

## MOLECULAR CHARACTERIZATION OF V-TYPE H<sup>+</sup>-ATPase (B-SUBUNIT) IN GILLS OF EURYHALINE CRABS AND ITS PHYSIOLOGICAL ROLE IN OSMOREGULATORY ION UPTAKE

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### Summary

The vacuolar-type H<sup>+</sup>-ATPase (V-ATPase) has been implicated in osmoregulatory ion uptake across external epithelia of a growing variety of species adapted to life in fresh water. In the present study, we investigated whether the V-ATPase may also function in a euryhaline species that tolerates brackish water (8‰ salinity) but not fresh water, the shore crab *Carcinus maenas*. cDNA coding for the regulatory B-subunit of the V-ATPase was amplified and sequenced from *C. maenas* gills and partially sequenced from four other crab species. Two isoforms differing in the 3'-untranslated region were found in *C. maenas*. In this species, the abundance of B-subunit mRNA was greater in the respiratory anterior gills than the ion-transporting posterior gills and was not increased by acclimation to dilute salinity. Immunocytochemical

analysis showed that the B-subunit protein is not targeted to the apical membrane but is distributed throughout the cytoplasmic compartment. Physiological studies of isolated perfused gills indicated that the V-ATPase inhibitor bafilomycin had no effect on transepithelial potential difference. Thus, in contrast to the freshwater-tolerant Chinese crab *Eriocheir sinensis*, in which the V-ATPase appears to play an important osmoregulatory role, the V-ATPase in *C. maenas* probably functions in acidification of intracellular organelles but not in transbranchial NaCl uptake.

Key words: vacuolar-type ATPase, cDNA sequence analysis, mRNA expression analysis, gill, osmoregulation, *Carcinus maenas*, *Eriocheir sinensis*, crab.

### Introduction

Together with P-ATPase and F-ATP synthase, the proton-translocating vacuolar-type ATPase (V-ATPase) is one of three major classes of the so-called ion-motive ATPases in eukaryotic cells (Forgac, 1989; Harvey, 1992; Wiczeorek et al., 1999). The V-ATPase is composed of two structural protein domains, the cytoplasmic V<sub>1</sub> domain and the membrane-spanning V<sub>0</sub> domain (Nelson, 1992). The V<sub>1</sub> domain, with a molecular mass of approximately 570 kDa, comprises the catalytic sector and is composed of at least eight different subunits (A, B, C, D, E, F, G, H) with subunits A (67–73 kDa) and B (55–60 kDa) as the catalytic and regulatory subunits, respectively. The stoichiometry found in the V<sub>1</sub> complex of yeast is A<sub>3</sub>B<sub>3</sub>C<sub>1</sub>D<sub>1</sub>E<sub>1</sub>F<sub>1</sub>G<sub>3</sub>H<sub>1</sub> (Forgac, 2000). The membrane-embedded V<sub>0</sub> domain, with a molecular mass of approximately 250 kDa, contains the proton-translocating structure. It is composed of at least four different subunits (M100, M40, M9.7, M17) with a probable stoichiometry of M100<sub>1</sub>M40<sub>1</sub>M9.7<sub>1</sub>M17<sub>6</sub> (Wiczeorek et al., 2000).

The diversity of cellular functions of the V-ATPase is attributed to its properties of generating both electrochemical

and pH gradients across endo- and exomembranes of eukaryotes by utilizing ATP to move protons unidirectionally against steep H<sup>+</sup> gradients. V-ATPases are involved in acidifying endosomes, lysosomes and other compartments of the cellular vacuolar system, where they play a role in the complex mechanisms of endo- and exocytosis. In proton-excreting cells such as osteoclasts or renal intercalated cells, the V-ATPase is distributed in high densities in the apical membrane of these polarized cells (Nelson et al., 1992). In midgut goblet cells of larvae of the lepidopteran *Manduca sexta*, apically situated V-ATPase energizes transepithelial secondary transport processes, including amino acid absorption, by producing a high luminal pH (Wiczeorek et al., 1999).

In freshwater-inhabiting species such as trout (Lin and Randall, 1993), toad (Larsen et al., 1992) and crayfish (Zare and Greenaway, 1998), the activity of V-ATPase is thought to complement Na<sup>+</sup>/K<sup>+</sup>-ATPase in energizing osmoregulatory ion uptake from highly diluted media. Such a role of gill V-ATPase has been explored in previous investigations employing the

extremely euryhaline Chinese mitten crab *Eriocheir sinensis*. Tracer-flux experiments on isolated perfused gills (Gilles et al., 1988; Riestenpatt et al., 1995), electrophysiological studies on split gill lamellae (Onken and Putzenlechner, 1995) and immunohistochemical experiments (Putzenlechner, 1994) suggest that the activity of an apically situated V-ATPase is necessary to regulate internal hemolymph osmolarity during exposure of *E. sinensis* to low external salinity.

In this study, we investigated whether the V-ATPase may also be involved in ion uptake in organisms with moderate osmoregulatory abilities such as the shore crab *Carcinus maenas*, in which the lower tolerance of external salinity is limited to 8‰ (Siebers et al., 1982). Weihrauch and Towle (1997) found the first molecular evidence for the expression of a V-ATPase in the gills of the shore crab by cloning a partial mRNA sequence of the B-subunit. To explore in more detail the presence and function of the V-ATPase in *C. maenas*, *E. sinensis* and other euryhaline crab species, molecular biological techniques, immunohistochemical approaches and experiments employing isolated perfused gills were performed. We present direct molecular evidence for the expression of a V-ATPase (B-subunit) in the gills of *Carcinus maenas*, *Eriocheir sinensis*, *Callinectes sapidus*, *Chasmagnathus granulatus* and *Cancer irroratus*. Both immunolocalization of the B-subunit in *Carcinus maenas* gills and mRNA expression analysis of this subunit in *Carcinus maenas* and *Eriocheir sinensis* support the suggestion that a V-ATPase is involved in osmoregulatory ion uptake in the extremely euryhaline Chinese crab but not in the moderately euryhaline shore crab.

## Materials and methods

### Animals

For experiments conducted in Maine or Illinois, green shore crabs *Carcinus maenas* (L) were collected from the intertidal zone at Oak Point, Trenton, Maine, or were supplied by the Marine Biological Laboratory (Woods Hole, MA, USA). For experiments conducted in Germany, *Carcinus maenas* were obtained from Kiel Bay, Baltic Sea. *Carcinus maenas* were maintained in recirculating aquaria at 15–16 °C in 35 or 10‰ salinity and were fed cleaned squid or bovine heart 2–3 times weekly. Chinese mitten crabs *Eriocheir sinensis* (H. Milne Edwards) were obtained from the Eider River (approximately 0.5‰ salinity) in Germany and were maintained at 16 °C in running fresh water or in recirculating aquaria at 35 or 20‰ salinity. The gills were removed from *E. sinensis* and stored in RNAlater (Ambion) for transport to Illinois. Blue crabs *Callinectes sapidus* (Rathbun) were obtained from the Waukegan (IL, USA) Oriental Food Store or from Gulf Specimen (Panacea, FL, USA) and were kept in recirculating aquaria at 20 °C in 35 or 5‰ salinity. South American estuarine crabs *Chasmagnathus granulatus* (Dana) were obtained from the coast of Argentina, through the University of Buenos Aires, and were kept in small aquaria at 20 °C in 2–3‰ salinity. Rock crabs *Cancer irroratus* (Say) were obtained by divers at the Mount Desert Island Biological

Laboratory and were kept briefly in flowing natural sea water at approximately 12 °C. Crabs were anesthetized by immersion in ice prior to removal of the gills.

### Molecular cloning of V-type ATPase B-subunit cDNA

Total RNA was extracted from gill tissue under RNase-free conditions (Chomczynski and Sacchi, 1987) using materials obtained from Promega Corporation. Reverse transcription of poly(A<sup>+</sup>) mRNA was initiated with oligo(dT) primer and Superscript II reverse transcriptase (Gibco-BRL). Amplification of putative B-subunit sequences was achieved using the polymerase chain reaction (PCR; Sigma REDTaq) using degenerate and specific primers designed with the assistance of Primer Premier software. Following separation on 1% agarose gels, PCR products were extracted from gel slices (Qiagen QiaQuick) and sequenced directly by the dideoxynucleotide method (Sanger et al., 1977) either manually on 5% polyacrylamide gels or automatically at the Marine DNA Sequencing Center of Mount Desert Island Biological Laboratory. Fragment sequences were assembled using DNASTAR software and were analyzed for open reading frames using DNASIS. A search of GenBank using the BLAST algorithm (Altschul et al., 1997) revealed close matches with previously published sequences. Multiple alignments were accomplished with ClustalW (<http://pbil.ibcp.fr/ANTHEPROT>) and GeneDoc software (<http://www.psc.edu/biomed/genedoc/>), and a phylogenetic tree was generated with TreeView (<http://taxonomy.zoology.gla.ac.uk/rod/rod.html>).

### Western blot analysis of B-subunit protein

A crude membrane fraction was prepared from anterior and posterior gills of *Carcinus maenas* acclimated to 10‰ salinity, and western blot analysis was performed according to the methods of Lucu and Flik (Lucu and Flik, 1999), using as primary antibody a monoclonal antibody against yeast B-subunit (Molecular Probes). Visualization of putative B-subunit protein was accomplished with the Opti-4CN system (BioRad Laboratories).

### Immunocytochemical localization of V-ATPase B-subunit in gill lamellae of *Carcinus maenas*

Shore crabs were acclimated to 10‰ sea water. Posterior and anterior gills were immersed in and perfused with 20‰ sea water for several minutes. Subsequently, the gills were perfused with a fixation solution composed of a 1:1 mixture of 20‰ sea water and a solution containing 8% paraformaldehyde, 0.35 mol l<sup>-1</sup> sucrose, 150 mmol l<sup>-1</sup> NaCl, 0.2 mol l<sup>-1</sup> sodium cacodylate buffer (pH 7.3). After 5 min of perfusion, the external 20‰ sea water was replaced with fixation solution, and the gills were perfused for another 10 min. Individual gill lamellae were dissected and kept in fixation solution for 1 h. Cryofixation was performed by a method similar to that described previously (Tokuyasu, 1980). The lamellae were incubated in a solution containing 2.3 mol l<sup>-1</sup> sucrose in 0.1 mol l<sup>-1</sup> sodium cacodylate buffer (pH 7.3) for at least 2 h. Small pieces of tissue from the thick region of the lamellae

