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Gill-specific transcriptional regulation of Na^+/K^+-ATPase α-subunit in the euryhaline shore crab *Pachygrapsus marmoratus*: sequence variants and promoter structure

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Summary

The sodium pump (Na^+/K^+-ATPase) has been implicated in osmoregulatory ion transport in many aquatic animals. In the euryhaline hyper–hypoosmoregulating shore crab *Pachygrapsus marmoratus*, induction of Na^+/K^+-ATPase α-subunit mRNA varies between gills in response to osmotic stress. Following transfer of crabs from normal seawater (36‰ salinity) to diluted seawater (10‰), a condition in which gills exhibit net ion uptake, α-subunit mRNA expression is upregulated in all tested gills, albeit with differing time courses. By contrast, following transfer from seawater to hypertonic (45‰) seawater, a condition in which the animal is excreting ions, α-subunit mRNA is induced primarily in gill no. 7 (nine in total), suggesting that this gill may be associated specifically with ion excretion in *P. marmoratus*.

Full-length sequencing of α-subunit cDNA revealed the existence of two isoforms differing only in the inclusion of an 81-nucleotide segment within the N-terminal open reading frame of the long (D) form in comparison to the short (C) form. The 81-nucleotide segment encodes a 14-3-3 protein binding site that may facilitate movement of the α-subunit protein between intracellular compartments and the plasma membrane. mRNA expression of the two forms followed similar patterns upon salinity transfer. Genomic DNA sequencing of the putative promoter region of the α-subunit gene demonstrated a spectrum of predicted transcription factor binding sites that are likely associated with the complex expression pattern observed among gills following osmotic stress.

Key words: crab gill, osmoregulation, real-time quantitative PCR, salinity, sequence variants, sodium pump, α-subunit.

Introduction

The cellular mechanisms by which euryhaline animals adjust to changes in environmental salinity are beginning to be explored at the level of gene expression. A number of candidate ion transporter and transport-related genes have been identified in osmoregulatory tissues of euryhaline species and their cDNAs have been sequenced, including Na^+/K^+-ATPase α-subunit (Cutler et al., 1995; Towle et al., 2001), Na^+/H^+ exchangers (Towle et al., 1997; Choe et al., 2005), $Na^+/K^+/2Cl^-$ cotransporter (Pelis et al., 2001; Luquet et al., 2005; Scott et al., 2005), cystic fibrosis transmembrane regulator (Singer et al., 1998), V-type H^+-ATPase (Weihrauch et al., 2001; Luquet et al., 2005), aquaporins (Cutler and Cramb, 2002; Lignot et al., 2002) and carbonic anhydrase (Henry et al., 2003).

In this study, we examined the expression of mRNA encoding the catalytic α-subunit of Na^+/K^+-ATPase, a transmembrane protein localized in the basolateral membrane of most animal epithelial cells, where it uses energy derived from ATP hydrolysis to move three Na^+ ions from the cytosol to the cell exterior in exchange for two K^+ ions, thus establishing an electrochemical gradient across the cell membrane that drives secondary active transport processes (Skou and Esmann, 1992; Lopina, 2000; Kaplan, 2002). In hyperosmoregulatory epithelia, basolateral Na^+/K^+-ATPase is thought to drive apical uptake of Na^+ from a dilute medium into the epithelial cells and across the basolateral membrane into the blood (Lucu and Towle, 2003; Evans et al., 2005). In aquatic animals capable of hypoosmoregulation, such as some marine teleosts, basolateral Na^+/K^+-ATPase is believed to indirectly energize excretion of ions *via* a $Na^+/K^+/2Cl^-$ cotransporter or other secondary transport mechanisms (Perry, 1997; Evans et al., 2005).

