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MOLECULAR CHARACTERIZATION OF V-TYPE H^+-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^+-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; Wieczorek et al., 1999). The V-ATPase is composed of two structural protein domains, the cytoplasmic V_1 domain and the membrane-spanning V_0 domain (Nelson, 1992). The V_1 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_1 complex of yeast is $A_3B_3C_1D_1E_1F_1G_3H_1$ (Forgac, 2000). The membrane-embedded V_0 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 $M100_1M40_1M9.7_1M17_6$ (Wieczorek 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^+ 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 (Wieczorek 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^+/K^+-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 RNA*later* (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^+) 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^{-1} sucrose, 150 mmol l^{-1} NaCl, 0.2 mol l^{-1} 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^{-1} sucrose in 0.1 mol l^{-1} 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^{-1} sucrose in 0.1 mol l^{-1} 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^{-1} 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^{-1} 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^{-1} phosphate buffer containing 0.5 mol l^{-1} NaCl (PBS-500) and once in PBS, incubated for 1 h in biotinylated secondary antibody [anti-mouse $F(a,b')_2$ 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-It*gel* 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^{-1}): 248 NaCl, 5 $CaCl_2$, 5 KCl, 4 $MgCl_2$, 2 $NaHCO_3$ and 2.5 Tris at pH 7.8. During perfusion at a flow rate of 0.135 ml min^{-1}, transepithelial potential differences (PD_{te}) 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^+-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′-GGCCACGCGTCGACTA-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

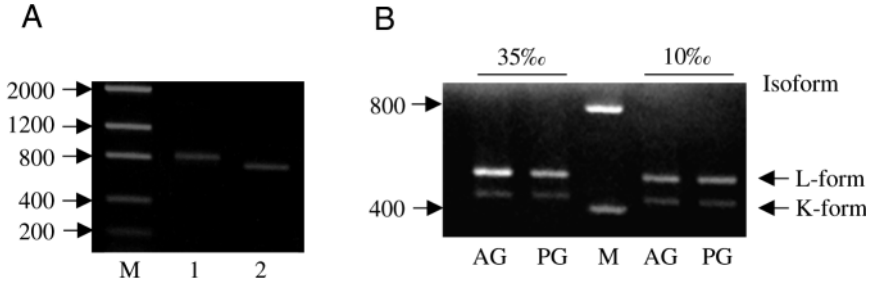

Fig. 1. Polymerase chain reaction (PCR) amplification of the 3′-untranslated region of V-ATPase B-subunit cDNA from the gills of shore crab *Carcinus maenas* using primers HATF1770 and AUAP (A) or HATF1550 and HATR1960 (B). In A, the two amplification products were separated by agarose gel electrophoresis, 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).