containing the ion-transporting cells were mounted on aluminum rods and frozen in liquid Freon cooled with liquid nitrogen. Semi-thin (0.5 μm) cryosections were obtained as described previously (Ziegler, 1997) using a Leica Ultracut S microtome equipped with an FCS cryochamber, glass knives and an antistatic device (Diatome) at temperatures of -70 °C. Sections were transferred to poly-L-lysine-covered glass slides with a droplet of 2.3 mol l<sup>-1</sup> sucrose in 0.1 mol l<sup>-1</sup> cacodylate buffer (pH 7.3). The sucrose was washed off with phosphate-buffered saline (PBS), and the sections were treated successively with 0.05 mol l<sup>-1</sup> glycine in PBS for 15 min, 1 % SDS in PBS for 5 min, PBS three times for 5 min, 0.001 % streptavidin for 15 min to block endogenous biotin, PBS for 5 min, 0.1 % biotin to block the biotin-binding sites of the streptavidin, PBS twice for 5 min and, finally, blocking solution (BS) containing 5 % bovine serum albumin (BSA) and 0.1 % cold-water fish gelatine (Biotrend) in PBS for 15 min. Small droplets (12 μl) of primary antibody (anti-yeast V-ATPase B-subunit, Molecular Probes) at 2.5 μg ml<sup>-1</sup> in incubation solution (IS) containing 1 % BSA and 0.1 % cold-water fish gelatine in PBS were placed on the sections and incubated overnight. Control sections were incubated in IS without primary antibody. The sections were washed three times in 0.1 mol l<sup>-1</sup> phosphate buffer containing 0.5 mol l<sup>-1</sup> NaCl (PBS-500) and once in PBS, incubated for 1 h in biotinylated secondary antibody [anti-mouse F(a,b')<sub>2</sub> fragments from sheep; Amersham], washed three times in PBS-500 for 5 min and once in PBS for 5 min. Sections were treated with TNB-blocking-solution (NEN) for 30 min, incubated with horseradish peroxidase (HRP)-linked streptavidin (NEN) at a dilution of 1:100 in TNB blocking solution for 1 h, and washed three times in PBS-500 for 5 min and once in PBS for 5 min. The sections were incubated in the fluorescent HRP substrate Tyramid-CY3 (NEN) at a dilution of 1:40 in amplification buffer (NEN) for 10 min in the dark, washed three times in PBS-500 for 5 min and once in PBS for 5 min. Subsequently, the sections were mounted in 80 % glycine, 20 % PBS plus 2 % *N*-propyl-gallate (to retard fading) and examined with a Zeiss Axiophot microscope. Micrographs were taken on Kodak Elite 400 film.

#### Analysis of B-subunit mRNA expression in gills of *Carcinus maenas*

Relative levels of B-subunit mRNA in gill RNA extracts

pooled from three animals per treatment were estimated using quantitative reverse transcriptase (RT)-PCR by incorporating biotin-dUTP during logarithmic amplification according to previously published methods (Towle et al., 1997). Single-tube duplex PCR was performed using primer pairs for both V-ATPase B-subunit and arginine kinase (Table 1), the latter serving as an unchanging control with respect to salinity and gill type (Kotlyar et al., 2000). The biotinylated products were separated on 1 % agarose gels, transferred to nylon membranes and visualized with the PhotoTope detection system (New England Biolabs). Intensities of B-subunit products were compared with those for arginine kinase mRNA by digitizing the bands using Un-Scan-Itgel software (Silk Scientific).

#### Functional analysis of V-type ATPase in isolated gills of *Carcinus maenas*

Gills were perfused according to previously published methods (Siebers et al., 1985; Weihrauch et al., 1998) using symmetrical saline solutions containing (in mmol l<sup>-1</sup>): 248 NaCl, 5 CaCl<sub>2</sub>, 5 KCl, 4 MgCl<sub>2</sub>, 2 NaHCO<sub>3</sub> and 2.5 Tris at pH 7.8. During perfusion at a flow rate of 0.135 ml min<sup>-1</sup>, transepithelial potential differences (*PD*<sub>te</sub>) were monitored using a millivolt meter (Keithley, model 197) connected to the perfusate and bath solutions *via* Ag/AgCl electrodes.

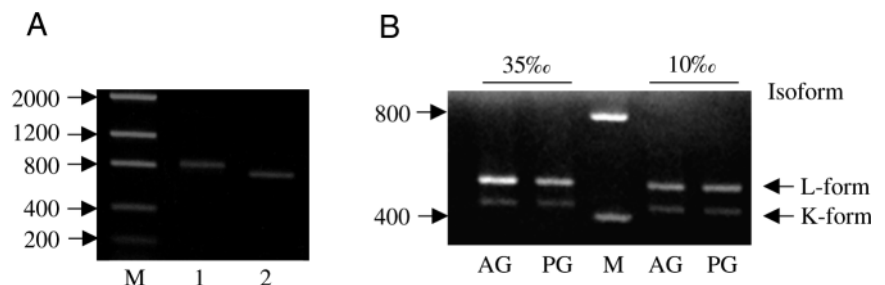
#### Results

To identify expression of V-type H<sup>+</sup>-ATPase in gill epithelium of the shore crab *Carcinus maenas*, we designed degenerate oligonucleotide primers (HATF2, HATR4; Table 1) on the basis of conserved regions in published cDNA sequences coding for the B-subunit of the V-ATPase in other species. Starting with an initial cDNA sequence of 360 nucleotides identified *via* BLAST search of GenBank as a putative B-subunit fragment (Weihrauch and Towle, 1997), we designed additional degenerate and specific sense and antisense primers (Table 1) to amplify and sequence the entire B-subunit cDNA from *Carcinus maenas*, using the RACE technique and AUAP primer (5'-GGCCACGCGTCTACTA-GTAC-3' (Gibco-BRL) to obtain the 5' and 3' regions of the cDNA.

Amplification of the 3'-untranslated region (UTR) employing the primer combination HATF1770/AUAP gave

Fig. 1. Polymerase chain reaction (PCR) amplification of the 3'-untranslated region of V-ATPase B-subunit cDNA from the gills of shore crab *Carcinus maenas* using primers HATF1770 and AUAP (A) or HATF1550 and HATR1960 (B). In A, the two amplification products were separated by agarose gel electrophoresis, extracted and purified, and run in separate lanes (1, 2) in a second electrophoresis in parallel with a DNA marker (M) with size indicated in base pairs (bp).

In B, the two products were amplified from anterior gills (AG) and posterior gills (PG) obtained from crabs acclimated to 35 or 10‰ salinity. The larger L-form and shorter K-form are shown in relation to a DNA marker (M).



1	ATT	GAT	CAG	CTG	ACA	TCA	TAT	GAC	AGA	CGC	CGC	TCA	GCC	<u>ACA</u>	<u>ATG</u>	<u>ACG</u>	48
<b>1</b>															<b>M</b>	<b>T</b>	<b>2</b>
49	AGT	GTA	GGC	CAC	ATG	GAG	CAG	ATC	GCA	GCG	GTG	CAG	CGT	GAC	TAC	ATC	96
<b>3</b>	<b>S</b>	<b>V</b>	<b>G</b>	<b>H</b>	<b>M</b>	<b>E</b>	<b>Q</b>	<b>I</b>	<b>A</b>	<b>A</b>	<b>V</b>	<b>Q</b>	<b>R</b>	<b>D</b>	<b>Y</b>	<b>I</b>	<b>18</b>
97	GCA	CAG	CCA	CGC	CTC	TGC	TAC	AAG	ACT	GTC	ACC	GGG	GTC	AAT	GGG	CCG	144
<b>19</b>	<b>A</b>	<b>Q</b>	<b>P</b>	<b>R</b>	<b>L</b>	<b>C</b>	<b>Y</b>	<b>K</b>	<b>T</b>	<b>V</b>	<b>T</b>	<b>G</b>	<b>V</b>	<b>N</b>	<b>G</b>	<b>P</b>	<b>34</b>
145	CTT	GTC	ATT	CTC	GAT	GAT	GTC	AAG	TTC	CCA	AAG	TTT	GCC	GAG	ATT	GTC	192
<b>35</b>	<b>L</b>	<b>V</b>	<b>I</b>	<b>L</b>	<b>D</b>	<b>D</b>	<b>V</b>	<b>K</b>	<b>F</b>	<b>P</b>	<b>K</b>	<b>F</b>	<b>A</b>	<b>E</b>	<b>I</b>	<b>V</b>	<b>50</b>
193	AAC	CTT	CAC	CTG	GCG	GAC	GGG	ACG	GAG	CGG	AAG	GGT	CAG	GTG	TTG	GAG	240
<b>51</b>	<b>N</b>	<b>L</b>	<b>H</b>	<b>L</b>	<b>A</b>	<b>D</b>	<b>G</b>	<b>T</b>	<b>E</b>	<b>R</b>	<b>K</b>	<b>G</b>	<b>Q</b>	<b>V</b>	<b>L</b>	<b>E</b>	<b>66</b>
241	GTC	AGC	GGC	TCC	AAG	GCT	GTG	GTG	CAG	GTG	TTT	GAG	GGA	ACA	TCA	GGT	288
<b>67</b>	<b>V</b>	<b>S</b>	<b>G</b>	<b>S</b>	<b>K</b>	<b>A</b>	<b>V</b>	<b>V</b>	<b>Q</b>	<b>V</b>	<b>F</b>	<b>E</b>	<b>G</b>	<b>T</b>	<b>S</b>	<b>G</b>	<b>82</b>
289	GTG	GAT	GCC	AAA	TAC	ACG	GTG	TGC	GAG	TTC	ACC	GGG	GAC	ATT	CTG	AGG	336
<b>83</b>	<b>V</b>	<b>D</b>	<b>A</b>	<b>K</b>	<b>Y</b>	<b>T</b>	<b>V</b>	<b>C</b>	<b>E</b>	<b>F</b>	<b>T</b>	<b>G</b>	<b>D</b>	<b>I</b>	<b>L</b>	<b>R</b>	<b>98</b>
337	ACC	CCG	GTA	TCT	GAG	GAC	ATG	CTG	GGC	CGA	GTG	TTT	AAC	GGG	TCC	GGC	384
<b>99</b>	<b>T</b>	<b>P</b>	<b>V</b>	<b>S</b>	<b>E</b>	<b>D</b>	<b>M</b>	<b>L</b>	<b>G</b>	<b>R</b>	<b>V</b>	<b>F</b>	<b>N</b>	<b>G</b>	<b>S</b>	<b>G</b>	<b>114</b>
385	AAA	CCC	ATC	GAC	CAG	GGC	CCC	TCA	GTG	CTG	GCA	GAG	GAC	TTC	CTT	GAC	432
<b>115</b>	<b>K</b>	<b>P</b>	<b>I</b>	<b>D</b>	<b>Q</b>	<b>G</b>	<b>P</b>	<b>S</b>	<b>V</b>	<b>L</b>	<b>A</b>	<b>E</b>	<b>D</b>	<b>F</b>	<b>L</b>	<b>D</b>	<b>130</b>
433	ATC	CAG	GGC	CAG	CCC	ATC	AAC	CCC	TGG	TCC	CGC	ACA	TAC	CCT	GAG	GAG	480
<b>131</b>	<b>I</b>	<b>Q</b>	<b>G</b>	<b>Q</b>	<b>P</b>	<b>I</b>	<b>N</b>	<b>P</b>	<b>W</b>	<b>S</b>	<b>R</b>	<b>T</b>	<b>Y</b>	<b>P</b>	<b>E</b>	<b>E</b>	<b>146</b>
481	ATG	ATT	CAG	ACC	GGC	ATC	TCT	TCC	ATT	GAT	GTC	ATG	AAC	TCC	ATT	GCC	528
<b>147</b>	<b>M</b>	<b>I</b>	<b>Q</b>	<b>T</b>	<b>G</b>	<b>I</b>	<b>S</b>	<b>S</b>	<b>I</b>	<b>D</b>	<b>V</b>	<b>M</b>	<b>N</b>	<b>S</b>	<b>I</b>	<b>A</b>	<b>162</b>
529	AGA	GGG	CAG	AAG	ATC	CCC	ATA	TTC	TCG	GCG	GCC	GGT	CTT	CCC	CAC	AAT	576
<b>163</b>	<b>R</b>	<b>G</b>	<b>Q</b>	<b>K</b>	<b>I</b>	<b>P</b>	<b>I</b>	<b>F</b>	<b>S</b>	<b>A</b>	<b>A</b>	<b>G</b>	<b>L</b>	<b>P</b>	<b>H</b>	<b>N</b>	<b>178</b>
577	GAA	ATC	GCC	GCC	CAG	ATC	TGT	CGT	CAG	GCG	GGT	CTG	GTG	AAG	CTG	CCC	624
<b>179</b>	<b>E</b>	<b>I</b>	<b>A</b>	<b>A</b>	<b>Q</b>	<b>I</b>	<b>C</b>	<b>R</b>	<b>Q</b>	<b>A</b>	<b>G</b>	<b>L</b>	<b>V</b>	<b>K</b>	<b>L</b>	<b>P</b>	<b>194</b>
625	AGC	AAG	GGA	GTC	CAT	GAT	GAC	CAC	AAG	GAT	AAC	TTT	GCC	ATC	GTG	TTT	672
<b>195</b>	<b>S</b>	<b>K</b>	<b>G</b>	<b>V</b>	<b>H</b>	<b>D</b>	<b>D</b>	<b>H</b>	<b>K</b>	<b>D</b>	<b>N</b>	<b>F</b>	<b>A</b>	<b>I</b>	<b>V</b>	<b>F</b>	<b>210</b>
673	GCC	GCC	ATG	GGT	GTC	AAC	ATG	GAG	ACT	GCC	AGG	TTC	TTC	AAG	CAG	GAC	720
<b>211</b>	<b>A</b>	<b>A</b>	<b>M</b>	<b>G</b>	<b>V</b>	<b>N</b>	<b>M</b>	<b>E</b>	<b>T</b>	<b>A</b>	<b>R</b>	<b>F</b>	<b>F</b>	<b>K</b>	<b>Q</b>	<b>D</b>	<b>226</b>
721	TTT	GAG	GAG	AAC	GGG	TCC	ATG	GAG	AAC	GTG	TGT	CTC	TTC	CTC	AAC	CTT	768
<b>227</b>	<b>F</b>	<b>E</b>	<b>E</b>	<b>N</b>	<b>G</b>	<b>S</b>	<b>M</b>	<b>E</b>	<b>N</b>	<b>V</b>	<b>C</b>	<b>L</b>	<b>F</b>	<b>L</b>	<b>N</b>	<b>L</b>	<b>242</b>
769	GCC	AAC	GAC	CCC	ACC	ATT	GAG	CGC	ATC	ATC	ACC	CCT	CGC	CTC	GCC	CTC	816
<b>243</b>	<b>A</b>	<b>N</b>	<b>D</b>	<b>P</b>	<b>T</b>	<b>I</b>	<b>E</b>	<b>R</b>	<b>I</b>	<b>I</b>	<b>T</b>	<b>P</b>	<b>R</b>	<b>L</b>	<b>A</b>	<b>L</b>	<b>258</b>
817	ACC	ACC	GCT	GAG	TAC	CTC	GCC	TAT	CAG	TGT	GAG	AAG	CAC	GTG	CTC	ATT	864
<b>259</b>	<b>T</b>	<b>T</b>	<b>A</b>	<b>E</b>	<b>Y</b>	<b>L</b>	<b>A</b>	<b>Y</b>	<b>Q</b>	<b>C</b>	<b>E</b>	<b>K</b>	<b>H</b>	<b>V</b>	<b>L</b>	<b>I</b>	<b>274</b>
865	ATC	CTC	ACC	GAC	ATG	TCC	TCC	TAT	GCT	GAG	GCT	CTT	CGT	GAG	GTT	TCT	912
<b>275</b>	<b>I</b>	<b>L</b>	<b>T</b>	<b>D</b>	<b>M</b>	<b>S</b>	<b>S</b>	<b>Y</b>	<b>A</b>	<b>E</b>	<b>A</b>	<b>L</b>	<b>R</b>	<b>E</b>	<b>V</b>	<b>S</b>	<b>290</b>
913	GCT	GCC	CGA	GAG	GAG	GTG	CCC	GGC	CGC	CGT	GGT	TTC	CCA	GGT	TAC	ATG	960
<b>291</b>	<b>A</b>	<b>A</b>	<b>R</b>	<b>E</b>	<b>E</b>	<b>V</b>	<b>P</b>	<b>G</b>	<b>R</b>	<b>R</b>	<b>G</b>	<b>F</b>	<b>P</b>	<b>G</b>	<b>Y</b>	<b>M</b>	<b>306</b>