It has been known for many years that the enzymatic activity of Na^+/K^+-ATPase in gills of euryhaline species is

responsive to environmental salinity, generally increasing in situations that demand greater osmoregulatory ion transport. For example, in the killifish *Fundulus heteroclitus*, Na^+/K^+-ATPase activity in gills is substantially higher in hypoosmoregulating seawater-acclimated animals than in hyperosmoregulating freshwater-acclimated animals (Epstein et al., 1967), and enzymatic activity in both conditions is higher than in animals acclimated to brackish water where little net salt transport is required (Towle et al., 1977). Among euryhaline crustaceans, gill Na^+/K^+-ATPase activity also responds to salinity, the highest activity being generally observed in reduced salinities in which the animals hyperosmoregulate their hemolymph (e.g. Towle et al., 1976; Neufeld et al., 1980; Siebers et al., 1982; Harris and Bayliss, 1988; Lucu and Flik, 1999). In particular, the posterior gills of euryhaline brachyurans exhibit significantly higher Na^+/K^+-ATPase activity than anterior gills, in agreement with the observed abundance of basolateral membrane infoldings in epithelial cells of posterior gills (Copeland and Fitzjarrell, 1968; Goodman and Cavey, 1990).

It has been unclear whether the adaptive changes in Na^+/K^+-ATPase activity were the result of enzymatic activation or synthesis and/or recruitment of new membrane-bound protein. Increases in the abundance of mRNA encoding the Na^+/K^+-ATPase α-subunit have been noted in gills of brown trout following seawater transfer (Madsen et al., 1995), in European sea bass following transfer from 15‰ salinity to either freshwater or seawater (Jensen et al., 1998), and in the killifish transferred from 10‰ salinity to freshwater, whereas a transient increase was observed after transfer to seawater in this species (Scott et al., 2004). Na^+/K^+-ATPase α-subunit mRNA levels have been shown to increase in posterior gills of the South American crab *Chasmagnathus granulatus* following transfer from isosmotic conditions (30‰ salinity) to either dilute (2‰) or concentrated (45‰) seawater (Luquet et al., 2005) and in posterior gills of the blue crab *Callinectes sapidus* after transfer from 32‰ to 10‰ (Lovett et al., 2006).

However, little attention has been given to ascertaining differences in gene expression in individual gills, although measurements of transport function and Na^+/K^+-ATPase activity following salinity challenge in euryhaline crabs, suggest important functional differences between gills (Siebers et al., 1982; Wanson et al., 1984; Welcomme and Devos, 1988; Martinez et al., 1998). In addition, there is little information regarding possible transcription factors and their binding sites that may be associated with transcriptional regulation of the Na^+/K^+-ATPase in crustaceans.

The marble shore crab *Pachygrapsus marmoratus* is strongly euryhaline, transporting environmental NaCl across the gill from low salinities and thus maintaining a hyperosmotic hemolymph in dilute seawater. At 33‰ salinity, the hemolymph of *P. marmoratus* is essentially isosmotic with the medium. At 10‰ salinity, however, the hemolymph of *P. marmoratus* is regulated at approximately 550 mOsmol kg^{-1} above the osmolality of the medium (Pierrot et al., 1995). Unlike many brachyuran crabs, this species is also capable of excreting NaCl into high salinity environments, thereby maintaining a hypoosmotic hemolymph in concentrated seawater. At 45‰ salinity, for example, the osmotic concentration of the hemolymph is regulated at approximately 250 mOsmol kg^{-1} below that of the medium (Pierrot et al., 1995).

The capacity for ion transport in isolated gills of this species is enhanced by treatment with neurohormonal extracts that may mediate the organismal response to salinity change, in part by stimulating changes in Na^+/K^+-ATPase activity (Eckhardt et al., 1995; Spanings-Pierrot et al., 2000). Like other brachyuran crabs, the gills are morphologically and functionally distinct: the anterior gills are thought to be specialized for gas exchange and the posterior gills for ion transport (Pierrot, 1996). Therefore, *P. marmoratus* is an excellent model in which to study the dynamics of environmentally influenced transporter gene expression in osmoregulatory tissues that show functional differentiation.

