no V-ATPase activity is observed.

Northern blots showed decreased transcript levels for almost all investigated V-ATPase subunits upon starvation and during the moult. These findings suggest that the larvae are very economical in dealing with their anabolic resources since they downregulate V-ATPase subunit synthesis during periods when no enzyme activity is needed because of the cessation of food intake. Since transcript levels correlated with the haemolyph titres of 20-HE and JHIII, we injected both hormones into feeding fifth-instar larvae at times when haemolymph titres of both hormones are known to be very low. In contrast to JHIII, which had no effect, injection of the

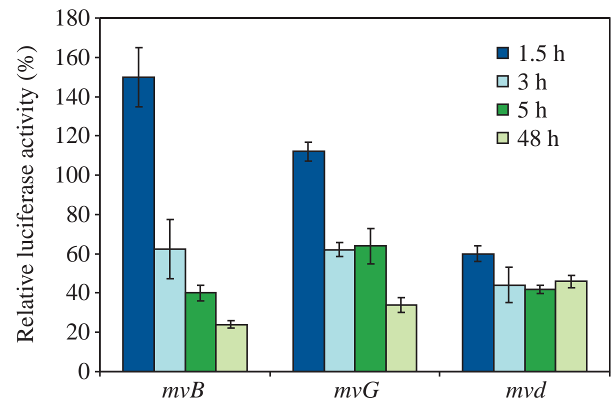

Fig. 7. Influence of 20-hydroxyecdysone on transcriptional activities of the *mvB*, *mvG* and *mvd* 5′ upstream regions. 976 bp of the 5′ upstream regions was ligated in front of the firefly luciferase gene of the pGL2-basic vector. Thus, expression of the luciferase gene was brought under the control of the V-ATPase gene promoters. *Sf*21 cells were co-transfected with these constructs and pRl-CMV, a control vector that constitutionally expresses *Renilla* luciferase and allows internal standardization. Expression of both luciferases was monitored luminometrically. The effect of the steroid hormone on reporter gene expression was determined after incubation of the transfected *Sf*21 cells with 2.5 μg ml^{-1} 20-hydroxyecdysone for the times indicated. Values (± S.E.M., N=3) are given as relative units compared with untreated cells and are normalized to the luciferase activity of the control vector.

moulting hormone 20-HE resulted in a short-term increase and a long-term decrease in levels of V-ATPase transcripts, implying that either transcription rates or transcript stabilities are regulated by the steroid hormone. Indeed, all 5′ upstream regions of the genes investigated, *mvB*, *mvG* and *mvd*, contained putative ecdysone response elements (Luo et al., 1991), and reporter gene assays also demonstrated the influence of 20-HE.

Ecdysteroids are known to be key regulatory factors for gene transcription, activating a nuclear receptor heterodimer consisting of the ecdysone receptor EcR and the *Drosophila* retinoid X receptor homologue USP, the ultraspiracle protein (Yao et al., 1993). Ecdysteroid-mediated control of transcriptional activities may be positive or negative depending on the hormone concentration (for a review, see Spindler et al., 2001). Upregulation of transcriptional activities during insect development by ecdysteroids is well documented in the literature. For instance, *Eips 28/29* are *Drosophila* genes that are controlled tissue- and stage-specifically by ecdysone-responsive elements present in the upstream and downstream flanking regions (Andres and Cherbas, 1994). Other genes that are likely to be regulated by the activated ecdysterone receptor are the *Drosophila* genes encoding the yolk protein (Bownes et al., 1996), the heat-shock proteins hsp23 and hsp27 (Luo et al., 1991) and the caspase DRONC (Dorstyn et al., 1999; Hawkins et al., 2000) and the *M. sexta* genes *EcR-A* and *EcR-B1*, which encode two ecdysone receptor isoforms (Jindra et al., 1996).

Downregulation of transcriptional activities by ecdysteroids was observed too, but there was no evidence for direct transcriptional repression. For instance, it has been suggested that the ecdysteroid-regulated gene *esr20*, which is expressed in the trachea of *M. sexta*, is downregulated at ecdysis, probably because of a decline in transcript stability that might be triggered indirectly by 20-HE (Meszaros and Morton, 1997). Characterization of the dopamine decarboxylase gene (*DDC*) of *M. sexta* revealed that it may be indirectly suppressed by 20-HE *via* an ecdysteroid-induced transcription factor that itself suppresses DDC transcription (Hiruma et al., 1995).