Fig. 2. Nucleotide sequence and predicted amino acid sequence of V-ATPase B-subunit amplified from gills of the shore crab *Carcinus maenas*. The longer L-form is shown here. The shorter K-form is identical except that it lacks 74 nucleotides at position 1870–1944 (shown bracketed in red). A Kozak (Kozak, 1991) initiation sequence surrounding the putative start codon (ACAATGA) is underlined in green. A red asterisk indicates the probable stop codon. ARE (adenosine- and uridine-rich element) degradation motifs (ATTTA) in the 3'-untranslated region are underlined in blue. Splicing consensus sequences are shown in green rectangles. A polyadenylation signal (AATAAA) is indicated within an elongated black rectangle. The locations of the degenerate primers used in the initial PCR amplification are shown as blue arrows.

two distinct cDNA fragments of different sizes (Fig. 1). The possibility that these products resulted from genomic DNA contamination was discounted by observing that no double bands appeared after PCR amplification with any of the oligonucleotide primers targeted towards regions other than the 3'-UTR. The complete cDNA sequences of both isoforms – the larger isoform called the L-form (2520 bp; GenBank Accession Number AF247971), the shorter isoform called the K-form (2446 bp; GenBank Accession Number AF189779) – are identical except for the insertion of 74 nucleotides at position 1870–1944 in the L-form. Both forms contain an identical open reading frame coding for a protein of 489 amino acid residues (Fig. 2) with a deduced molecular mass of 54.5 kDa. PCR reactions employing the specific primer combination

HATF1550/HATR1960 to amplify sequences from both isoforms revealed that both the L-form and the K-form are expressed in anterior and posterior gills of brackish-water and seawater-adapted crabs. It also appears that the L-form is more highly expressed than the K-form in all tissues (Fig. 1B).

In both B-subunit isoforms, the cDNA sequence contains a motif with an adenine residue at the –3 position from the start codon, resembling a Kozak initiation sequence (Kozak, 1991) with an adenine instead of a guanine residue at the +4 position (ACAATGA; Fig. 2). In the 3'-UTR 20 base pairs (bp) upstream from the poly(A<sup>+</sup>) site, a polyadenylation signal (AATAAA) was found; this is the most frequently observed polyadenylation signal in eukaryotic mRNAs (Graber et al., 1999). In the 3'-UTR, several adenosine- and uridine-rich



961	TAC	ACT	GAC	TTG	GCC	ACC	ATC	TAT	GAG	CGT	GCG	GGC	AGA	GTG	GAA	GGT	1008
307	Y	T	D	L	A	T	I	Y	E	R	A	G	R	V	E	G	322
1009	CGC	CAG	GGC	TCC	ATC	ACC	CAG	ATC	CCC	ATC	CTT	ACT	ATG	CCT	AAT	GAT	1056
323	R	Q	G	S	I	T	Q	I	P	I	L	T	M	P	N	D	338
	<u>HATR4</u>																
1057	GAC	ATC	ACT	CAT	CCC	ATC	CCT	GAT	CTG	ACG	GGT	TAC	ATC	ACG	GAG	GGA	1104
339	D	I	T	H	P	I	P	D	L	T	G	Y	I	T	E	G	354
1105	CAG	ATC	TAC	GTG	GAG	CGA	CAG	TTG	CAC	AAC	CGT	CAA	ATT	TAC	CCT	CCC	1152
355	Q	I	Y	V	E	R	Q	L	H	N	R	Q	I	Y	P	P	370
1153	ATC	AAC	GTG	CTG	CCT	TCT	CTC	TCC	CGA	CTC	ATG	AAG	TCT	GCC	ATC	GGT	1200
371	I	N	V	L	P	S	L	S	R	L	M	K	S	A	I	G	386
1201	GAG	GGC	ATG	ACC	CGC	AAG	GAC	CAC	TCT	GAC	GTG	TGC	AAC	CAG	CTG	TAT	1248
387	E	G	M	T	R	K	D	H	S	D	V	C	N	Q	L	Y	402
1249	GCC	TGC	TAT	GCC	ATT	GCC	AAG	GAT	CTG	CAG	GCC	ATG	AAA	GCC	GTG	GTG	1296
403	A	C	Y	A	I	A	K	D	L	Q	A	M	K	A	V	V	418
1297	GGT	GAG	GAG	GCT	CTT	ACC	GCC	GAT	GAT	TTG	CTC	TAC	CTG	GAG	TTC	TTG	1344
419	G	E	E	A	L	T	A	D	D	L	L	Y	L	E	F	L	434
1345	GCC	AAG	TTT	GAG	AAG	TCT	TTC	ATC	TCT	CAA	GGG	TCG	TAC	ATG	AAG	CGC	1392
435	A	K	F	E	K	S	F	I	S	Q	G	S	Y	M	K	R	450
1393	ACC	ATC	TTT	GAG	TCC	CTA	GAC	ATT	GGG	TGG	CAG	CTG	TTG	CGT	ATC	TTC	1440
451	T	I	F	E	S	L	D	I	G	W	Q	L	L	R	I	F	466
1441	CCC	AAG	GAG	ATG	CTG	AAG	CGT	ATC	CCG	GCC	TCC	ACG	CTG	GCC	GAG	TTC	1488
467	P	K	E	M	L	K	R	I	P	A	S	T	L	A	E	F	482
1489	TAC	CCT	CGA	GAC	CGT	CCT	CAG	TAA	GGG	GAT	GAG	AGA	GGC	TCC	AGT	ATT	1536
483	Y	P	R	D	R	P	Q	*									489
1537	TGG	GTG	GAT	TTG	AAA	TCA	GCT	GGT	TGT	TTG	TTG	TGT	CTC	AAG	TGT	CTT	1584
1585	GTT	GAT	CCA	CAG	TTG	TGG	TAA	AAC	ACG	GCT	AGC	AGC	ATG	CTA	TGT	ATG	1632
1633	TGT	GTG	TGT	GTG	TGG	AAT	TGT	TAT	AAG	GTA	CTC	GAA	CTT	ACA	TTC	ATG	1680
1681	TGT	CCA	GTA	GTA	GGG	ATG	TAC	TGT	AAA	AAA	TTT	AAT	ATT	GTT	GCC	ATT	1728
1729	TTA	TGT	AAG	ATT	TTA	TTT	CTA	AAT	TAC	GGA	GAT	AAA	CCC	TGT	TGT	ATT	1776
1777	TAT	ACA	GAG	TAT	ATT	TTA	TTT	ATT	TTG	GAT	GTT	GAC	ATC	ACA	ATT	GTA	1824
1825	TAG	AAG	CAA	TTT	GCA	GTC	ATG	TTA	TCT	GTA	GTA	AGA	TAC	AAT	GAA	G[CT]	1872
1873	GCA	GAA	GAA	AGA	CCA	TTT	ATG	TTA	AAG	TTT	TAT	GCC	TTC	AGG	GAA	ATT	1920
1921	CTA	AGT	ACT	TAT	TAC	TGA	CTA	GAG	GTA	TGT	CTG	TAC	TAT	AAT	AAT	GAT	1968
1969	TGT	AGC	AAC	CTG	TGG	AAG	CAT	AAT	GTC	CTA	CAC	TCC	CTC	CTT	CCT	TCA	2016
2017	CTT	GTC	ATG	GTC	ATT	ACT	TAA	CAT	GGA	CTC	AGC	AGG	CCA	CCT	GAC	ACA	2064
2065	GAC	CTG	CTT	ACC	CCA	GCA	GGT	CAA	GTC	CTT	CAT	GAA	GAG	TGG	TGT	AGG	2112
2113	CTG	GGC	CCA	GGT	GTG	TGG	TGG	GGA	GGG	TGC	CTC	CTT	AAC	AAC	TGT	CCT	2160
2161	TCA	TGA	TGA	TTA	AAG	TTT	TGT	GTA	GGT	TGT	AGA	CAT	AAT	GCT	GTT	GTT	2208
2209	GCT	GAG	CAC	AAG	TTG	TTG	TAG	TTA	GGT	GCA	GTT	GTA	TAT	ATA	AAG	TAG	2256
2257	AGA	AAG	AGT	TGT	GTG	GTG	AGC	ATA	ATG	GGT	TCG	AGG	GTC	TGT	GTC	TGC	2304
2305	ATT	AGT	TTG	TTG	GAG	GAA	ATT	CTT	TGT	GTA	CAT	TCC	AGA	TGT	TGA	TGC	2352
2353	AGC	TGC	ATT	TAA	TTA	AGA	GAT	TAC	TTT	GTC	ATC	TCT	GTT	GTT	CTG	TTA	2400
2401	ATA	CTG	TGT	ATA	ATG	AGG	TCT	CGG	TTA	CAG	TCT	GGG	TGG	GTT	AGT	GTT	2448
2449	TTA	ATC	TTG	TGG	AAA	CAC	TGA	CTC	TGT	GGT	ATT	AAT	AAA	GTG	TAT	AGT	2496
2497	AAA	TGA	AAA	AAA	AAA	AAA	AAA	AAA									2520

