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Induction of branchial ion transporter mRNA expression during acclimation to salinity change in the euryhaline crab *Chasmagnathus granulatus*

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Summary

Using quantitative real-time PCR, the expression of mRNAs encoding three transport-related proteins and one putative housekeeping protein was analyzed in anterior and posterior gills of the euryhaline crab *Chasmagnathus granulatus* following transfer from isosmotic conditions (30‰ salinity) to either dilute (2‰) or concentrated (45‰) seawater. Modest changes were observed in the abundance of mRNAs encoding the housekeeping protein arginine kinase and the vacuolar-type H^+-ATPase B-subunit, both of which were highly expressed under all conditions. By contrast, the expression of Na^+/K^+-ATPase α-subunit mRNA and $Na^+/K^+/2Cl^-$ cotransporter mRNA was strongly responsive to external salinity. During acclimation to dilute seawater, cotransporter mRNA increased 10–20-fold in posterior gills within the first 24 h while Na^+/K^+-ATPase α-subunit mRNA increased 35–55-fold. During acclimation to concentrated seawater, cotransporter mRNA increased 60-fold by 96 h and Na^+/K^+-ATPase α-subunit increased approximately 25-fold in posterior gills. Our results indicate a complex pattern of transcriptional regulation dependent upon the direction of salinity change and the developmental background of the gills.

Key words: Na^+/K^+-ATPase, $Na^+/K^+/2Cl^-$ cotransporter, V-type H^+-ATPase, arginine kinase, crab, gill, gene expression, quantitative PCR.

Introduction

Physiological mechanisms by which euryhaline organisms adapt to changing salinities remain largely unexplored at the molecular level, particularly regarding the regulation of gene expression. The organism selected for the present studies, the semi-terrestrial euryhaline crab *Chasmagnathus granulatus*, is found in abundance along estuaries of the Atlantic coast of Brazil, Uruguay and Argentina, where it faces rapid changes of environmental salinity due to rains and tides. Adults of this species strongly hyper-osmoregulate in salinities less than normal seawater (35‰) and hypo-osmoregulate effectively in salinities more concentrated than seawater (Charmantier et al., 2002; Luquet et al., 1992). For example, following transfer from 35 to 10‰ seawater, hemolymph osmolality of *C. granulatus* declined only about 20%, primarily due to controlling the loss of $[Na^+]$ and $[Cl^-]$ (Schleich et al., 2001). This strong osmoregulatory response to salinity dilution suggests the presence of effective NaCl uptake and retention mechanisms, processes that are believed to occur primarily across posterior gills (Lucu, 1993; Péqueux, 1995; Taylor and Taylor, 1992; Towle and Weihrauch, 2001). Conversely, hypo-osmoregulation in concentrated seawater indicates an ability to excrete NaCl across the gill epithelium against an osmotic gradient, through mechanisms that are not well understood for crustacean species.

Ultrastructural studies of *C. granulatus* gills show that a morphology characteristic of ion-transporting cells predominates in posterior gills (Luquet et al., 2000). Within 25 days following transfer from seawater to 12‰ salinity, the depth of septate junctions between epithelial cells in posterior gills increased significantly, suggesting that the gill epithelium is less permeable in reduced salinity (Luquet et al., 2002a). In addition, the abundance of the ion-transporting cell type increased, notably through a process of differentiation and specialization rather than proliferation of cells (Genovese et al., 2000). Following transfer to concentrated seawater, the sub-apical space expanded and septate junction depth was even less than that in normal seawater (Luquet et al., 2002a), indicating further specialization for hypo-osmoregulation.

Superimposed upon these long-term changes in morphology are likely to be short-term changes in the function and/or expression of transport systems within the gill epithelium, controlled by hormonal processes or by direct response to osmotic changes. Indeed, we have shown that alteration of the osmotic concentration of perfusion media leads rapidly to adaptive changes in the transport capacity of isolated posterior gills of *C. granulatus* (Tresguerres et al., 2003). Furthermore, dopamine administration to isolated gills results in rapid changes in transport as well, probably acting through two different receptors, one activating and one inhibitory (Halperin et al., 2004).

Possible targets of these regulatory processes have been tentatively identified through an analysis of the transport properties of isolated split-gill lamellae mounted in Ussing-type chambers (Onken et al., 2003). Ion substitution and inhibitor application strongly supported a significant role of basolateral Na^+/K^+-ATPase in hyper-osmoregulatory ion uptake in *C. granulatus*, confirming related studies in this (Genovese et al., 2004) and several other crab species (Burnett and Towle, 1990; Lucu and Towle, 2003; Towle and Kays, 1986). In addition, experiments with split gill lamellae pointed toward possible roles in ion uptake for an apical Na^+/H^+ exchanger, $Na^+/K^+/2Cl^-$ cotransporter and Cl^-/HCO_3^- exchanger, as well as intracellular carbonic anhydrase (Onken et al., 2003), in line with other models of NaCl absorption across crustacean gill (Lucu, 1993; Onken and Riestenpatt, 1998; Towle and Weihrauch, 2001). In addition, recent experiments support a role for the V-type H^+-ATPase in energizing Cl^- uptake across *C. granulatus* gills (G. Genovese and C. M. Luquet, unpublished).