From our experiments, we conclude that 20-HE influences V-ATPase gene expression in more than one way. Injection of 20-HE into fifth-instar larvae (days 2–3) resulted in both a short-term increase and a long-term decrease in V-ATPase transcript levels. At first sight, this seems to be contradictory because V-ATPase activity is not initially upregulated upon moulting or starvation. However, pump deactivation appears not to be directly related to transcriptional control of subunit synthesis because reassembly of the V_1 and V_o complex has been shown previously to be independent of biosynthesis in yeast and in *M. sexta* (Kane, 1995; Merzendorfer et al., 1997a). Thus, short-term upregulation of transcription does not necessarily lead to upregulation of V-ATPase activity, especially since translation rates do not have to follow transcription rates strictly.

In contrast to 20-HE, injection of JHIII had only negligible effects on transcript levels. However, we cannot exclude the possibility that treatment with other JH isoforms could influence mRNA levels of V-ATPase subunits because, in lepidopterans, the haemolymph titres of JHI and JHII are significantly higher than that of JHIII (Baker et al., 1987). Results similar to those of the injection experiments were obtained in reporter gene assays with upstream regions of the V-ATPase genes *mvB*, *mvG* and *mvD*, indicating that transcription rates are influenced by 20-HE in both directions, depending on the duration of 20-HE treatment. In principle, the decrease in V-ATPase gene expression could be due to a general repression of transcript levels, as has been observed in *M. sexta* during the fifth instar between days 2 and 3 and upon ecdysteroid treatment of day 1 epidermis by RNA labelling experiments (Shaaya and Riddiford, 1988). However, we believe the decrease to be specific since we normalized all our assays either to ribosomal S7 mRNA or to the expression of a constitutive promoter.

The observation that levels of V-ATPase transcripts also decline during starvation independent of moulting processes suggests that, during these periods, ecdysteroids may be involved in the control of transcript levels. Unfortunately, to our knowledge, ecdysteroid titres have not been measured in the haemolymph of starving larvae. However, there might be a further level of control that is dependent upon feeding and independent of edysteroids, as was suggested for the *Drosophila melanogaster* yolk protein gene transcription (Bownes et al., 1988). Thus, both the lack of nutrients and the rising titre of ecdysteroids may contribute to the decrease in V-ATPase mRNA levels upon starvation and during the moult. This resembles the expression pattern of arylphorin in *M. sexta*, where both the lack of nutrients and the rising ecdysteroid titre contribute to the decrease in arylphorin mRNA levels during moults and during the wandering stage (Webb and Riddiford, 1988).

This work was supported by Grant Wi 698 from the Deutsche Forschungsgemeinschaft. We thank Ulla Mädler and Margret Düvel for excellent technical assistance. The nucleotide sequences reported in this paper have been submitted to the GenBanke4/EMBL Data Bank with accession numbers AJ315143, AJ315144 and AJ315145.

References

Andres, A. J. and Cherbas, P. (1994). Tissue-specific regulation by ecdysone: Distinct patterns of *Eip28/29* expression are controlled by different ecdysone response elements. *Dev. Genet.* **15**, 320–331.

Azuma, M., Harvey, W. R. and Wieczorek, H. (1995). Stoichiometry of K^+/H^+ antiport helps to explain extracellular pH 11 in a model epithelium. *FEBS Lett.* **361**, 153–156.

Baker, F. C., Tsai, L. W., Reuter, C. C. and Schooley, D. A. (1987). *In vivo* fluctuation of JH, JH acid and ecdysteroid titer and JH esterase activity during development of fifth stadium *Manduca sexta*. *Insect Biochem.* **17**, 989–996.

Baldwin, B. M. and Hakim, R. S. (1991). Growth and differentiation of the larval midgut epithelium during moult in the moth, *Manduca sexta*. *Tissue Cell* **23**, 411–422.

Bell, R. A. and Joachim, F. G. (1974). Techniques for rearing laboratory colonies of tobacco hornworms and pink bollworms. *Ann. Entomol. Soc. Am.* **69**, 365–373.

Bollenbacher, W. E., Smith, S. L., Goodman, W. and Gilbert, L. I. (1981). Ecdysteroid titer during larval–pupal–adult development of the tobacco hornworm, *Manduca sexta*. *Gen. Comp. Endocrinol.* **44**, 302–306.

Bownes, M., Ronaldson, E. and Mauchline, D. (1996). 20-Hydroxyecdysone, but not juvenile hormone, regulation of yolk protein gene expression can be mapped to *cis*-acting DNA sequences. *Dev. Biol.* **173**, 475–489.