elements (AREs) with the sequence ATTTA were found. The abundance of ARE sequences in the 3'-UTR appears to be inversely related to the lifetime of eukaryotic mRNAs (Chen and Shyu, 1995). An additional ATTTA motif was found within the additional 74 bases of the L-form. This 74-base segment appears to represent an unspliced intron, carrying the typical splicing consensus motifs with AGCT at the 5' splicing site, TACTGAC within the sequence and AGG at the 3' splicing site (Padgett et al., 1986) (Fig. 2). Retention of the 74-nucleotide insert with its additional ARE may relate to regulation of mRNA degradation rates, but the functional significance of the two isoforms is completely unknown.

Employing the putative B-subunit nucleotide sequence in a BLAST search of GenBank (Altschul et al., 1997) revealed

extremely high similarities to sequences all identified as the B-subunit of a V-ATPase. The derived *Carcinus maenas* B-subunit amino acid sequence showed a slightly higher similarity to the so-called 'brain-type' isoform B2 from human and cow (82% identity) than to the 'kidney-type' isoform B1 (78% identity; Fig. 3A). However, a repeated motif (GGGGG)<sub>3</sub> found in human 'brain-type' B-subunit cDNA approximately 72 bp upstream from the putative polyadenylation signal (Bernasconi et al., 1990) is not present in the crab 3'-UTR region. Alignment of the predicted *Carcinus maenas* B-subunit amino acid sequence with representatives of invertebrate, plant and fungal species revealed a high degree of sequence conservation, ranging from 71 to 89% (Fig. 3B).

Table 1. Oligonucleotide primers employed to amplify B-subunit (V-ATPase) and arginine kinase cDNA

Primer	Nucleotide sequence (5'→3')	Position in final cDNA
(A) V-ATPase		
Degenerate sense primer		
HATF2	GCN ATG GGN GTN AAY ATG GA	677–696
Specific sense primers		
HATF1550	CCA GTA TTT GGG TGG ATT TGA	1529–1549
HATF1770	AAA TTA CGG AGA TAA ACC CTG	1749–1769
HATF1950	CCC TCC TTC CTT CAC TTG ACA	2003–2023
HATESF1	GCA GGA CTT TGA GGA GAT GG	27–47
Degenerate antisense primer		
HATR4	TGN GTD ATR TCR TCG TTN GG	1048–1067
Specific antisense primers		
HATR392	ACC CAG ACT GTA ACC GAG AC	2418–2437
HATESR1	TGT AAC CAG GGA AAC CAC GAC	251–271
HATR1960	GTA ATG CCC ATG TCA AGT GAA	2012–2032
(B) Arginine kinase		
Specific sense primer		
AKF51	CGC TGA GTC TAA GAA GGG ATT	7–27
Specific antisense primers		
AKR31	GAT ACC GTC CTG CAT CTC CTT	1075–1095
AKESR1	GCT GTA CCT GCC AGC GAC CT	925–944

Locations of V-ATPase primers in the final B-subunit cDNA sequence from *Carcinus maenas* are presented.

Positions of arginine kinase primers are based on published sequences (GenBank Accession Numbers AF167313 and AF233356). D, A/G/T; H, A/C/T; I, A/C/G/T; N, A/C/G/T; R, A/G; Y, C/T.

The degenerate primers HATF2 and HATR4 were used to amplify putative fragments of B-subunit cDNA from several crab species differing in their degree of euryhalinity, including *Callinectes sapidus* (GenBank Accession Number AF189780), *Eriocheir sinensis* (GenBank Accession Number AF189782), *Chasmagnathus granulatus* (GenBank Accession Number AF189783) and *Cancer irroratus* (GenBank Accession Number AF189781). These sequences were used as query sequences for gapped BLAST searches (Altschul et al., 1997) and were all identified as B-subunit cDNAs with high bit-scores greater than 200. A phylogenetic analysis of the B-subunit cDNA sequences revealed the expected relationship between these species, with *Carcinus* and *Callinectes* in the family of the Portunidae, *Eriocheir* and *Chasmagnathus* in the family of the Grapsidae, and *Cancer* relatively distant from the other species in the family of the Cancridae (Fig. 4).

To determine whether the predicted B-subunit protein can be found in the shore crab, western blot analysis was performed employing membrane fractions from anterior and posterior gills from brackish-water-adapted (10‰ salinity) *Carcinus maenas*. After SDS-PAGE electrophoresis and transfer to nitrocellulose membranes, a monoclonal antibody against yeast 60 kDa B-subunit cross-reacted with proteins from the gill membrane fraction, producing two distinct bands with calculated sizes of approximately 59 kDa and 48 kDa (Fig. 5). Since the molecular mass of the B-subunit predicted from the *Carcinus maenas* cDNA sequence was calculated to be 54.5 kDa, we assume that the 59 kDa band on the western blot represents the B-subunit from crab gill. The smaller 48 kDa

band is probably a proteolytic product of the B-subunit, even though aprotinin was included in the medium used to prepare the membrane fraction (Lucu and Flik, 1999). The monoclonal antibody against yeast 57 kDa B-subunit probably recognizes the corresponding target in *Carcinus maenas* because the amino acid sequences of the two proteins are 76% identical (Nelson et al., 1989).

To investigate the subcellular location of the V-ATPase protein in epithelial cells of *Carcinus maenas* gill, the monoclonal antibody against the B-subunit was used to probe semi-thin cryosections (0.5 µm) from anterior and posterior gills of brackish-water-acclimated shore crabs. As analyzed by light microscopy, labeling by the antibody was found

Fig. 3. (A) Alignment of *Carcinus maenas* V-ATPase B-subunit amino acid sequence with mammalian isoforms including human brain-type (NP\_001684), bovine brain-type (AAA30400), human kidney-type (P15313), and bovine kidney-type (P31407). The background color indicates the degree of agreement between the five sequences: red 5/5, blue 4/5, green 3/5. Blue arrows indicate homologies of the *Carcinus maenas* amino acid sequence with brain-type isoforms and red arrows with kidney-type isoforms. (B) Alignment of *Carcinus maenas* V-ATPase B-subunit amino acid sequence with representatives of invertebrate, plant and fungal species, including *Drosophila melanogaster* (S25167), *Manduca sexta* (S24387), *Caenorhabditis elegans* (T34226), *Arabidopsis thaliana* (A31886), *Saccharomyces cerevisiae* (S45996) and *Plasmodium falciparum* (U03915). Accession numbers are given in parentheses. Alignments were produced with ClustalW and GeneDoc software.