However, very little is known about possible mechanisms of NaCl excretion resulting in hypo-osmoregulation. A study of the hyper-/hypo-osmoregulating mangrove crab *Ucides cordatus* identified differences between individual gills in their capacity for ion absorption *versus* ion excretion (Martinez et al., 1998), suggesting that the molecular machinery implementing absorption and excretion is functionally unique. By contrast, Luquet et al. (2002b) have recorded similar ouabain-sensitive potential differences in the three posterior gills of *C. granulatus*. Although models for ion excretion across fish gills are quite well accepted (Evans, 2002; Perry, 1997), no similar conceptual basis exists for hypo-osmoregulating crustaceans.

To further elucidate the molecular physiology of bidirectional ion transport across gills of a strongly euryhaline crab, we sought to identify and characterize the expression of candidate transporter genes in 30‰-acclimated *C. granulatus* transferred for varying lengths of time to dilute (2‰) or concentrated (45‰) seawater, hypothesizing that transporters playing an essential role in hyper- or hypo-osmoregulation might be upregulated *via* transcriptional induction. Several candidate transporters in osmoregulatory tissues of euryhaline crustaceans have been identified and characterized at the molecular level, including the Na^+/H^+ exchanger (Towle et al., 1997a), Na^+/K^+-ATPase α-subunit (Towle et al., 2001), V-type H^+-ATPase B-subunit (Weihrauch et al., 2001) and $Na^+/K^+/2Cl^-$ cotransporter (Towle et al., 1997b). In the present study, we used quantitative polymerase chain reaction (QPCR) techniques to analyze mRNA transcript abundance for the latter three transporters in gills of *C. granulatus* challenged by salinity change, to determine if transcriptional expression of transporter-encoding genes is altered in response to salinity stress. A predicted housekeeping mRNA, that coding for arginine kinase (Kotlyar et al., 2000), was used as a reference transcript.

Materials and methods

Adult male specimens in intermolt stage C (Drach and Tchernigovtzeff, 1967) of the South American rainbow crab, *Chasmagnathus granulatus* (deHaan 1835), were caught by net at Punta Rasa Beach, San Clemente del Tuyú, Buenos Aires Province, Argentina. Crabs were kept at 20±1°C with a 12 h:12 h L:D photoperiod in aerated artificial seawater of 30‰. Crabs were fed twice a week with pellets of rabbit food, and the water was changed the following day.

For salinity acclimation experiments, animals were transferred from 30‰ seawater, in which the hemolymph is essentially isosmotic to the medium (Luquet et al., 1992), to salinities of either 2‰ or 45‰. Crabs were rapidly sacrificed at timed intervals following the transfer. After removing the dorsal carapace, gill pairs 3–8 were excised at their base and placed into RNase-free vials containing a large excess of RNA*later* (Ambion, Austin, TX, USA) to inactivate endogenous RNases in the gill tissue. Tissues in RNA*later* were stored at –20°C until air transport to Mount Desert Island Biological Laboratory, where they were returned to –20°C.

Total RNA was prepared by pooling the gills of each pair from four animals (Chomczynski and Sacchi, 1987) using RNase-free disposable labware with the RNAgents Total RNA kit (Promega, Madison, WI, USA). RNA quality and quantity were determined by microfluidic electrophoresis with the RNA 6000 Nano Assay system and 2100 Bioanalyzer (Agilent, Waldbronn, Germany). Ribosomal RNA produced three sharp peaks (one 18S rRNA and two 28S fragments) characteristic of crustacean and other arthropod species (Skinner, 1968).

cDNA was reverse transcribed from mRNA in 2.0 μg of total RNA using SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA) and oligo-dT as primer. Degenerate oligonucleotide primers for the polymerase chain reaction were based on conserved regions identified by multiple alignments of target amino acid sequences from other species and were synthesized by Integrated DNA Technologies (Coralville, IA, USA) (Table 1). Target cDNAs were those encoding arginine kinase, a putative housekeeping gene (Kotlyar et al., 2000), plus candidate ion transporters V-type H^+-ATPase (B-subunit), $Na^+/K^+/2Cl^-$ cotransporter and Na^+/K^+-ATPase (α-subunit). Conventional PCR was performed at an annealing temperature of 45°C using RedTaq polymerase (Sigma, St Louis, MO, USA), and amplification products were isolated electrophoretically on 0.8% agarose gels in TBE buffer.