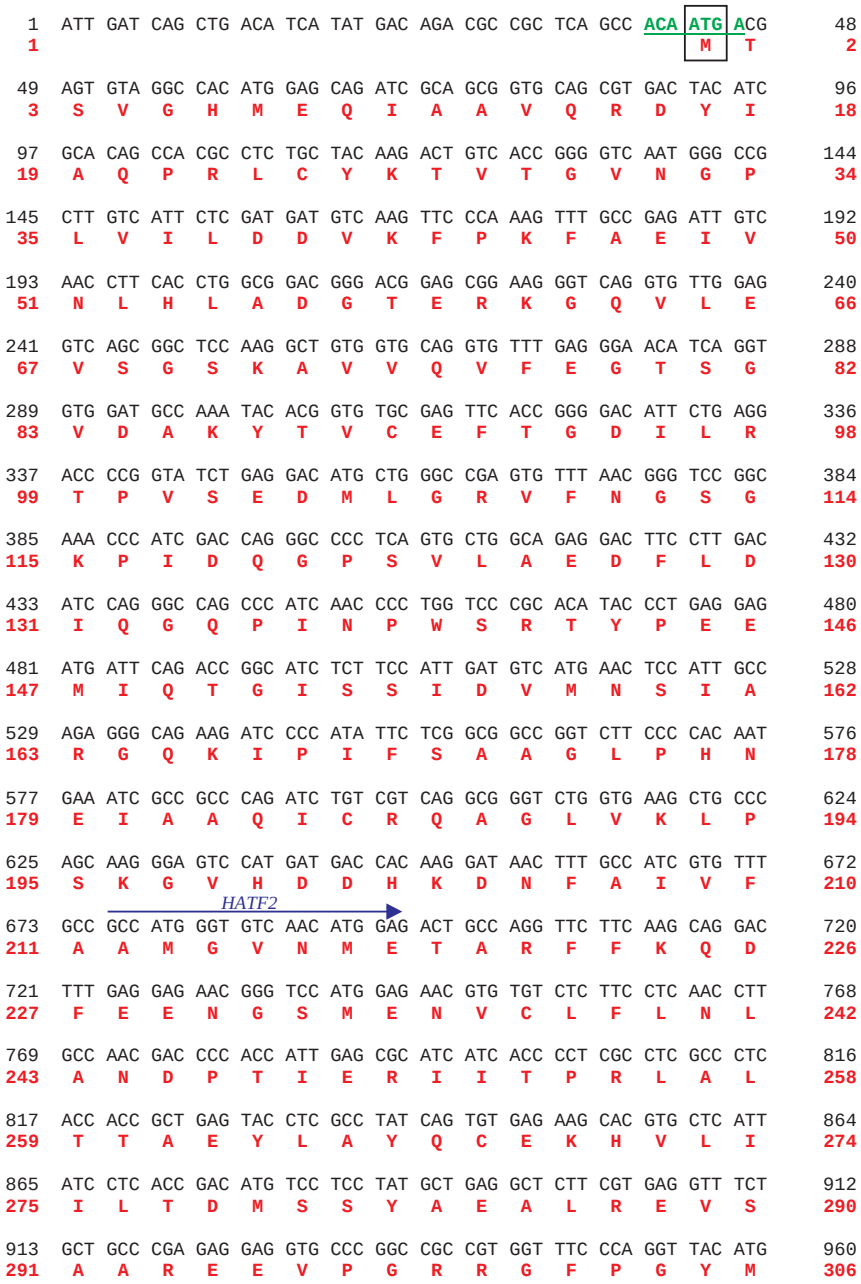

Fig. 2. Nucleotide sequence and predicted amino acid sequence of V-ATPase B-subunit amplified from gills of the shore crab *Carcinus maenas*. The longer L-form is shown here. The shorter K-form is identical except that it lacks 74 nucleotides at position 1870–1944 (shown bracketed in red). A Kozak (Kozak, 1991) initiation sequence surrounding the putative start codon (ACA*ATG*A) 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 (ACA*ATG*A; Fig. 2). In the 3′-UTR 20 base pairs (bp) upstream from the poly(A^+) 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

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 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
       HATR4
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 ATG 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
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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 $(GGGGGN)_3$ 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.