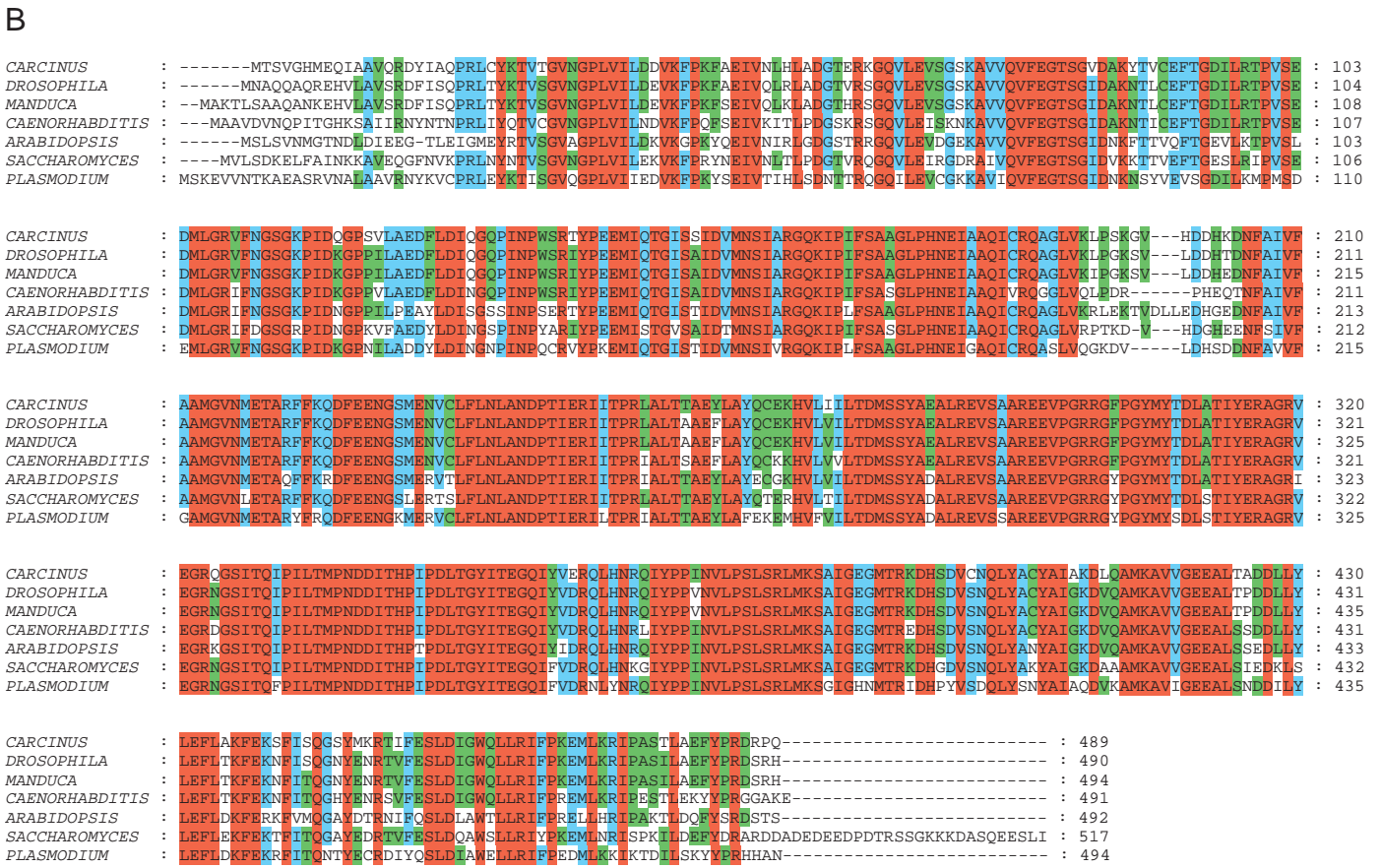
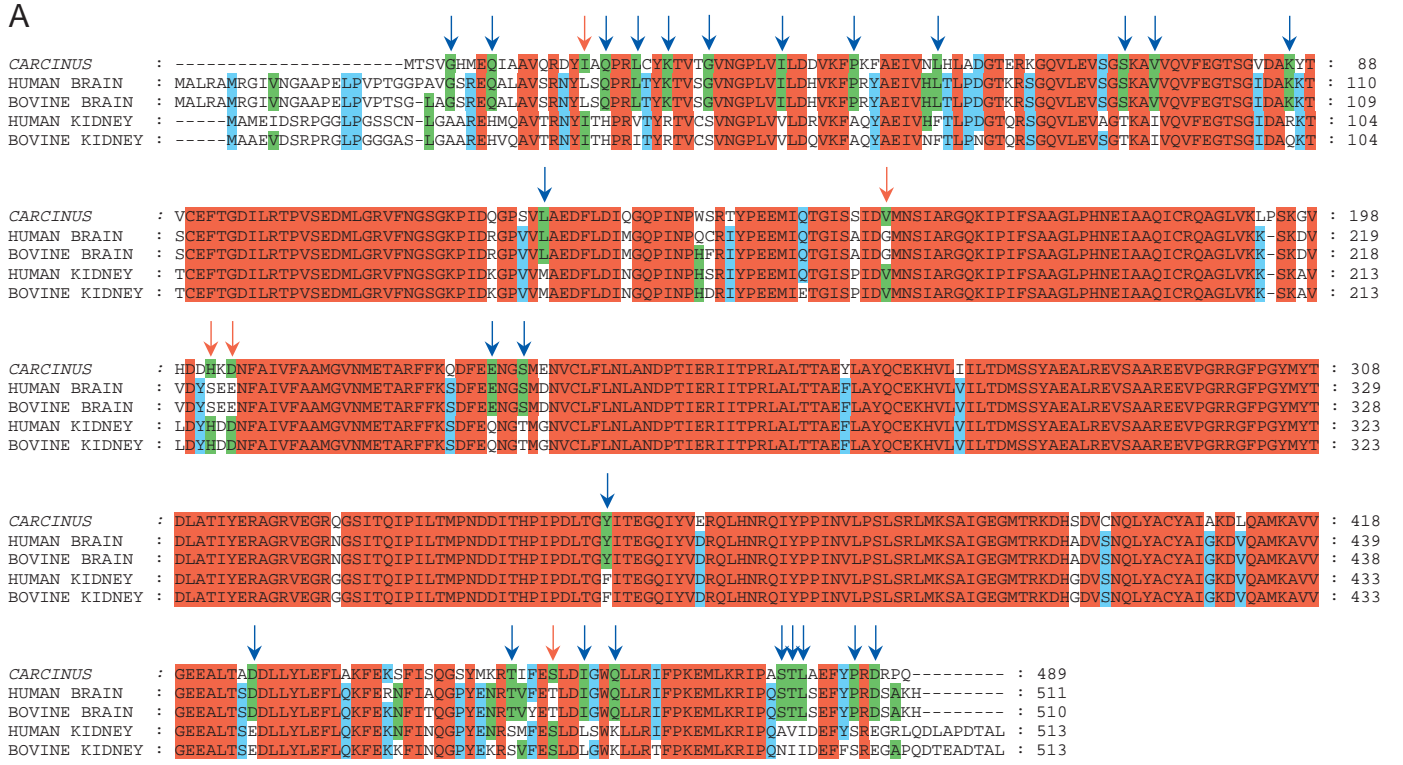


Fig. 3

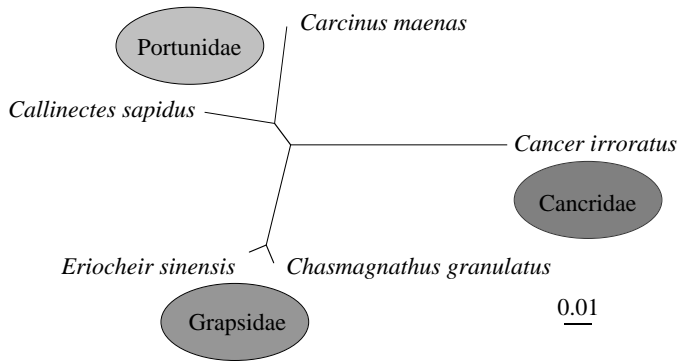


Fig. 4. Phylogenetic relationships of partial V-ATPase B-subunit nucleotide sequences obtained from five crab species: shore crab (*Carcinus maenas*), blue crab (*Callinectes sapidus*), Chinese mitten crab (*Eriocheir sinensis*), South American estuarine crab (*Chasmagnathus granulatus*) and rock crab (*Cancer irroratus*). Representative sequences of identical size were compared. The phylogenetic tree was produced using ClustalW and TreeView software. The scale represents a 1% nucleotide difference.

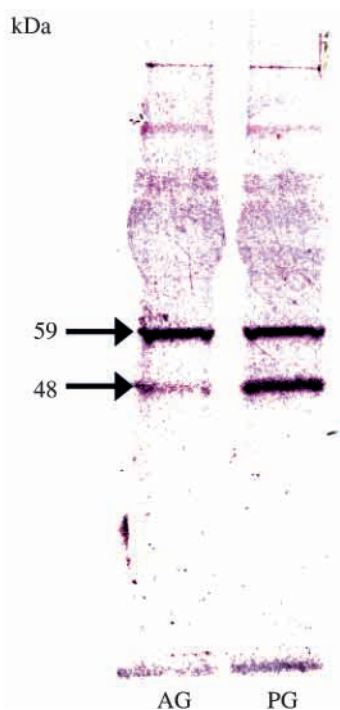


Fig. 5. Western blot analysis of V-ATPase B-subunit protein in a crude membrane fraction prepared from anterior (AG) and posterior (PG) gills of shore crab (*Carcinus maenas*). Two bands of approximately 59 and 48 kDa were evident, the larger probably corresponding to the amino acid sequence predicted from the B-subunit cDNA.

throughout the cytoplasmic compartment of both pillar cells and epithelial cells (Fig. 6). No particular accumulation of V-ATPase was detected at the apical membrane; indeed, the region adjacent to the apical membrane appeared to be deficient in V-ATPase reactivity. The distribution of V-ATPase in posterior gills, believed to function primarily in

osmoregulatory ion transport, was similar to that in anterior gills, thought to function predominantly in gas exchange (Taylor and Taylor, 1992).

To clarify whether V-ATPase gene expression is affected by osmoregulatory challenge, B-subunit mRNA abundance in gills of brackish-water- (10‰ salinity) and seawater- (35‰ salinity) adapted crabs was compared with the mRNA abundance of arginine kinase, whose mRNA expression and enzyme activity are not affected by salinity change (Kotlyar et al., 2000). The results of this semi-quantitative analysis using biotinylated PCR products in the linear phase of amplification (Towle et al., 1997) show that the method is quite sensitive to template availability, easily revealing differences in mRNA abundance of 25% or less (Fig. 7A). The estimated relative abundance of V-ATPase B-subunit mRNA in anterior gill was shown to be somewhat higher than in posterior gills in both brackish-water- and seawater-adapted *Carcinus maenas*, with increasing differences noted in low salinity (Fig. 7B,C). These results contrast with the relative expression of B-subunit mRNA in gills of Chinese crab *E. sinensis*, in which posterior gills of crabs acclimated to salinity extremes (35‰ sea water and fresh water) demonstrate higher levels of B-subunit mRNA than anterior gills (Fig. 7B,C).

To explore the possible involvement of V-ATPase in osmoregulatory ion uptake at a physiological level in *Carcinus maenas*, isolated posterior gills from brackish-water-adapted animals were perfused and bathed with identical hemolymph-like salines, and the transepithelial potential differences ( $PD_{te}$ ) were monitored with and without the application of bafilomycin A<sub>1</sub>, a specific inhibitor of the V-ATPase (Bowman et al., 1988). Under control conditions, a  $PD_{te}$  of  $-5.9 \pm 0.7$  mV (mean  $\pm$  S.E.M.,  $N=5$ ) was measured with respect to the apical bath, indicating active uptake of NaCl across the gill epithelium (Lucu, 1990). After symmetrical application of  $1 \mu\text{mol l}^{-1}$  bafilomycin, no change in  $PD_{te}$  could be observed. A reduction in  $PD_{te}$  of approximately 50% resulted when  $\text{Na}^+/\text{K}^+$ -ATPase was blocked by application of  $5 \text{ mmol l}^{-1}$  ouabain (in the presence of bafilomycin) in the basolateral saline. After wash-out, a partial recovery of the  $PD_{te}$  was detected, indicating that the gills had not been damaged by the treatments (Fig. 8).