The objective of the present work was to focus on one of the main ion transporters, the Na^+/K^+-ATPase, by amplifying and sequencing Na^+/K^+-ATPase α-subunit cDNA from gills of *P. marmoratus* and by measuring salinity-related α-subunit mRNA expression in individual gills from crabs subjected to salinity stress using real-time quantitative PCR, a technique for profiling gene expression quantitatively (Bustin et al., 2005). Sequencing of upstream promoter regions in the α-subunit gene revealed potential transcription factor binding sites that may function in transcriptional regulation.

Materials and methods

Animals and tissue sampling

Specimens of the marble shore crab *Pachygrapsus marmoratus* Fabricius 1787 were caught on rocky areas of the Mediterranean coast of France near Montpellier and transported to Université Montpellier II where they were kept at 20±1°C in individual compartments containing recirculating natural seawater at 36‰ salinity. A 12 h:12 h light:dark photoperiod was maintained. They were fed three times a week with pieces of mussels. Prior to experimentation, food was withdrawn for 48 h. Crabs in intermolt stage C (Drach and Tchernigovtzeff, 1967) were transferred to either 10‰ salinity (seawater diluted with dechlorinated fresh water) or 45‰ salinity (seawater supplemented with synthetic sea salts, Reef Crystals, Instant Ocean, Sarrebourg, France). At intervals of 2, 4, 6, 24 and 48 h, crabs were sacrificed following anesthesia on ice and individual gills were dissected and immediately placed into RNA*later* (Ambion, Cambridge, UK), yielding separate samples of the largest gills in the branchial chamber, i.e. the last two pairs of anterior gills (numbers 5 and 6) and the three pairs of posterior gills (7, 8 and 9). Gills 1–4 were of insufficient size to work with individually and were not used in this study. Dissected gills were transported in RNA*later* to the Mount Desert Island Biological Laboratory where molecular analyses were carried out.

Table 1. *Composition of oligonucleotide primers employed in initial amplification, genomic DNA sequencing, and quantitative PCR analysis of Na^+/K^+-ATPase α-subunit isoforms and arginine kinase cDNA in gills of Pachygrapsus marmoratus*

Name of primer	Nucleotide sequence 5′–3′	Target position
Na^++K^+-ATPase α-subunit		
NAK10F	atg aci gti gci cay atg tgg	1507–1527
NAK16R	ggr tgr tci cci gti acc at	2194–2212
NAKPMF1	cag agg agg agc cca aca ac	713–732
NAKPMR1	cca taa tga agg caa taa cga aga	1268–1291
PMNAKSPF (for C form)	gca acc atg gcc gac tca aaa aag a	304–318, 400–409
PMNAKUSF (for D form)	ccg aca cgg gca gga cag att c	314–335
PMNAK238R	tca cgg atg aca atg gca tac tga	849–872
PMNAK583R	gag tct gct tgg gag gag tga g	589–610
Arginine kinase		
PMAKF1	gca gat ggg cgg tga ctt	718–735
PMAKR1	ggg tgg agc cga act tta ct	1089–1108

Position numbers refer to NCBI accession no. DQ173924 (D form of α-subunit) or AF288785 (arginine kinase).

RNA and DNA extraction, cDNA synthesis and sequencing

Total RNA was extracted from numbered gills pooled from three to four animals under RNAse-free conditions (Chomczynski and Sacchi, 1987) with materials supplied by Promega (RNAgents Total RNA Isolation System, Madison, WI, USA). Because high quality RNA and normalization to total RNA are essential to producing biologically relevant and reliable data using real-time PCR (Bustin et al., 2005), each RNA extract was analyzed for quality and quantity by microfluidic electrophoresis with an Agilent 2100 Bioanalyzer (Waldbronn, Germany). Two micrograms of each sample of total RNA were reverse transcribed to single-stranded cDNA with SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA) using oligo(dT) as primer.