Table 1. *Nucleotide sequence of degenerate and non-degenerate primers used for conventional and quantitative PCR amplification of arginine kinase, V-type H^+-ATPase B-subunit, $Na^+/K^+/2Cl^-$ cotransporter, and Na^+/K^+-ATPase α-subunit cDNAs from gills of* Chasmagnathus granulatus

Name	Sequence (5′→3′)	Target cDNA
Primers used in initial amplification by conventional PCR		
AKF51	CGC TGA GTC TAA GAA GGG ATT	Arginine kinase
AKR31	GAT ACC GTC CTG CAT CTC CTT	
HATF2	GCN ATG GGN GTN AAY ATG GA	V-type H^+-ATPase B-subunit
HATR4	TGN GTD ATR TCR TCG TTN GG	
COTFX	TNA AYA THT GGG GNG TNA TG	$Na^+/K^+/2Cl^-$ cotransporter
COTRX	CCR TCR TCR TAN ARC CAC CA	
NAK10F	ATG ACI GTI GCI CAY ATG TGG	Na^+/K^+-ATPase α-subunit
NAK16R	GGR TGR TCI CCI GTI ACC AT	
Species-specific primers used in quantitative PCR		
CgAKF1	GTT TCA AGC AGA CCG ACA AG	Arginine kinase
CgAKR2	CTT CGT TGC ACC ATA CCA G	
CgHATF1	CCG ATT CTT CAA GCA GGA C	V-type H^+-ATPase B-subunit
CgHATR2	AAC CAG GGA AAC CAC GAC	
CgCOTF1	TGG CTC GCA GAT TGA CTT	$Na^+/K^+/2Cl^-$ cotransporter
CgCOTR2	TGA AGC ATC CCT CAG TGT AA	
CgNAKF1	TCC CTT CAA CTC CAC CAA	Na^+/K^+-ATPase α-subunit
CgNAKR2	ATA CCA GCA GAA CGG CAC	

D=A/G/T; H=A/C/T; I=A/C/G/T; N=A/C/G/T; R=A/G; Y=C/T.

Following gel extraction (Qiagen, Valencia, CA, USA), amplification products were sequenced on an ABI 3100 automated sequencer (Applied Biosystems, Foster City, CA, USA) at the Marine DNA Sequencing and Analysis Center at the Mount Desert Island Biological Laboratory. Raw sequence traces were analyzed and trimmed using Chromas software (http://www.technelysium.com.au/chromas.html) and were submitted to BLASTX analysis for tentative functional identification (Altschul et al., 1997). Species-specific primers based on the resulting sequences were designed with Primer Premier software (Premier Biosoft, Palo Alto, CA, USA) (Table 1). Open reading frames were identified, and nucleotide sequences were translated to their most likely amino acid sequences using DNASIS software (Molecular Biology Insights, Cascade, CO, USA). Multiple alignments were generated with Multalin (Corpet, 1988) and GeneDoc software (http://www.psc.edu/biomed/genedoc).

QPCR was accomplished with species-specific primers (Table 1) at an annealing temperature of 55°C on a Stratagene MX4000 real-time sequence detection instrument, using reagents in the Brilliant SYBR Green QPCR kit (Stratagene, LaJolla, CA, USA). mRNA expression levels were measured in triplicate samples of cDNA reverse transcribed from 0.10 μg total RNA, thus normalizing to total RNA levels in each preparation, an accepted method of normalization for gene expression studies (Bustin, 2002). Relative mRNA abundance was calculated by comparison to a dilution series of a selected reference cDNA (usually gill 6 from 30‰-acclimated animals). Means of relative expression values for anterior gills 3, 4 and 5 and posterior gills 6, 7 and 8 were pooled for calculation of overall anterior and posterior gill means and standard errors. Differences in relative abundance were compared by two-way analysis of variance (ANOVA) and *post hoc* comparisons, taking sampling time and gill group (anterior *vs* posterior) as factors.

Results

Conventional PCR using degenerate primers produced single amplification products for each target transporter and housekeeping transcript, starting with cDNA prepared from posterior gills of *C. granulatus* (Fig. 1). The nucleotide sequences of these amplification products were translated to open reading frames that yielded high-scoring BLASTX matches to known sequences in GenBank, corresponding to each target transporter or housekeeping transcript (Fig. 2). Alignment of the amino acid sequences from *C. granulatus* with sequences from a selection of other species revealed conserved regions likely to be essential for protein function (Fig. 3).

Following transfer of crabs from 30‰ seawater to a dilute salinity of 2‰, a condition in which *C. granulatus* is a strong hyper-osmoregulator, QPCR analysis showed that mRNA encoding a presumed housekeeping gene, arginine kinase (Kotlyar et al., 2000), increased in abundance by 3–4-fold both in anterior and posterior gills, beginning at 24 h following the transfer ($P<0.0001$, $N=3$) (Fig. 4A). The relative abundance of arginine kinase mRNA was significantly higher in posterior gills.

V-type H^+-ATPase B-subunit mRNA levels were 5–8-fold higher in all gills at 24 h following transfer to dilute salinity ($P<0.001$, $N=3$). After 8 days in 2‰, V-type H^+-ATPase