## Discussion

Electrophysiological and tracer flux studies on osmoregulatory ion-uptake mechanisms in *Carcinus maenas* have led to the suggestion that in this crab the activity of the basolateral  $\text{Na}^+/\text{K}^+$ -ATPase (Towle and Kays, 1986) is the major driving force for electrogenic NaCl uptake across the gill epithelium. Both net NaCl influx and  $\text{Cl}^-$ -dependent short-circuit current ( $I_{sc}$ ) are dramatically inhibited by basolateral application of ouabain, a specific inhibitor of the  $\text{Na}^+/\text{K}^+$ -ATPase (Lucu and Siebers, 1987; Riestenpatt et al., 1996). The failure to identify the presence of a V-ATPase in *Carcinus maenas* gills by enzyme activity assays (Towle and Harding, 1993) questioned the importance of a proton pump



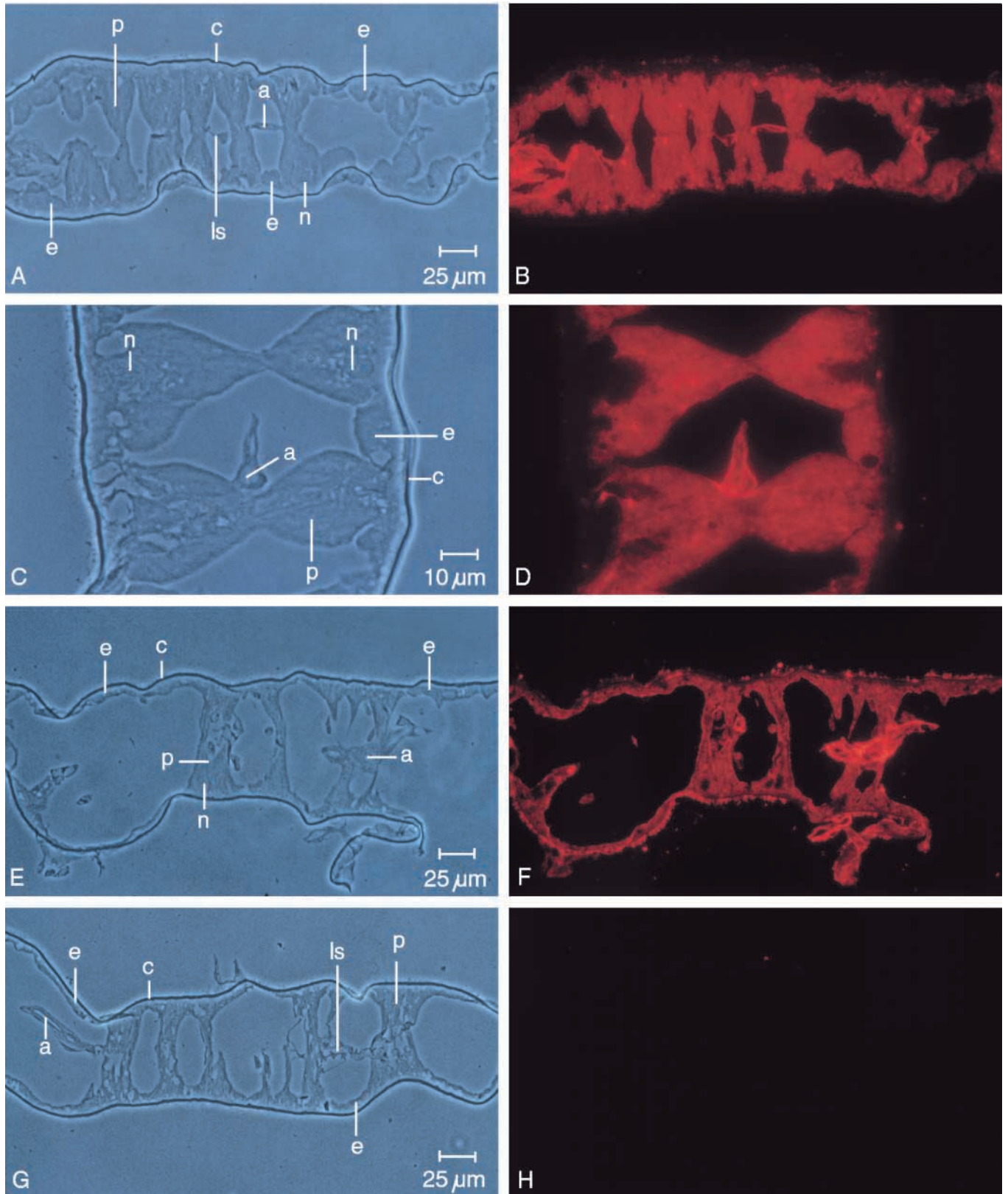


Fig. 6. Immunocytochemical analysis of V-ATPase B-subunit distribution in cryosections of posterior gill (A–D) and anterior gill (E–H) from *Carcinus maenas* acclimated to 10‰ salinity. Phase-contrast micrographs are presented in A, C, E and G. Immunocytochemical staining for B-subunit is presented in parallel micrographs B, D, F and H, with primary antibody omitted in H. p, pillar cell; c, cuticle; a, arteriole; ls, lamellar septum; e, epithelial cell; n, nucleus.

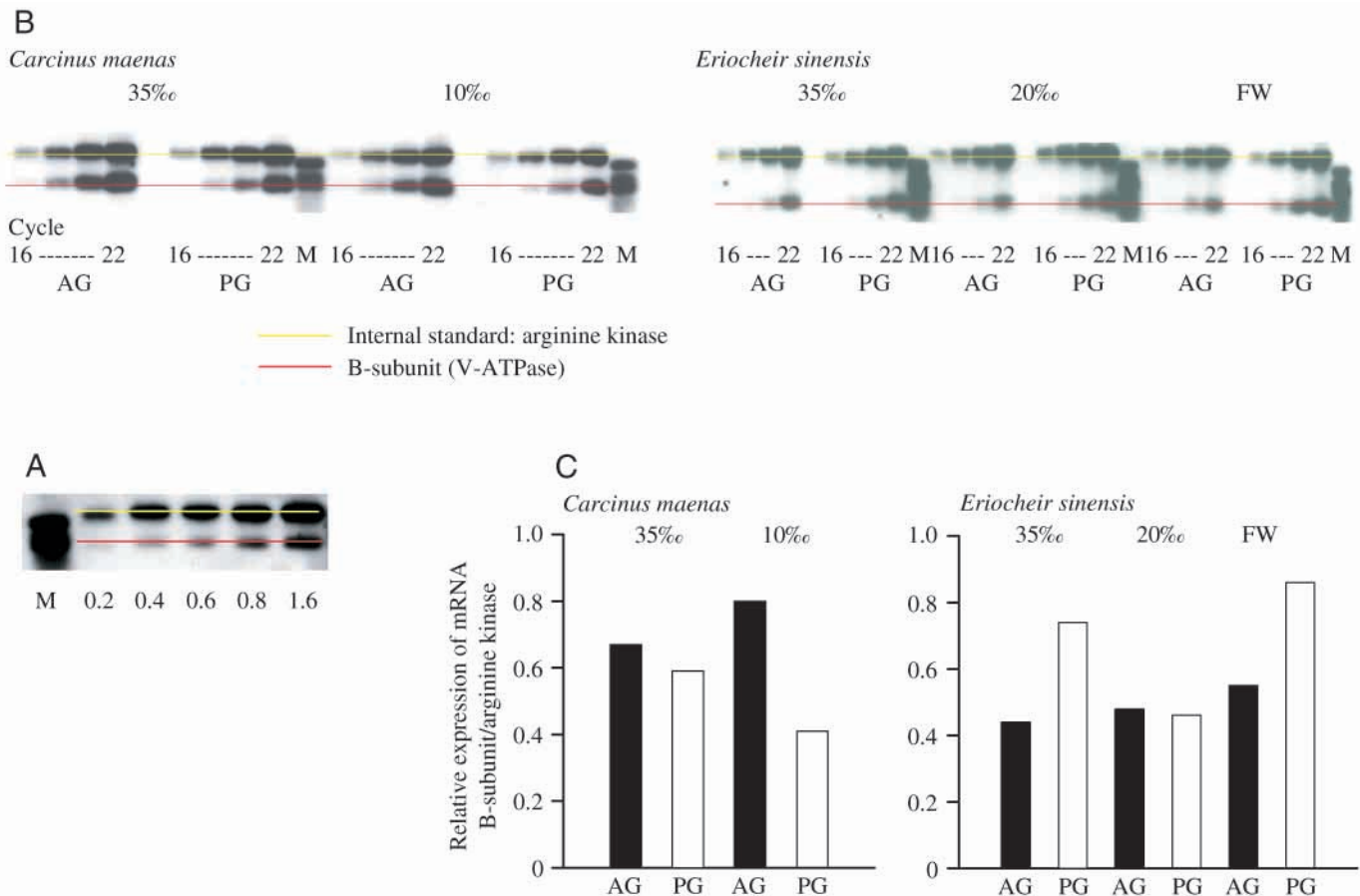


Fig. 7. Quantitative reverse transcriptase/polymerase chain reaction (RT-PCR) analysis of V-ATPase B-subunit and arginine kinase (internal standard) mRNA abundance in gill extracts obtained from *Carcinus maenas* and *Eriocheir sinensis* using duplex PCR with two primer pairs. For *Carcinus maenas*, primers HATF1950 and HATR392 amplified both V-ATPase B-subunit isoforms and AKF51 and AKR31 amplified arginine kinase cDNA. For *Eriocheir sinensis*, primers HATESF1 and HATESR1 amplified V-ATPase B-subunit and AKF51 and AKESR1 amplified arginine kinase cDNA. (A) Demonstration of template-dependent quantification of V-ATPase B-subunit (lower band) and arginine kinase (upper band) mRNA in posterior gills of *Carcinus maenas* acclimated to 10‰ salinity. Amplification proceeded with varying amounts of cDNA template (indicated as  $\mu$ l of the standard reverse transcription reaction) for 18 cycles of 92 °C (1 min), 45 °C (1 min) and 72 °C (2 min) with biotin-dUTP included in the nucleotide mixture. (B) Cycle-dependent quantification of V-ATPase B-subunit (lower band) and arginine kinase (upper band) mRNA in anterior (AG) and posterior (PG) gills of *Carcinus maenas* acclimated to 35 and 10‰ and *Eriocheir sinensis* acclimated to 35‰, 20‰ and fresh water (FW). Amplification proceeded for 16, 18, 20 and 22 cycles. (C) Digitization of biotinylated PCR product intensities using Un-Scan-Itgel software. The abundance of V-ATPase B-subunit mRNA was normalized to the abundance of arginine kinase mRNA at 18 cycles for *Carcinus maenas* and at 20 cycles for *Eriocheir sinensis*.

in osmoregulatory ion uptake in this moderately euryhaline species. Now that we have molecular evidence that the B-subunit of the catalytic  $V_1$  complex of the V-ATPase is indeed expressed in *Carcinus maenas* gills (Weihrauch and Towle, 1997), the role of this proton pump in transbranchial NaCl uptake has to be reconsidered.