Bownes, M., Scott, A. and Shirras, A. (1988). Dietary components modulate yolk protein gene transcription in *Drosophila melanogaster*. *Development* **103**, 119–128.

Bucher, P. (1990). Weight matrix descriptions of four eukaryotic RNA polymerase II promoter elements derived from 502 unrelated promoter sequences. *J. Mol. Biol.* **212**, 563–578.

Castagna, M., Shayakul, C., Trotti, D., Sacchi, V. F., Harvey, W. R. and Hediger, M. A. (1998). Cloning and characterization of a potassium-coupled amino acid transporter. *Proc. Natl. Acad. Sci. USA* **95**, 5395–5400.

Dorstyn, L., Colussi, P. A., Quinn, L. M., Richardson, H. and Kumar, S. (1999). DRONC, an ecdysone-inducible *Drosophila* caspase. *Proc. Natl. Acad. Sci. USA* **96**, 4307–4312.

Dow, J. A. T. and Peacock, J. M. (1989). Microelectrode evidence for the electrical isolation of goblet cell cavities in *Manduca sexta* middle midgut. *J. Exp. Biol.* **143**, 101–114.

Edgar, K. A., Noriega, F. G., Bonning, B. C. and Wells, M. A. (2000). Recombinant juvenile hormone esterase, an effective tool for modifying juvenile hormone-dependent expression of the early trypsin gene in mosquitoes. *Insect Mol. Biol.* **9**, 27–31.

Fain, M. J. and Riddiford, L. M. (1975). Juvenile hormone titers in the hemolymph during late larval development of the tobacco hornworm, *Manduca sexta* (L.). *Biol Bull.* **149**, 506–521.

Forgac, M. (2000). Structure, mechanism and regulation of the clathrin-coated vesicle and yeast vacuolar H^+-ATPases. *J. Exp. Biol.* **203**, 71–80.

Gräf, R., Harvey, W. R. and Wieczorek, H. (1996). Purification and properties of a cytosolic V_1 ATPase. *J. Biol. Chem.* **271**, 20908–20913.

Grüber, G., Radermacher, M., Ruiz, T., Godovac-Zimmermann, J., Canas, B., Kleine-Kohlbrecher, D., Huss, M., Harvey, W. R. and Wieczorek, H. (2000). Three-dimensional structure and subunit topology of the V_1 ATPase from *Manduca sexta* midgut. *Biochemistry* **39**, 8609–8616.

Hawkins, C. J., Yoo, S. J., Peterson, E. P., Wang, S. L., Vernooy, S. Y. and Hay, B. A. (2000). The *Drosophila* caspase DRONC cleaves following glutamate or aspartate and is regulated by DIAP1, HID and GRIM. *J. Biol. Chem.* **275**, 27084–27093.

Hewes, R. S. and Truman, J. W. (1994). Steroid regulation of excitability in identified insect neurosecretory cells. *J. Neurosci.* **14**, 1812–1819.

Hiruma, K., Carter, M. S. and Riddiford, L. M. (1995). Characterization of the dopa decarboxylase gene of *Manduca sexta* and its suppression by 20-hydroxyecdysone. *Dev. Biol.* **169**, 195–209.

Hiruma, K., Shinoda, T., Malone, F. and Riddiford, L. M. (1999). Juvenile hormone modulates 20-hydroxyecdysone inducible ecdysone receptor and ultraspiracle gene expression in the tobacco hornworm, *Manduca sexta*. *Dev. Genes Evol.* **209**, 18–30.

Jiang, H., Wang, Y. and Kanost, M. R. (1996). Primary structure of ribosomal proteins S3 and S7 from *Manduca sexta*. *Insect Mol. Biol.* **5**, 31–38.

Jindra, M., Malone, F., Hiruma, K. and Riddiford, L. M. (1996). Developmental profiles and ecdysteroid regulation of the mRNAs for two ecdysone receptor isoforms in the epidermis and wings of the tobacco hornworm, *Manduca sexta*. *Dev. Biol.* **180**, 258–272.

Kane, P. (1995). Disassembly and reassembly of the yeast vacuolar H^+-ATPase *in vivo*. *J. Biol. Chem.* **270**, 17025–17032.

Klein, U., Löffelmann, G. and Wieczorek, H. (1991). The midgut as a model system for insect K^+-transporting epithelia: immunocytochemical localization of a vacuolar-type H^+ pump. *J. Exp. Biol.* **161**, 61–75.