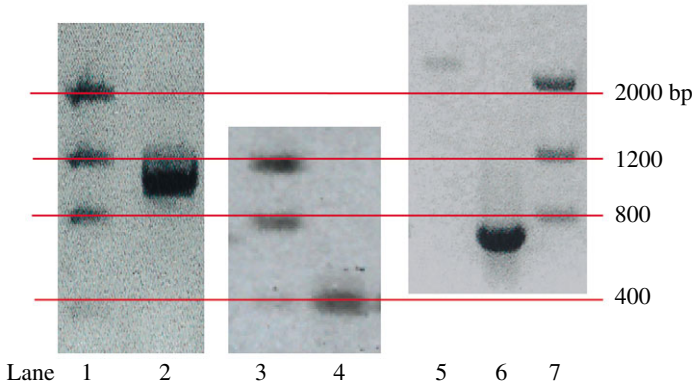

Fig. 1. Amplification of target transporter and housekeeping cDNAs from *Chasmagnathus granulatus* gill by conventional PCR. Expected sizes of amplification products are indicated in parentheses: lane 1, DNA ladder; lane 2, arginine kinase (1088 bp); lane 3, DNA ladder; lane 4, V-type H^+-ATPase B-subunit (390 bp); lane 5, $Na^+/K^+/2Cl^-$ cotransporter (2100 bp); lane 6, Na^+/K^+-ATPase α-subunit (703 bp); lane 7, DNA ladder. Sizes of ladder standards are indicated at the right; each gel image was adjusted to give parallel ladder bands.

mRNA levels in both anterior and posterior gills were about 4-fold higher than the 30‰ controls (Fig. 4B). There were no significant differences between anterior and posterior gills. It should be noted that mRNAs encoding arginine kinase and the V-type H^+-ATPase B-subunit were highly expressed under all conditions, relative to the $Na^+/K^+/2Cl^-$ cotransporter and Na^+/K^+-ATPase α-subunit (Table 2), and thus may represent transcripts that are primarily constitutively expressed.

By contrast, the expression of $Na^+/K^+/2Cl^-$ cotransporter transcripts increased 10–22-fold in posterior gills by 24 h of exposure to 2‰ salinity (P<0.001, N=3), with more modest increases in anterior gills (P<0.05 between gill groups) (Fig. 4C). The large standard error noted at 48 h for posterior gills reflects the disparate responses of the three gills to salinity reduction at that time. Gill 7 contained very high levels of cotransporter mRNA at 48 h, while gill 8 showed little change from the previous time sample. However, by 96 h, differences between posterior gills were smaller, and a 10-fold increase in expression relative to 30‰ controls was maintained through the 8-day time sample.

Within 6 h after transfer to low salinity, Na^+/K^+-ATPase α-subunit transcripts increased ~5-fold in the three posterior gills but remained unchanged in anterior gills. However, by 24 h after the transfer to 2‰ salinity, α-subunit mRNA levels in all tested gills increased between 20- and 50-fold (P<0.001, N=3), with anterior gills declining by 96 h but posterior gills remaining high (P<0.001 between groups) (Fig. 4D).

Following transfer of crabs from 30‰ to 45‰ seawater, a condition in which *C. granulatus* is a strong hypo-osmoregulator, transporter and housekeeping transcript levels showed little change until 96 h after the transfer (Fig. 5). At that time, major increases in mRNA abundance for all four target genes occurred, primarily in posterior gills 6 and 7. The degree of change in posterior gills was 2–3-fold for arginine kinase (P<0.0001 for time; P<0.05 between gill groups), 10-fold for V-type H^+-ATPase B-subunit (P<0.0001 for time; no significant differences between groups), 60-fold for $Na^+/K^+/2Cl^-$ cotransporter (P<0.0001 for time; P<0.005 between groups), and 28-fold for Na^+/K^+-ATPase α-subunit (P<0.001 for time; P<0.05 between groups) (Fig. 5A–D). After 8 days, the expression of all transcripts was similarly high in the three posterior gills and significantly higher than in anterior gills for $Na^+/K^+/2Cl^-$ cotransporter, Na^+/K^+-ATPase α-subunit and arginine kinase but not for V-type H^+-ATPase B-subunit.

Discussion

Our results using quantitative PCR clearly show that gills of *Chasmagnathus granulatus* respond to salinity change by inducing the synthesis and/or retention of messenger RNAs encoding at least two of the three candidate ion transporters, namely $Na^+/K^+/2Cl^-$ cotransporter and Na^+/K^+-ATPase α-subunit. The degree of change in mRNA expression from isosmotic conditions (30‰ salinity) to either 2‰ or 45‰ for these two transporters is substantially larger than changes in expression observed for either the putative housekeeping gene arginine kinase or the third candidate transporter V-type H^+-ATPase (B-subunit). Both of these mRNAs are highly expressed in gills compared with the cotransporter and Na^+/K^+-ATPase α-subunit and may represent constitutive genes that do not respond strongly to salinity stress but are required for other branchial functions. The V-type H^+-ATPase, for example, is thought to play an important role in ammonia excretion across the gill (Weihrauch et al., 2002, 2004), a function that is likely to be at least partly independent of environmental salinity. Arginine kinase, catalyzing the phosphorylation of ADP to ATP at the expense of phosphoarginine, is believed to play an essential role in maintaining intracellular ATP concentrations (Ellington, 2001) required for most cellular activity. The transcription and retention of arginine kinase-encoding mRNA would thus be expected to remain robust under most environmental conditions requiring energy expenditure.