Analysis of the deduced amino acid sequence of the *Carcinus maenas* V-ATPase B-subunit revealed a higher homology to the so-called 'brain-type' or  $B_2$  isoform than to the 'kidney-type' or  $B_1$  isoform (Fig. 3). In mammalian kidney, the 'brain-type' isoform is found in proximal tubules but not in intercalated cells. In contrast, the 'kidney-type' isoform is not abundant in proximal tubules but is abundant in intercalated cells (Nelson, 1991), which are specialized in  $H^+$  transport and where the V-ATPase resides in high densities in

a polarized distribution in the apical plasma membrane (Brown et al., 1992). Thus, the *Carcinus maenas* gill B-subunit resembles a mammalian isoform that is not part of an apically localized V-ATPase. But, even if the B-subunit is a very highly conserved protein (multiple alignment of the *Carcinus maenas* amino acid sequence with B-subunit sequences from other phyla showed 78–82% identities to vertebrate sequences, 77–80% identity to plant sequences, 76% identity to fungal sequences and 59% identity to Archaea A-ATPase B-subunit), the function and the location of the crustacean gill V-ATPase probably differ from those in mammalian tissues. Further evidence of high sequence homology was obtained by western blot analysis of a *Carcinus maenas* gill membrane fraction in which a monoclonal antibody against yeast V-ATPase B-subunit detected a 59 kDa protein (Fig. 5), which probably

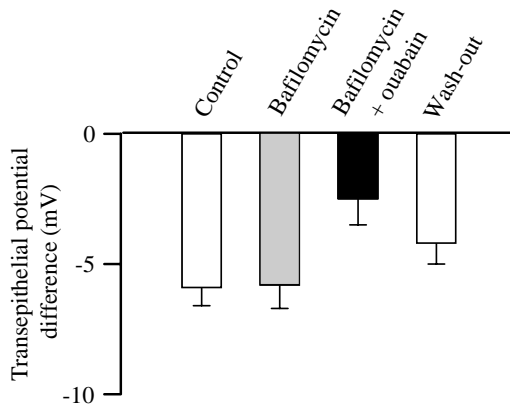


Fig. 8. Effects of the V-ATPase inhibitor bafilomycin ( $1\mu\text{mol l}^{-1}$ ) and the  $\text{Na}^+/\text{K}^+$ -ATPase inhibitor ouabain ( $5\text{mmol l}^{-1}$ ) on transepithelial potential differences measured in perfused posterior gills of *Carcinus maenas* acclimated to 10‰ salinity. Transepithelial potential differences are presented as mean + S.E.M. ( $N=5$ ).

represents the crab B-subunit whose molecular mass deduced from the cDNA sequence was calculated to be 54.5 kDa.

To localize the V-ATPase B-subunit within the gills of *Carcinus maenas*, antibody against the yeast B-subunit was employed in semi-thin cryosections of the so-called 'dark areas' representing highly differentiated transporting epithelium (Taylor and Taylor, 1992). These antibodies successfully cross-reacted with the B-subunit in the sternal epithelium of another crustacean, the land-inhabiting isopod *Porcellio scaber* (Ziegler et al., 2000). In *Carcinus maenas*, the V-ATPase B-subunit was found throughout the cytoplasm, but not in the apical membrane, in epithelial and pillar cells of both anterior and posterior gills (Fig. 6), suggesting vesicular rather than apical localization. The distribution of the V-ATPase throughout the gill epithelium is in contrast to the findings in seawater-acclimated *Eriocheir sinensis*, in which the V-ATPase is situated predominantly in the apical membrane of pillar cells (Putzenlechner, 1994). Our localization results indicate that, in *Carcinus maenas* gills, the V-ATPase probably serves in acidification of intracellular vesicles rather than in generating an electrochemical proton gradient across the apical membrane that would be expected if the V-ATPase were to function in osmoregulatory  $\text{NaCl}$  transport (Larsen et al., 1992; Lin and Randall, 1993; Zare and Greenaway, 1998).

It cannot be excluded, however, that the cytoplasmic distribution of the B-subunit may be related to the regulation of V-ATPase activity by disassembly and reassembly of the  $V_1$  and  $V_0$  domains (Kane, 1995; Merzendorfer et al., 1997; Sumner et al., 1995). During molting or starvation of *Manduca sexta* larvae, the V-ATPase situated in the apical membrane of midgut goblet cells and energizing active  $\text{K}^+$  secretion is inactivated by disassembling of the cytoplasmic  $V_1$  domain from the membrane-associated  $V_0$  domain (Wieczorek and Harvey, 1995). Therefore, during inactivation, a major proportion of the  $V_1$  is found throughout the cytoplasm (Gräf et al., 1996; Sumner et al., 1995). Since the B-subunit is part of the  $V_1$  complex, detection of this subunit in the cytoplasm

of *Carcinus maenas* gills using specific antibodies could lead to inaccurate interpretations of our findings.

Comparison of the relative expression of the B-subunit in *Carcinus maenas* gills reveals a higher abundance of the B-subunit mRNA in the respiratory anterior gills than in the osmoregulatory posterior gills, with an anterior/posterior ratio of approximately 2 for shore crabs acclimated to low salinity and 1.1 for shore crabs acclimated to full-strength sea water (Fig. 7). This is of particular interest considering the greater metabolic activities of the posterior gills, especially in diluted media, as demonstrated by the sevenfold higher activity of carbonic anhydrase (Böttcher et al., 1995) and the approximately 3.5-fold higher activity of  $\text{Na}^+/\text{K}^+$ -ATPase (Siebers et al., 1982) following a reduction of external salinity. The enhanced abundance of V-ATPase B-subunit mRNA in anterior gills may relate to our previous observation that the anterior gills are more proficient in active ammonia excretion than the posterior gills (Weihrach et al., 1998). Expression analysis shows that, in the osmoregulatory posterior gills of *Carcinus maenas*, mRNA abundance decreases slightly following acclimation from full-strength sea water to brackish water (Fig. 7), a trend that is in opposition to the increase in the rates of net  $\text{NaCl}$  uptake expected for *Carcinus maenas* acclimated to low salinity, indicating once more that the V-ATPase is probably not involved in osmoregulatory ion uptake in this species.

In contrast, the Chinese crab *Eriocheir sinensis* shows a substantially higher abundance of V-ATPase B-subunit mRNA in the posterior gills than in the anterior gills in animals acclimated to full-strength sea water or fresh water (Fig. 7). These data are in line with enzyme activity measurements of bafilomycin- $A_1$ -sensitive ATPase in posterior and anterior gills of *Eriocheir sinensis* (Putzenlechner, 1994) and are consistent with the ion-transporting ability of posterior gills in this species. Strongly hyperosmotic in fresh water and slightly hypo-osmotic in sea water (Mantel and Farmer, 1983), the Chinese crab exhibits a wider range of osmoregulatory capacity than *Carcinus maenas*. V-ATPase mRNA abundance in posterior gills was lowest at 20‰ salinity, where the  $\text{NaCl}$  concentration of hemolymph most closely matches that of the medium (Onken, 1999). It is not known whether V-ATPase enzyme activity is also reduced under these conditions. The specific activity of the V-ATPase in seawater-acclimated *Eriocheir sinensis* was reported to be approximately three times higher than in freshwater-acclimated animals (Putzenlechner, 1994), whereas the relative V-ATPase mRNA expression measured in this study was somewhat higher in fresh water than in sea water (Fig. 7). A possible explanation for this discrepancy could be a different translation rate or stability of the mRNA, which may lead to different protein abundance, or the V-ATPase may be regulated by assembly/disassembly as noted above. In any case, the substantial levels of V-ATPase mRNA and enzyme activity in the posterior gills of seawater-acclimated animals may reflect a possible involvement of the V-ATPase in  $\text{NaCl}$  excretion, as suggested for the Malpighian tubules of insects (Maddrell and O'Donnell, 1992).



Measurements of transepithelial potential difference ( $PD_{te}$ ) across symmetrically perfused crab gills have been interpreted extensively to indicate active ion transport. The steady-state  $PD_{te}$  of *Carcinus maenas* and *Eriocheir sinensis* posterior gills is altered by inhibitors of  $Na^+$  transport, including ouabain (an inhibitor of  $Na^+/K^+$ -ATPase) and amiloride (an inhibitor of the  $Na^+/H^+$  exchanger and the epithelial  $Na^+$  channel) (Lucu and Siebers, 1986; Pequeux and Gilles, 1988; Siebers et al., 1985; present study). In addition,  $Cl^-$  channel blockers applied to the basolateral side depolarize the epithelium of *Carcinus maenas* gills (Siebers et al., 1990). However,  $PD_{te}$  of isolated *Carcinus maenas* gills is not affected by the V-ATPase inhibitor bafilomycin, indicating that the components of active NaCl transport remain intact under these conditions. In contrast, active ammonia excretion by *Carcinus maenas* gills is inhibited by 66% by bafilomycin at  $1\text{ mol l}^{-1}$ , the same concentration employed in the present experiments (Weihrach et al., 2000).

In conclusion, the V-type  $H^+$ -ATPase of *Eriocheir sinensis* gills appears to possess a suite of properties that suggests its intimate involvement in osmoregulatory ion transport. However, several lines of evidence, both molecular and physiological, indicate that this is not the case for *Carcinus maenas*, a moderate osmoregulator. The salinity minimum of 8‰ observed for survival of *Carcinus maenas* may result in part from the lack of participation of V-ATPase in mediating NaCl uptake from more dilute medium. It will be interesting to discover whether freshwater-tolerant crab species other than *Eriocheir sinensis* utilize V-ATPase in this important function.