Lepier, A., Gräf, R., Azuma, M., Merzendorfer, H., Harvey, W. R. and Wieczorek, H. (1996). The peripheral complex of the tobacco hornworm V-ATPase contains a novel 13-kDa subunit G. *J. Biol. Chem.* **271**, 8502–8508.

Luo, Y., Amin, J. and Voellmy, R. (1991). Ecdysterone receptor is a sequence-specific transcription factor involved in the developmental regulation of heat shock genes. *Mol. Cell. Biol.* **11**, 3660–3675.

Merzendorfer, H., Gräf, R., Huss, M., Harvey, W. R. and Wieczorek, H. (1997a). Regulation of proton-translocating V-ATPases. *J. Exp. Biol.* **200**, 225–235.

Merzendorfer, H., Harvey, W. R. and Wieczorek, H. (1997b). Sense and antisense RNA for the membrane associated 40 kDa subunit M40 of the insect V-ATPase. *FEBS Lett.* **411**, 239–244.

Merzendorfer, H., Reineke, S., Zhao, X. F., Jacobmeier, B., Harvey, W. R. and Wieczorek, H. (2000). The multigene family of the tobacco hornworm V-ATPase: novel subunits a, C, D, H and putative isoforms. *Biochim. Biophys. Acta* **1467**, 369–379.

Meszaros, M. and Morton, D. B. (1997). Up- and downregulation of *esr20*, an ecdysteroid-regulated gene expressed in the tracheae of *Manduca sexta*. *Arch. Insect Biochem. Physiol.* **34**, 159–174.

Novak, F. J., Gräf, R., Waring, R. B., Wolfersberger, M. G., Wieczorek, H. and Harvey, W. R. (1992). Primary structure of V-ATPase subunit B from *Manduca sexta* midgut. *Biochim. Biophys. Acta* **1132**, 67–71.

Roesler, W. J., Vandenbark, G. R. and Hanson, R. W. (1988). Cyclic AMP and the induction of eukaryotic gene transcription. *J. Biol. Chem.* **263**, 9063–9066.

Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989). *Molecular Cloning: A Laboratory Manual*. New York: Cold Springer Harbor Laboratory Press.

Shaaya, E. and Riddiford, L. M. (1988). Depressed RNA synthesis in the early stages of the larval–pupal transformation of Lepidoptera: The role of 20-hydroxyecdysone. *J. Insect Physiol.* **34**, 655–659.

Spindler, K.-D., Przibilla, S. and Spindler-Barth, M. (2001). Moulting hormones of arthropods: Molecular mechanisms. *Zoology* **103**, 189–201.

Sumner, J. P., Dow, J. A., Earley, F. G., Klein, U., Jager, D. and Wieczorek, H. (1995). Regulation of plasma membrane V-ATPase activity by dissociation of peripheral subunits. *J. Biol. Chem.* **270**, 5649–5653.

Svergun, D. I., Konrad, S., Huss, M., Koch, M. H., Wieczorek, H., Altendorf, K., Volkov, V. V. and Grüber, G. (1998). Quaternary structure of V_1 and F_1 ATPase: significance of structural homologies and diversities. *Biochemistry* **37**, 17659–17663.

Webb, B. A. and Riddiford, L. M. (1988). Regulation of expression of arylphorin and female-specific protein mRNAs in the tobacco hornworm, *Manduca sexta*. *Dev. Biol.* **130**, 682–692.

Wieczorek, H., Brown, D., Grinstein, S., Ehrenfeld, J. and Harvey, W. R. (1999). Animal plasma membrane energization by proton motive V-ATPases. *BioEssays* **21**, 637–648.

Wieczorek, H., Grüber, G., Harvey, W. R., Huss, M., Merzendorfer, H. and Zeiske, W. (2000). Structure and regulation of insect plasma membrane H^+ V-ATPase. *J. Exp. Biol.* **203**, 127–135.

Wieczorek, H., Putzenlechner, M., Zeiske, W. and Klein, U. (1991). A vacuolar-type proton pump energizes K^+/H^+ antiport in an animal plasma membrane. *J. Biol. Chem.* **266**, 15340–15347.

Yao, T. P., Forman, B. M., Jiang, Z., Cherbas, L., Chen, J. D., McKeown, M., Cherbas, P. and Evans, R. M. (1993). Functional ecdysone receptor is the product of *EcR* and *Ultraspiracle* genes. *Nature* **366**, 476–479.