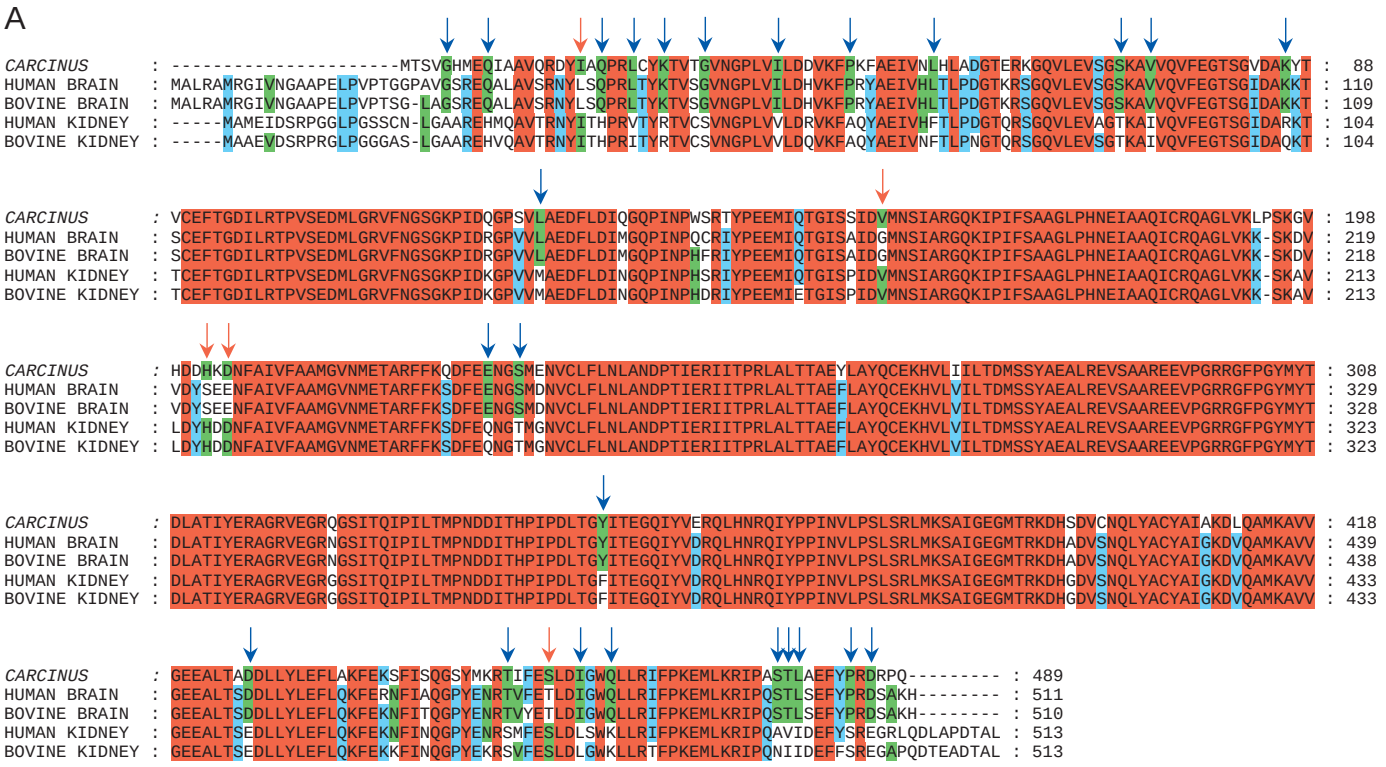
Positions of arginine kinase primers are based on published sequences (GenBank Accession Numbers AF167313 and AF233356). D, A/G/T; H, A/C/T; I, A/C/G/T; N, A/C/G/T; R, A/G; Y, C/T.