The $Na^+/K^+/2Cl^-$ cotransporter occurs in two major forms in vertebrate epithelial cells, an apical form involved in NaCl uptake and a basolateral form involved in NaCl excretion (Mount et al., 1998). Ion substitution experiments in split gill lamellae of *Carcinus maenas* support the existence of an apical $Na^+/K^+/2Cl^-$ cotransporter in this species (Riestenpatt et al., 1996). Previous molecular cloning experiments showed that a $Na^+/K^+/2Cl^-$ cotransporter is expressed in gills of crustacean species, including *C. granulatus* (Luquet et al., 2003; Towle and Peppin, 2002), but the resulting sequence information was insufficient to classify the product as apical or basolateral. The apparent induction of $Na^+/K^+/2Cl^-$ cotransporter mRNA accumulation in gills of *C. granulatus* transferred from 30‰ to 2‰ salinity suggests that an apical form may be induced in 2‰, where it could function in NaCl uptake from the medium. On the other hand, an even stronger apparent induction in 45‰, a condition in which the crab is excreting NaCl most likely *via* the gills, suggests recruitment of a basolateral

Fig. 2. Partial nucleotide and translated amino acid sequences of cDNAs amplified from *Chasmagnathus granulatus* gill, shown with identifications derived from BLASTX analysis. (A) arginine kinase; (B) V-type H^+-ATPase B-subunit; (C) $Na^+/K^+/2Cl^-$ cotransporter; (D) Na^+/K^+-ATPase α-subunit. GenBank accession numbers are given in parentheses. Locations of primers used in quantitative PCR are indicated in blue.