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### References

- Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W. and Lipman, D. J. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.* **25**, 3389–3402.
- Bernasconi, P., Rausch, T., Struve, I., Morgan, L. and Taiz, L. (1990). An mRNA from human brain encodes an isoform of the B-subunit of the vacuolar  $H^+$ -ATPase. *J. Biol. Chem.* **265**, 17428–17431.
- Bowman, E. J., Siebers, A. and Altendorf, K. (1988). Bafilomycins: A class of inhibitors of membrane-ATPases from microorganisms, animal cells and plant cells. *Proc. Natl. Acad. Sci. USA* **85**, 7972–7976.
- Böttcher, K., Siebers, D. and Sender, S. (1995). Carbonic anhydrase, a respiratory enzyme in the gills of the shore crab *Carcinus maenas*. *Helgoländer Meeresuntersuchungen* **49**, 737–745.
- Brown, D., Sabolic, I. and Gluck, S. (1992). Polarized targeting of V-ATPase in kidney epithelial cells. *J. Exp. Biol.* **172**, 231–243.
- Chen, C. Y. and Shyu, A. B. (1995). AU-rich elements: characterization and importance in mRNA degradation. *Trends Biochem. Sci.* **20**, 465–470.
- Chomczynski, P. and Sacchi, N. (1987). Single-step method of RNA isolation by acid guanidinium thiocyanate–phenol–chloroform extraction. *Analyt. Biochem.* **162**, 156–159.
- Forgac, M. (1989). Structure and function of vacuolar class of ATP-driven proton pumps. *Physiol. Rev.* **69**, 765–796.
- Forgac, M. (2000). Structure, mechanism and regulation of the clathrin-coated vesicle and yeast vacuolar  $H^+$ -ATPase. *J. Exp. Biol.* **203**, 71–80.
- Gilles, R., Pequeux, A. and Bianchini, A. (1988). Physiological aspects of NaCl movements in the gills of the euryhaline crab, *Eriocheir sinensis*, acclimated to fresh water. *Comp. Biochem. Physiol.* **90A**, 201–207.
- Graber, J. H., Cantor, C. R., Mohr, S. C. and Smith, T. F. (1999). In silico detection of control signals: mRNA 3'-end-processing sequences in diverse species. *Proc. Natl. Acad. Sci. USA* **96**, 14055–14060.
- Gräf, R., Harvey, W. R. and Wiczorek, H. (1996). Purification and properties of a cytosolic  $V_1$ -ATPase. *J. Biol. Chem.* **271**, 20908–20913.
- Harvey, W. R. (1992). Physiology of V-ATPases. *J. Exp. Biol.* **172**, 1–17.
- Kane, P. M. (1995). Disassembly and reassembly of the yeast vacuolar  $H^+$ -ATPase *in vivo*. *J. Biol. Chem.* **270**, 17025–17032.
- Kotlyar, S., Weihrach, D., Paulsen, R. S. and Towle, D. W. (2000). Expression of arginine kinase enzymatic activity and mRNA in gills of the euryhaline crabs *Carcinus maenas* and *Callinectes sapidus*. *J. Exp. Biol.* **203**, 2395–2404.
- Kozak, M. (1991). Structural features in eukaryotic mRNAs that modulate the initiation of translation. *J. Biol. Chem.* **266**, 19867–19870.
- Larsen, E. H., Willumsen, N. J. and Christoffersen, B. C. (1992). Role of proton pump of mitochondria-rich cells for active transport of chloride ions in toad skin epithelium. *J. Physiol., Lond.* **450**, 203–216.
- Lin, H. and Randall, D. J. (1993).  $H^+$ -ATPase activity in crude homogenates of fish gill tissue: inhibitor sensitivity and environmental and hormonal regulation. *J. Exp. Biol.* **180**, 163–174.
- Lucu, C. (1990). Ionic regulatory mechanisms in crustacean gill epithelia. *Comp. Biochem. Physiol.* **97A**, 297–306.
- Lucu, C. and Flik, G. (1999).  $Na^+$ - $K^+$ -ATPase and  $Na^+$ / $Ca^{2+}$  exchange activities in gills of hyperregulating *Carcinus maenas*. *Am. J. Physiol.* **276**, R490–R499.
- Lucu, C. and Siebers, D. (1986). Amiloride-sensitive sodium flux and potentials in perfused *Carcinus* gill preparations. *J. Exp. Biol.* **122**, 25–35.
- Lucu, C. and Siebers, D. (1987). Linkage of  $Cl^-$  fluxes with ouabain sensitive Na/K exchange through *Carcinus* gill epithelia. *Comp. Biochem. Physiol.* **87A**, 807–811.
- Maddrell, S. H. P. and O'Donnell, M. J. (1992). Insect Malpighian tubules: V-ATPase action in ion and fluid transport. *J. Exp. Biol.* **172**, 417–429.
- Mantel, L. H. and Farmer, L. L. (1983). Osmotic and ionic regulation. In *The Biology of Crustacea*, vol. 5, *Internal Anatomy*



- and *Physiological Regulation* (ed. L. H. Mantel), pp. 53–161. New York: Academic Press.
- Merzendorfer, H., Gräf, R., Huss, M., Harvey, W. R. and Wieczorek, H.** (1997). Regulation of proton-translocating V-ATPases. *J. Exp. Biol.* **200**, 225–235.
- Nelson, H., Mandiyan, S. and Nelson, N.** (1989). A conserved gene encoding the 57-kDa subunit of the yeast vacuolar H<sup>+</sup>-ATPase. *J. Biol. Chem.* **264**, 1775–1778.
- Nelson, N.** (1991). Structure and pharmacology of the proton-ATPases. *Trends Pharmac. Sci.* **12**, 71–74.
- Nelson, N.** (1992). The vacuolar H<sup>+</sup>-ATPase – one of the most fundamental ion pumps in nature. *J. Exp. Biol.* **172**, 19–27.
- Nelson, R. D., Guo, X. L., Masood, K., Brown, D., Kalkbrenner, M. and Gluck, S.** (1992). Selectively amplified expression of an isoform of the vacuolar H<sup>+</sup>-ATPase 56-kilodalton subunit in renal intercalated cells. *Proc. Natl. Acad. Sci. USA* **89**, 3541–3545.
- Onken, H.** (1999). Active NaCl absorption across split lamellae of posterior gills of Chinese crabs (*Eriocheir sinensis*) adapted to different salinities. *Comp. Biochem. Physiol.* **123A**, 377–384.
- Onken, H. and Putzenlechner, M.** (1995). A V-ATPase drives active, electrogenic and Na<sup>+</sup>-independent Cl<sup>-</sup> absorption across the gills of *Eriocheir sinensis*. *J. Exp. Biol.* **198**, 767–774.
- Padgett, R. A., Grabowski, P. J., Konarska, M. M., Seiler, S. and Sharp, P. A.** (1986). Splicing of messenger RNA precursors. *Annu. Rev. Biochem.* **55**, 1119–1150.
- Pequeux, A. and Gilles, R.** (1988). The transepithelial potential difference of isolated perfused gills of the Chinese crab *Eriocheir sinensis* acclimated to fresh water. *Comp. Biochem. Physiol.* **89A**, 163–172.
- Putzenlechner, M.** (1994). Charakterisierung und Lokalisation einer Protonen-ATPase des V-Typs in den Kiemen dekapoder Krebse (Crustacea, Decapoda). Dissertation. Berlin: Freien Universität. 119pp.
- Riestenpatt, S., Onken, H. and Siebers, D.** (1996). Active absorption of Na<sup>+</sup> and Cl<sup>-</sup> across the gill epithelium of the shore crab *Carcinus maenas*: voltage-clamp and ion-flux studies. *J. Exp. Biol.* **199**, 1545–1554.
- Riestenpatt, S., Petrausch, G. and Siebers, D.** (1995). Cl<sup>-</sup> influx across posterior gills of the Chinese crab (*Eriocheir sinensis*): Potential energization by a V-type H<sup>+</sup>-ATPase. *Comp. Biochem. Physiol.* **110A**, 235–241.
- Sanger, F., Nicklen, S. and Coulson, A. R.** (1977). DNA sequencing with chain terminating inhibitors. *Proc. Natl. Acad. Sci. USA* **74**, 5463–5467.
- Siebers, D., Leweck, K., Markus, H. and Winkler, A.** (1982). Sodium regulation in the shore crab *Carcinus maenas* as related to ambient salinity. *Mar. Biol.* **69**, 37–43.
- Siebers, D., Böttcher, K., Petrausch, G. and Hamann, A.** (1990). Effects of some chloride channel blockers on potential differences and ion fluxes in isolated perfused gills of shore crabs *Carcinus maenas*. *Comp. Biochem. Physiol.* **97A**, 9–15.
- Siebers, D., Winkler, A., Lucu Thedens, G. and Weichart, D.** (1985). Na–K-ATPase generates an active transport potential in the gills of the hyperregulating shore crab *Carcinus maenas*. *Mar. Biol.* **87**, 185–192.
- Sumner, J. P., Dow, J. A., Earley, F. G., Klein, U., Jäger, D. and Wieczorek, H.** (1995). Regulation of plasma membrane V-ATPase activity by dissociation of peripheral subunits. *J. Biol. Chem.* **270**, 5649–5653.
- Taylor, H. H. and Taylor, E. W.** (1992). Gills and lungs: The exchange of gases and ions. In *Microscopic Anatomy of Invertebrates*, vol. 10, *Decapod Crustacea* (ed. F. W. Harrison and A. G. Humes), pp. 203–293. New York: Wiley-Liss.
- Tokuyasu, K. T.** (1980). Immunocytochemistry on ultrathin frozen sections. *Histochem. J.* **12**, 381–403.
- Towle, D. W. and Harding, S.** (1993). Crab gill epithelial cells do not express a V-type H<sup>+</sup>-ATPase. *Am. Zool.* **33**, 13A (Abstract).
- Towle, D. W. and Kays, W. T.** (1986). Basolateral localization of Na<sup>+</sup>+K<sup>+</sup>-ATPase in gill epithelium of two osmoregulating crabs, *Callinectes sapidus* and *Carcinus maenas*. *J. Exp. Zool.* **239**, 311–318.
- Towle, D. W., Rushton, M. E., Heidysch, D., Magnani, J. J., Rose, M. J., Amstutz, A., Jordan, M. K., Shearer, D. W. and Wu, W. S.** (1997). Sodium–proton antiporter in the euryhaline crab *Carcinus maenas*: molecular cloning, expression and tissue distribution. *J. Exp. Biol.* **200**, 1003–1014.
- Weihrauch, D., Becker, W., Postel, U., Riestenpatt, S. and Siebers, D.** (1998). Active excretion of ammonia across the gills of the shore crab *Carcinus maenas* and its relation to osmoregulatory ion uptake. *J. Comp. Physiol. B* **168**, 364–376.
- Weihrauch, D. and Towle, D. W.** (1997). Molecular evidence for a V-type H<sup>+</sup>-ATPase in gills of the euryhaline crabs *Carcinus maenas* and *Callinectes sapidus*. *Am. Zool.* **37**, 87A (Abstract).
- Weihrauch, D., Ziegler, A., Siebers, D. and Towle, D. W.** (2000). Exocytosis, a clever mechanism for active ammonia excretion in aquatic animals. *Comp. Biochem. Physiol.* **126B**, S103 (Abstract).
- 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., Gruber, R., Harvey, W. R., Huss, M., Merzendorfer, H. and Zeiske, W.** (2000). Structure and regulation of insect plasma membrane H<sup>+</sup> V-ATPase. *J. Exp. Biol.* **203**, 127–135.
- Wieczorek, H. and Harvey, W. R.** (1995). Energization of animal plasma membranes by the proton-motive force. *Physiol. Zool.* **68**, 15–23.
- Zare, S. and Greenaway, P.** (1998). The effect of molting and sodium depletion on sodium transport and the activities of Na<sup>+</sup>K<sup>+</sup>-ATPase and V-ATPase in the freshwater crayfish *Cherax destructor* (Crustacea: Parastacidae). *Comp. Biochem. Physiol.* **119A**, 739–745.
- Ziegler, A.** (1997). Immunocytochemical localization of Na<sup>+</sup>,K<sup>+</sup>-ATPase in the calcium-transporting sternal epithelium of the terrestrial isopod *Porcellio scaber* L. (Crustacea). *J. Histochem. Cytochem.* **45**, 437–446.
- Ziegler, A., Weihrauch, D. and Bleher, R.** (2000). A V-type H<sup>+</sup>-ATPase in the calcium transporting anterior sternal epithelia cells of *Porcellio scaber*. *Eur. J. Cell Biol.* **79** (Suppl. 50), 80 (Abstract).