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.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HUMAN BRAIN : MALRAMRGIVNGAAPELPVPTGGPAVGSREQALAVSRNYLSQPRLTYKTVSGVNGPLVILDHVKFPRYAEIVHLTLPDGTKRSGQVLEVSGSKAVVQVFEGTSGIDAKKT : 110
BOVINE BRAIN : MALRAMRGIVNGAAPELPVPTSG-LAGSREQALAVSRNYLSQPRLTYKTVSGVNGPLVILDHVKFPRYAEIVHLTLPDGTKRSGQVLEVSGSKAVVQVFEGTSGIDAKKT : 109
HUMAN KIDNEY : -----MAMEIDSRPGGLPGSSCN-LGAAREHMQAVTRNYITHPRVTYRTVCSVNGPLVVLDRVKFAQYAEIVHFTLPDGTQRSGQVLEVAGTKAIVQVFEGTSGIDARKT : 104
BOVINE KIDNEY : -----MAAEVDSRPRGLPGGGAS-LGAAREHVQAVTRNYITHPRITYRTVCSVNGPLVVLDQVKFAQYAEIVNFTLPNGTQRSGQVLEVSGTKAIVQVFEGTSGIDAQKT : 104
CARCINUS : VCEFTGDILRTPVSEDMLGRVFNGSGKPIDQGPSVLAEDFLDIQGQPINPWSRTYPEEMIQTGISSIDVMNSIARGQKIPIFSAAGLPHNEIAAQICRQAGLVKLPSKGV : 198
HUMAN BRAIN : SCEFTGDILRTPVSEDMLGRVFNGSGKPIDRGPVVLAEDFLDIMGQPINPQCRIYPEEMIQTGISAIDGMNSIARGQKIPIFSAAGLPHNEIAAQICRQAGLVKK-SKDV : 219
BOVINE BRAIN : SCEFTGDILRTPVSEDMLGRVFNGSGKPIDRGPVVLAEDFLDIMGQPINPHFRIYPEEMIQTGISAIDGMNSIARGQKIPIFSAAGLPHNEIAAQICRQAGLVKK-SKDV : 218
HUMAN KIDNEY : TCEFTGDILRTPVSEDMLGRVFNGSGKPIDKGPVVMAEDFLDINGQPINPHSRIYPEEMIQTGISPIDVMNSIARGQKIPIFSAAGLPHNEIAAQICRQAGLVKK-SKAV : 213
BOVINE KIDNEY : TCEFTGDILRTPVSEDMLGRVFNGSGKPIDKGPVVMAEDFLDINGQPINPHDRIYPEEMIETGISPIDVMNSIARGQKIPIFSAAGLPHNEIAAQICRQAGLVKK-SKAV : 213
CARCINUS : HDDHKDNFAIVFAAMGVNMETARFFKQDFEENGSMENVCLFLNLANDPTIERIITPRLALTTAEYLAYQCEKHVLIILTDMSSYAEALREVSAAREEVPGRRGFPGYMYT : 308
HUMAN BRAIN : VDYSEENFAIVFAAMGVNMETARFFKSDFEENGSMDNVCLFLNLANDPTIERIITPRLALTTAEFLAYQCEKHVLVILTDMSSYAEALREVSAAREEVPGRRGFPGYMYT : 329
BOVINE BRAIN : VDYSEENFAIVFAAMGVNMETARFFKSDFEENGSMDNVCLFLNLANDPTIERIITPRLALTTAEFLAYQCEKHVLVILTDMSSYAEALREVSAAREEVPGRRGFPGYMYT : 328
HUMAN KIDNEY : LDYHDDNFAIVFAAMGVNMETARFFKSDFEQNGTMGNVCLFLNLANDPTIERIITPRLALTTAEFLAYQCEKHVLVILTDMSSYAEALREVSAAREEVPGRRGFPGYMYT : 323
BOVINE KIDNEY : LDYHDDNFAIVFAAMGVNMETARFFKSDFEQNGTMGNVCLFLNLANDPTIERIITPRLALTTAEFLAYQCEKHVLVILTDMSSYAEALREVSAAREEVPGRRGFPGYMYT : 323
CARCINUS : DLATIYERAGRVEGRQGSITQIPILTMPNDDITHPIPDLTGYITEGQIYVERQLHNRQIYPPINVLPSLSRLMKSAIGEGMTRKDHSDVCNQLYACYAIAKDLQAMKAVV : 418
HUMAN BRAIN : DLATIYERAGRVEGRNGSITQIPILTMPNDDITHPIPDLTGYITEGQIYVDRQLHNRQIYPPINVLPSLSRLMKSAIGEGMTRKDHADVSNQLYACYAIGKDVQAMKAVV : 439
BOVINE BRAIN : DLATIYERAGRVEGRNGSITQIPILTMPNDDITHPIPDLTGYITEGQIYVDRQLHNRQIYPPINVLPSLSRLMKSAIGEGMTRKDHADVSNQLYACYAIGKDVQAMKAVV : 438
HUMAN KIDNEY : DLATIYERAGRVEGRGGSITQIPILTMPNDDITHPIPDLTGFITEGQIYVDRQLHNRQIYPPINVLPSLSRLMKSAIGEGMTRKDHGDVSNQLYACYAIGKDVQAMKAVV : 433
BOVINE KIDNEY : DLATIYERAGRVEGRGGSITQIPILTMPNDDITHPIPDLTGFITEGQIYVDRQLHNRQIYPPINVLPSLSRLMKSAIGEGMTRKDHGDVSNQLYACYAIGKDVQAMKAVV : 433
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HUMAN BRAIN : GEEALTSDDLLYLEFLQKFERNFIAQGPYENRTVFETLDIGWQLLRIFPKEMLKRIPQSTLSEFYPRDSAKH-------- : 511