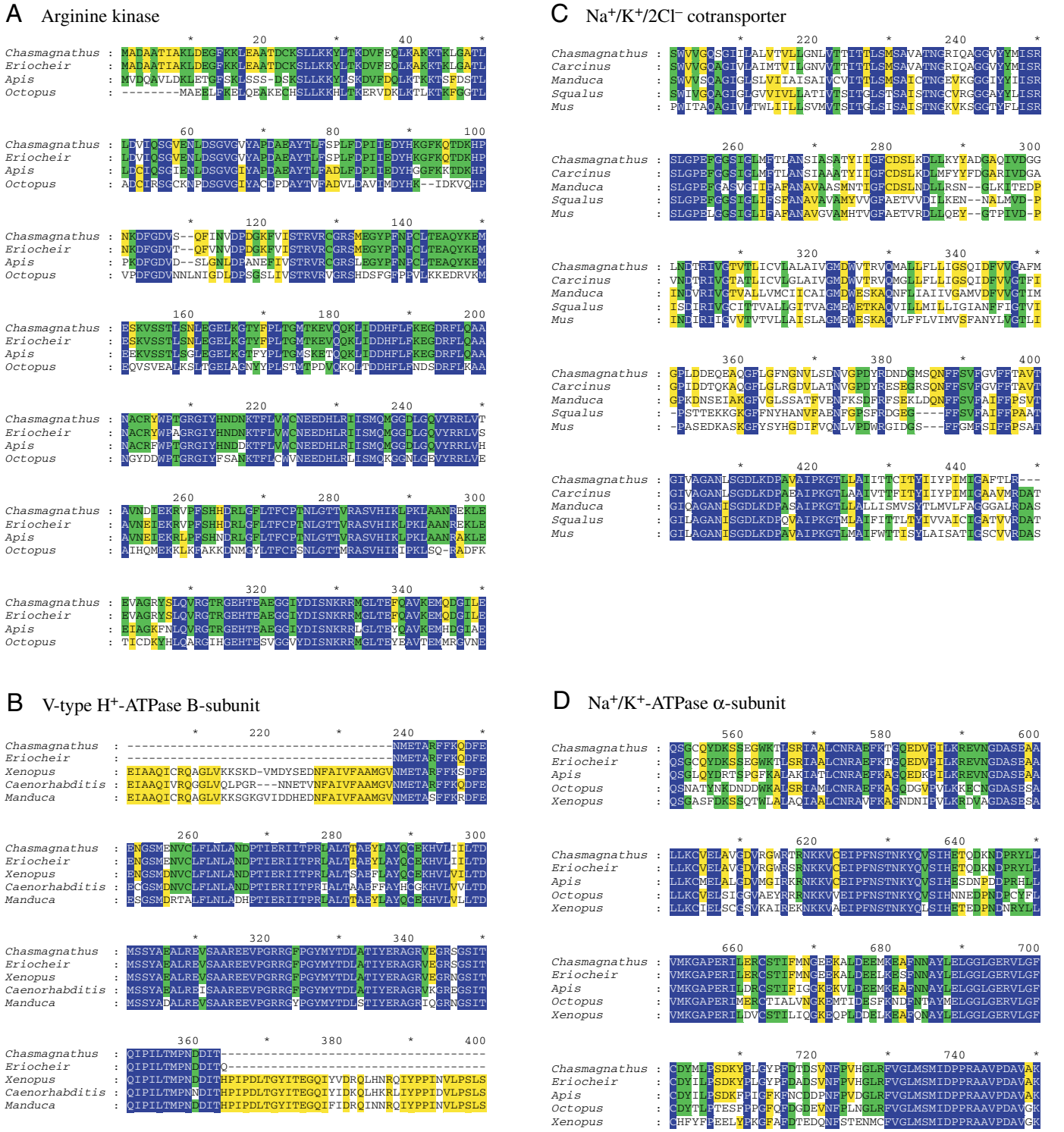

Fig. 3. Multiple alignment of translated amino acid sequences of transporter and housekeeping cDNAs from *Chasmagnathus granulatus* with corresponding fragments obtained from GenBank, indicated by species names and accession numbers. Blue background, 100% agreement; green background, 75–80% agreement, yellow background, 50–67% agreement. (A) Arginine kinase: *Chasmagnathus granulatus* (present study, AF233357), *Eriocheir sinensis* (AAF43437), *Apis mellifera* (AF023619), *Octopus vulgaris* (AB042331); (B) V-type H^+-ATPase B-subunit: *Chasmagnathus granulatus* (present study, AF189783), *Eriocheir sinensis* (AAF08284), *Xenopus laevis* (AAH46738), *Caenorhabditis elegans* (AAF60418), *Manduca sexta* (AAS38817); (C) $Na^+/K^+/2Cl^-$ cotransporter: *Chasmagnathus granulatus* (present study, AF548368), *Carcinus maenas* (AAG62044), *Manduca sexta* (AAA75600), *Squalus acanthias* (AAM74966), *Mus musculus* (AAH38612); (D) Na^+/K^+-ATPase α-subunit: *Chasmagnathus granulatus* (present study, AF548369), *Eriocheir sinensis* (AAG39936), *Apis mellifera* (XP_392363), *Octopus rubescens* (AAQ72761), *Xenopus laevis* (AAH43743). Asterisks indicate position markers between the numbered sites.

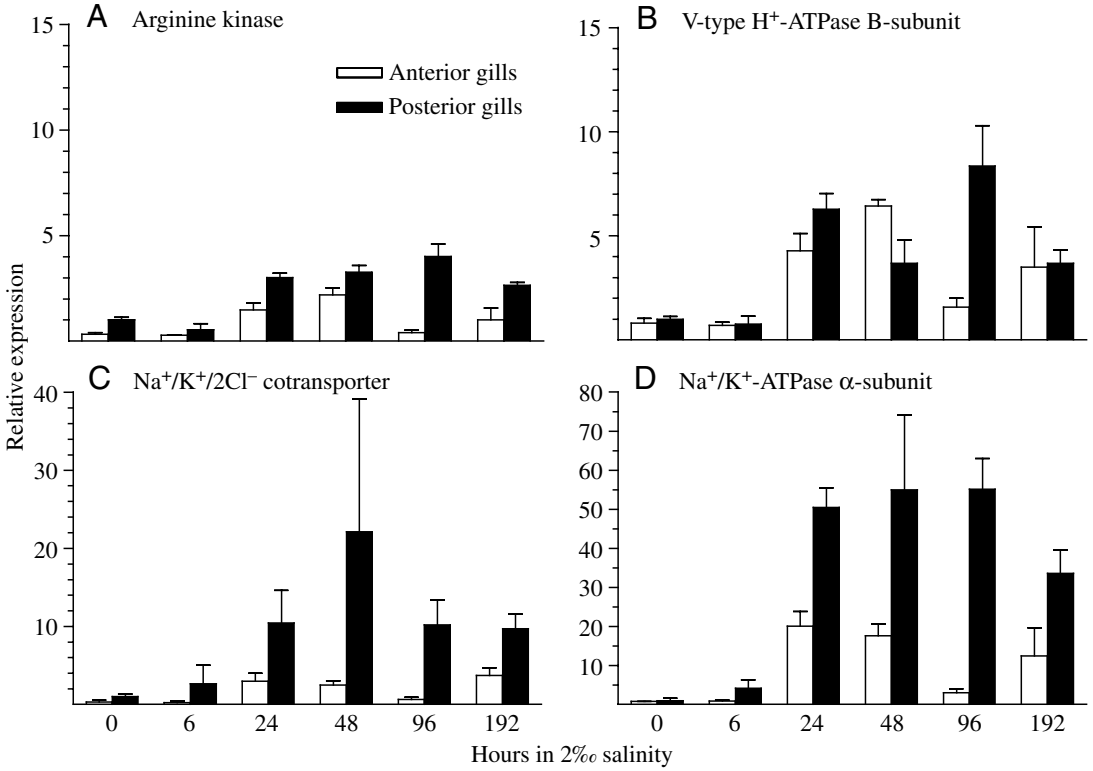

Fig. 4. Quantitative PCR analysis of mRNA transcript abundance in anterior (3, 4, 5) and posterior (6, 7, 8) gills of *Chasmagnathus granulatus* following transfer from 30‰ salinity to 2‰, normalized to the mean of posterior gills from crabs acclimated to 30‰ seawater (zero-time value). (A) Arginine kinase; (B) V-type H^+-ATPase B-subunit; (C) $Na^+/K^+/2Cl^-$ cotransporter; (D) Na^+/K^+-ATPase α-subunit. Means and standard errors were calculated from gills 3, 4 and 5 (anterior) and gills 6, 7 and 8 (posterior), with triplicate determinations for each gill preparation. Results of statistical analysis by ANOVA and *post hoc* comparisons are presented in the text.

$Na^+/K^+/2Cl^-$ cotransporter, functioning to move NaCl from hemolymph into branchial epithelial cells, which would then excrete Na^+ or Cl^- into the medium. Further work is required to distinguish between these possibilities.