BOVINE BRAIN : GEEALTSDDLLYLEFLQKFEKNFITQGPYENRTVYETLDIGWQLLRIFPKEMLKRIPQSTLSEFYPRDSAKH-------- : 510
HUMAN KIDNEY : GEEALTSEDLLYLEFLQKFEKNFINQGPYENRSMFESLDLSWKLLRIFPKEMLKRIPQAVIDEFYSREGRLQDLAPDTAL : 513
BOVINE KIDNEY : GEEALTSEDLLYLEFLQKFEKKFINQGPYEKRSVFESLDLGWKLLRTFPKEMLKRIPQNIIDEFFSREGAPQDTEADTAL : 513
B
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DROSOPHILA : ------MNAQQAQREHVLAVSRDFISQPRLTYKTVSGVNGPLVILDEVKFPKFAEIVQLRLADGTVRSGQVLEVSGSKAVVQVFEGTSGIDAKNTLCEFTGDILRTPVSE : 104
MANDUCA : --MAKTLSAAQANKEHVLAVSRDFISQPRLTYKTVSGVNGPLVILDEVKFPKFSEIVQLKLADGTHRSGQVLEVSGSKAVVQVFEGTSGIDAKNTLCEFTGDILRTPVSE : 108
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ARABIDOPSIS : ------MSLSVNMGTNDLDIEEG-TLEIGMEYRTVSGVAGPLVILDKVKGPKYQEIVNIRLGDGSTRRGQVLEVDGEKAVVQVFEGTSGIDNKFTTVQFTGEVLKTPVSL : 103
SACCHAROMYCES : ----MVLSDKELFAINKKAVEQGFNVKPRLNYNTVSGVNGPLVILEKVKFPRYNEIVNLTLPDGTVRQGQVLEIRGDRAIVQVFEGTSGIDVKKTTVEFTGESLRIPVSE : 106
PLASMODIUM : MSKEVVNTKAEASRVNALAAVRNYKVCPRLEYKTISGVQGPLVIEDVKFPKYSEIVTIHLSDNITRQGQILEVCGKKAVIQVFEGTSGIDNKNSYVEVSGDILKMPMSD : 110
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SACCHAROMYCES : DMLGRIFDGSGRPIDNGPKVFAEDYLDINGSPINPYARIYPEEMISTGVSAIDTMNSIARGQKIPIFSASGLPHNEIAAQICRQAGLVRPTKD-V---HDGHEENFSIVF : 212
PLASMODIUM : EMLGRVFNGSGKPIDKGPNILADDYLDINGNPINPQCRVYPKEMIQTGISTIDVMNSIVRGQKIPLFSAAGLPHNEIGAQICRQASLVQGKDV-----LDHSDDNFAVVF : 215
CARCINUS : AAMGVNMETARFFKQDFEENGSMENVCLFLNLANDPTIERIITPRLALTTAEYLAYQCEKHVLIILTDMSSYAEALREVSAAREEVPGRRGFPGYMYTDLATIYERAGRV : 320
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CARCINUS : EGRQGSITQIPILTMPNDDITHPIPDLTGYITEGQIYVERQLHNRQIYPPINVLPSLSRLMKSAIGEGMTRKDHSDVCNQLYACYAIAKDLQAMKAVVGEEALTADDLLY : 430
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ARABIDOPSIS : EGRKGSITQIPILTMPNDDITHPTPDLTGYITEGQIYIDRQLHNRQIYPPINVLPSLSRLMKSAIGEGMTRKDHSDVSNQLYANYAIGKDVQAMKAVVGEEALSSEDLLY : 433
SACCHAROMYCES : EGRNGSITQIPILTMPNDDITHPIPDLTGYITEGQIFVDRQLHNKGIYPPINVLPSLSRLMKSAIGEGMTRKDHGDVSNQLYAKYAIGKDAAAMKAVVGEEALSIEDKLS : 432
PLASMODIUM : EGRNGSITQFPILTMPNDDITHPIPDLTGYITEGQIFVDRNLYNRQIYPPINVLPSLSRLMKSGIGHNMTRIDHPYVSDQLYSNYAIAQDVKAMKAVIGEEALSNDDILY : 435
CARCINUS : LEFLAKFEKSFISQGSYMKRTIFESLDIGWQLLRIFPKEMLKRIPASTLAEFYPRDRPQ---------- : 489
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CAENORHABDITIS : LEFLTKFEKNFITQGHYENRSVFESLDIGWQLLRIFPREMLKRIPESTLEKYYPRGGAKE---------- : 491
ARABIDOPSIS : LEFLDKFERKFVMQGAYDTRNIFQSLDLAWTLLRIFPRELLHRIPAKTLDQFYSRDSTS---------- : 492
SACCHAROMYCES : LEFLEKFEKTFITQGAYEDRTVFESLDQAWSLLRIYPKEMLNRISPKILDEFYDRARDDADEDEEDPDTRSSGKKKDASQEESLI : 517
PLASMODIUM : LEFLDKFEKRFITQNTYECRDIYQSLDIAWELLRIFPEDMLKKIKTDILSKYYPRHHAN---------- : 494

Fig. 3

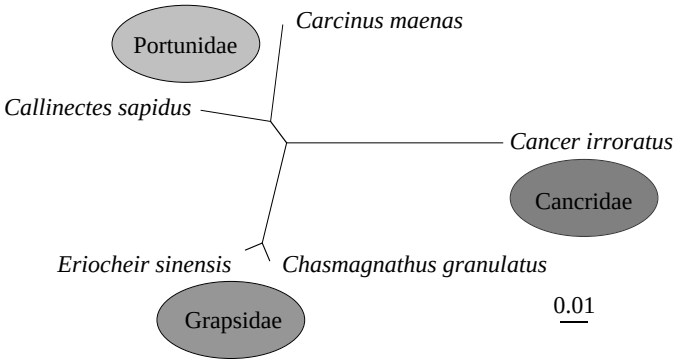

Fig. 4. Phylogenetic relationships of partial V-ATPase B-subunit nucleotide sequences obtained from five crab species: shore crab (*Carcinus maenas*), blue crab (*Callinectes sapidus*), Chinese mitten crab (*Eriocheir sinensis*), South American estuarine crab (*Chasmagnathus granulatus*) and rock crab (*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.

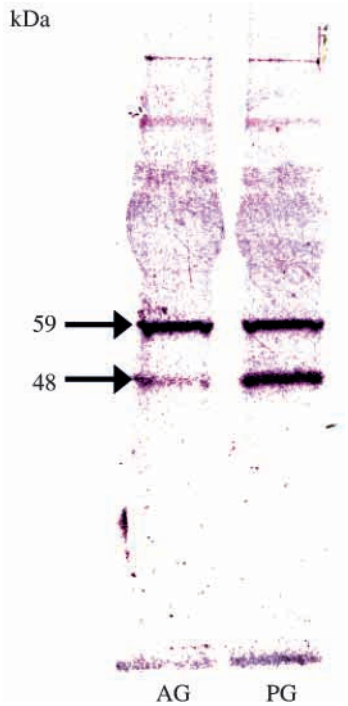

Fig. 5. Western blot analysis of V-ATPase B-subunit protein in a crude membrane fraction prepared from anterior (AG) and posterior (PG) gills of shore crab (*Carcinus maenas*). Two bands of approximately 59 and 48 kDa were evident, the larger probably corresponding to the amino acid sequence predicted 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_1, a specific inhibitor of the V-ATPase (Bowman et al., 1988). Under control conditions, a PD_{te} of −5.9±0.7 mV (mean ± 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 μmol l^{-1} bafilomycin, no change in PD_{te} could be observed. A reduction in PD_{te} of approximately 50 % resulted when Na^+/K^+-ATPase was blocked by application of 5 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 Na^+/K^+-ATPase (Towle and Kays, 1986) is the major driving force for electrogenic NaCl uptake across the gill epithelium. Both net NaCl influx and Cl^--dependent short-circuit current (I_{sc}) are dramatically inhibited by basolateral application of ouabain, a specific inhibitor of the Na^+/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