The most dramatic change in transcript abundance was observed for the Na^+/K^+-ATPase α-subunit. Between 6 h and 24 h following transfer from 30 to 2‰ salinity, the mRNA abundance for the α-subunit reached maximum levels, an approximately 50-fold increase in posterior gills, and remained at those levels for at least 4 days. Anterior gills also showed large increases, as much as 20-fold higher than the 30‰ controls, beginning at 24 h. By contrast, major changes in Na^+/K^+-ATPase α-subunit transcript quantity following transfer to 45‰ occurred only after 96 h of exposure, similar to the time frame observed for the $Na^+/K^+/2Cl^-$ cotransporter.

Table 2. *Relative mRNA abundance in anterior gills (gills 3, 4 and 5) and posterior gills (gills 6, 7 and 8) of* Chasmagnathus granulatus *acclimated to 30‰ seawater, normalized to the level of arginine kinase mRNA in gill 6*

	Relative mRNA abunsdance	
Encoded protein	Anterior gills	Posterior gills
Arginine kinase	0.2704±0.0218	0.8125±0.0686
V-type H^+-ATPase	0.0430±0.0047	1.6464±0.6713
$Na^+/K^+/2Cl^-$ cotransporter	0.0001±0.0000	0.0091±0.0002
Na^+/K^+-ATPase	0.0613±0.0055	0.2279±0.0311

Values are means ± S.E.M. (N=3).

The Na^+/K^+-ATPase of crustacean gills is restricted to the basolateral membrane (Towle and Kays, 1986), where it can be inhibited in perfused gills by the specific inhibitor ouabain (Burnett and Towle, 1990). In *C. granulatus*, we have shown that ouabain produces a large decrease in the transepithelial potential in symmetrically perfused posterior gills, indicating that the Na^+/K^+-ATPase is essential in energizing transbranchial ion transport (Luquet et al., 2002b). A recent study from our laboratory showed that posterior gills 6, 7 and 8 of *C. granulatus* contain about 82% of the total gill Na^+/K^+-ATPase activity (Genovese et al., 2004), a finding that is reflected in the current study showing a substantially higher content of Na^+/K^+-ATPase-encoding mRNA in posterior gills compared with anterior gills.

However, measurements of Na^+/K^+-ATPase enzymatic activity have not shown large differences in *C. granulatus* gills in relation to acclimation salinity (Genovese et al., 2004). Homogenates of posterior gills from animals acclimated to 30 or 45‰ seawater exhibit approximately the same specific Na^+/K^+-ATPase activity, and animals acclimated to 10‰ show only a modest increase (Genovese

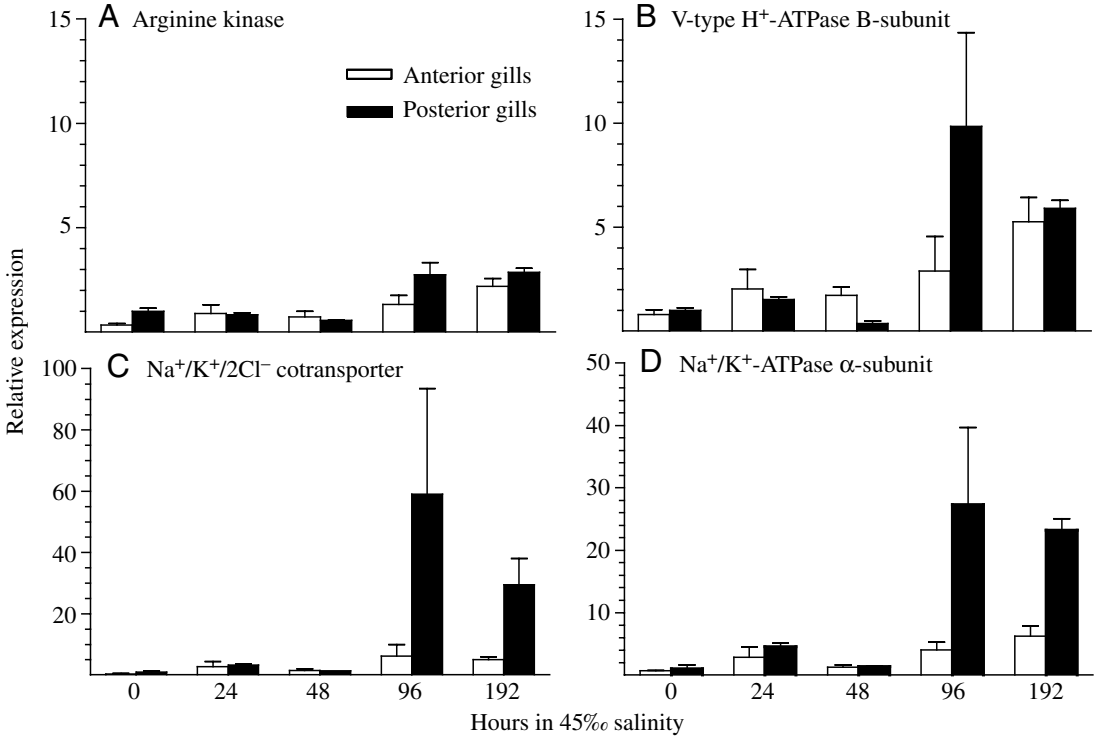

Fig. 5. Quantitative PCR analysis of mRNA transcript abundance in anterior (3, 4, 5) and posterior (6, 7, 8) gills of *Chasmagnathus granulatus* following transfer from 30‰ salinity to 45‰, normalized to the mean of posterior gills from crabs acclimated to 30‰ seawater (zero-time value). (A) Arginine kinase; (B) V-type H^+-ATPase B-subunit; (C) $Na^+/K^+/2Cl^-$ cotransporter; (D) Na^+/K^+-ATPase α-subunit. Means and standard errors were calculated from gills 3, 4 and 5 (anterior) and gills 6, 7 and 8 (posterior), with triplicate determinations for each gill preparation. Results of statistical analysis by ANOVA and *post hoc* comparisons are presented in the text.

et al., 2004). The discrepancy between activity measurements and α-subunit mRNA abundance as detected by quantitative PCR requires explanation. It is known that catalytic activity of the Na^+/K^+-ATPase in other species may be modified by protein kinase A and protein kinase C, as well as by interaction with a regulatory γ-subunit (Therien and Blostein, 2000). In addition, access to the active site and/or the ouabain binding site in vesicular forms of the enzyme may not occur fully in assays employing homogenates without detergent treatment (Lucu and Flik, 1999). Hydrolytic activity measured *in vitro* may therefore not reflect accurately the functional rate of transport mediated by the Na^+/K^+-ATPase. Furthermore, transepithelial potential differences and ^{22}Na transport in posterior gills of crabs acclimated to 45‰ seawater are strongly inhibited by ouabain, suggesting that ion excretion through *C. granulatus* gills is indeed energized by Na^+/K^+-ATPase (Luquet et al., 2002b), a function that would be enhanced by the accumulation and translation of α-subunit mRNA. The large apparent increases in Na^+/K^+-ATPase α-subunit mRNA observed in this study may reflect a rapid turnover of Na^+/K^+-ATPase protein in gill plasma membranes of *C. granulatus*, requiring a high level of mRNA to support efficient replacement of the protein.

The rapid response of $Na^+/K^+/2Cl^-$ cotransporter and Na^+/K^+-ATPase α-subunit mRNA levels to salinity dilution, compared with the relatively delayed response observed in hypersaline conditions, probably indicates the existence of distinctive regulatory mechanisms as well as transport geometry. In work in our laboratory with split gill lamellae from *C. granulatus* acclimated to 2‰ salinity (Onken et al., 2003), we have observed an almost complete inhibition of the short-circuit current both in Na^+-free and Cl^--free medium, along with a strong inhibitory effect of apical CsCl (an inhibitor of K^+ channels). We have concluded that coupled electrogenic NaCl absorption is mediated by an apically located $Na^+/K^+/2Cl^-$ cotransporter in parallel with K^+ channels and is energized by Na^+/K^+-ATPase at the basolateral membrane. The dramatic induction of $Na^+/K^+/2Cl^-$ cotransporter and Na^+/K^+-ATPase gives further support to this hypothetical mechanism.

In recent unpublished experiments, we have changed the perfusion conditions to hypo-osmotic and found an Na^+-independent transepithelial potential difference that is inhibited by the V-type H^+-ATPase inhibitor bafilomycin (G. Genovese and C. M. Luquet, unpublished). These preliminary data support a role for the V-type H^+-ATPase in ion uptake from extremely dilute salinity (2‰), supported by the modest increase in V-type H^+-ATPase mRNA measured in the present study.

The induction of the three candidate transporters after

acclimation to high salinity makes us speculate that they are intimately involved in ion excretion across the gill. The current model of NaCl excretion in marine fishes includes a basolateral $Na^+/K^+/2Cl^-$ cotransporter that mediates the flux of Cl^- from the blood to the cytosol of the ion-transporting cell, energized by the Na^+/K^+-ATPase (Evans, 2002). In this model, Cl^- leaves the cell through an apical channel, possibly the cystic fibrosis transmembrane regulator protein (Singer et al., 1998). This Cl^- flux generates an outside negative potential difference, which is believed to drive Na^+ through a paracellular route. Our electrophysiological and ion flux studies performed on isolated perfused gills of *C. granulatus* support the involvement of Na^+/K^+-ATPase. However, in most experiments we have recorded an outside positive potential difference, suggesting that Na^+ and not Cl^- is actively transported (Luquet et al., 2002b). Thus, further studies using split gill lamellae mounted in an Ussing chamber remain to be carried out in order to propose an ion excretion model for crab gill.

Three levels of response to salinity change have been observed in the gills of euryhaline crabs: rapid, over a period of minutes; moderately rapid, over a period of hours; and slow, over a period of days. The most rapid responses probably involve protein phosphorylation and/or recruitment of membrane proteins from intracellular stores (Halperin et al., 2004). The slowest responses appear to include structural remodeling of the gill epithelium associated with cellular differentiation (Genovese et al., 2000; Luquet et al., 2002a). It is clear that the responses noted here are in the order of hours to days, providing an intermediate time scale of regulation associated with a complex pattern of specific transcriptional events.

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