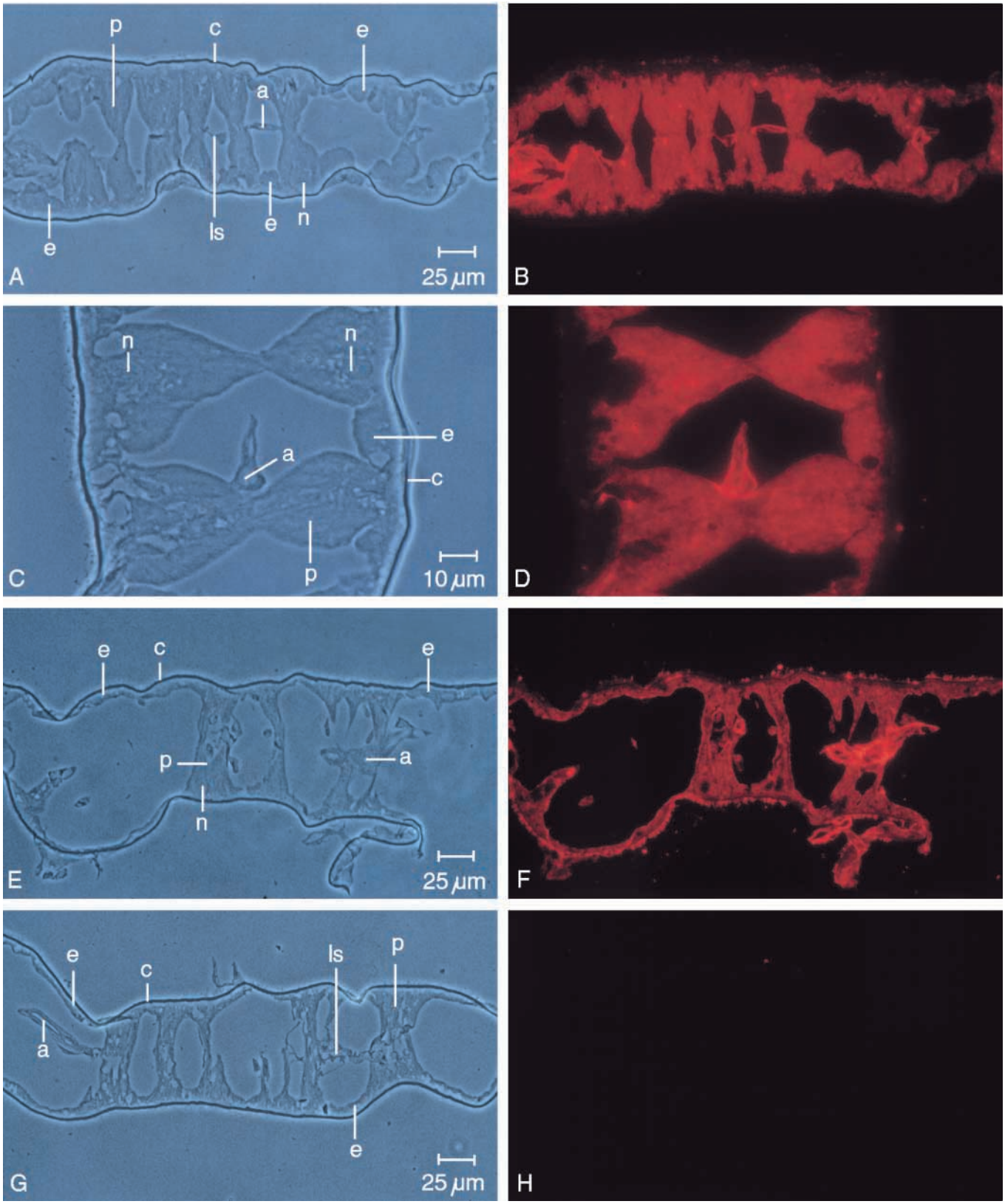

Fig. 6. Immunocytochemical analysis of V-ATPase B-subunit distribution in cryosections of posterior gill (A–D) and anterior gill (E–H) from *Carcinus maenas* acclimated to 10‰ salinity. Phase-contrast micrographs are presented in A, C, E and G. Immunocytochemical staining for B-subunit is presented in 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.

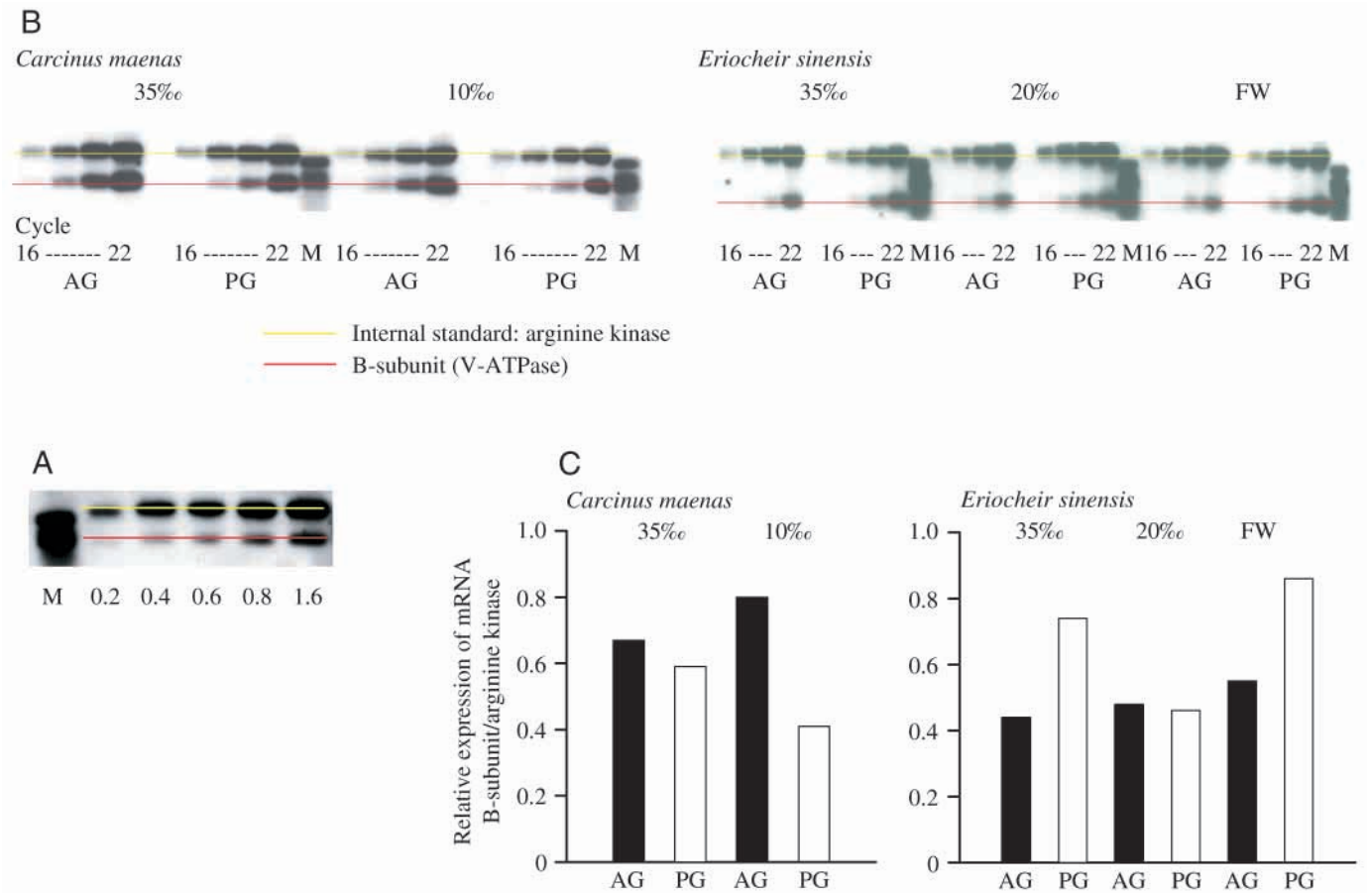

Fig. 7. Quantitative reverse transcriptase/polymerase chain reaction (RT-PCR) analysis of V-ATPase B-subunit and arginine kinase (internal standard) mRNA abundance in gill extracts obtained from *Carcinus maenas* and *Eriocheir sinensis* using duplex PCR with two primer pairs. For *Carcinus maenas*, 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 µl 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-It*gel* 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

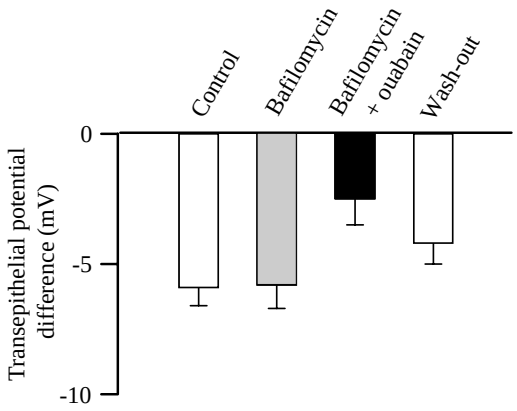

Fig. 8. Effects of the V-ATPase inhibitor bafilomycin (1 μmol l^{-1}) and the Na^+/K^+-ATPase inhibitor ouabain (5 mmol l^{-1}) on transepithelial potential differences measured in perfused posterior gills of *Carcinus maenas* acclimated to 10 ‰ salinity. Transepithelial potential differences are presented 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 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 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 Na^+/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 (Weihrauch 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 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 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 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 $1\,mol\,l^{-1}$, the same concentration employed in the present experiments (Weihrauch 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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