A partial cDNA sequence encoding the α-subunit of *P. marmoratus* Na^+/K^+-ATPase was initially amplified by PCR from gill cDNA employing degenerate primers NAK10F and NAK16R that were based on conserved sequences from other species (Towle et al., 2001) (Table 1). Following an initial hot start at 91°C for 5 min, amplification was performed for 30 cycles of 94°C for 1 min, 45°C for 1 min and 72°C for 2 min, followed by 1 cycle of 72°C for 5 min and storage at 4°C. Following gel purification (MinElute Gel Extraction, Qiagen, Valencia, CA, USA) and dideoxynucleotide sequencing (ABI Prism 3100, Foster City, CA, USA), a 707-nucleotide sequence was obtained, confirmed by BLAST analysis (Altschul et al., 1997) as a partial Na^+/K^+-ATPase α-subunit cDNA sequence. Primers specific to this sequence, designed using Primer Premier software, were employed in the completion of *P. marmoratus* α-subunit cDNA sequencing using 3′-RACE (Invitrogen) and 5′-RACE (Clontech, Palo Alto, CA, USA) techniques. To differentiate problematic amplifications in the 5′ region of the cDNA, PCR products were subcloned using TA cloning (Invitrogen) and inserts in individual plasmid preparations were then sequenced. A multiple alignment and phylogenetic tree of α-subunit nucleotide sequences were produced using the MegAlign component of DNASTAR software and open reading frames were predicted using DNASIS software.

Genomic DNA was isolated from *P. marmoratus* gills and testis using a DNeasy Tissue Kit (Qiagen). The segment of genomic DNA encoding the upstream promoter region, the probable transcription start site, and the 5′ region of the mRNA transcript of the Na^+/K^+-ATPase α-subunit were amplified and sequenced using a GenomeWalker Kit (Clontech) (Fors et al., 1990) with reverse primers (PMNAK238R and PMNAK583R; Table 1) based initially on the α-subunit cDNA sequence with subsequent primers designed as necessary. Putative transcription factor binding sites were predicted using MatInspector (Cartharius et al., 2005), transcription element search software (TESS) (Schug and Overton, 1997), P-Match (Chekmenev et al., 2005) and TFSearch (Akiyama, 1995). Because some predictions could represent false positives, we included in our analysis only putative binding sites predicted by at least three of these programs.

Quantification of gene expression

Analysis of Na^+/K^+-ATPase α-subunit mRNA expression was accomplished by real-time quantitative PCR (QPCR) using SYBR green binding with the Brilliant QPCR Master Mix and MX4000 instrumentation (Stratagene, Cedar Creek, TX, USA). Initial experiments used primers that were designed to quantify the total expression of α-subunit mRNA (NAKPMF1 and NAKPMR1, Table 1). Primers employed to differentiate between the two α-subunit isoforms were PMNAKUSF, which targeted a portion of the 81-nucleotide segment present in isoform D, and PMNAKSPF, which bridged the flanking sequences around this 81-nucleotide sequence, missing in isoform C. Both of these forward primers were used with reverse primer PMNAK238R (Table 1). The thermal profile for real-time PCR consisted of an activation step at 95°C for 15 min and 40 cycles of denaturing at 94°C for 40 s, annealing at 55°C for 40 s and elongation at 72°C for 1 min. After the last amplification cycle, the temperature was increased to 95°C

for 1 min and then decreased to 55°C to run 82 cycles, increasing by 0.5°C per cycle, to obtain melting curves, which confirmed the absence of non-specific PCR products and primer dimers.

mRNA expression levels were normalized to total RNA content by using triplicate 1 μl aliquots of each 20 μl cDNA reaction mixture that was produced with 2 μg total RNA. cDNA in each QPCR incubation was thus derived from 0.1 μg total RNA. One reference gill preparation shown to exhibit high expression levels (gill 6 at 48 h exposure to 10‰ salinity) served as the basis for a standard dilution series, demonstrating a linear relationship between threshold cycle (C_t) and $\log_{10}$ of template availability, and was used as the basis for calculating relative abundance values in the remaining samples. Expression of Na^+/K^+-ATPase α-subunit mRNA was compared to that of a putative housekeeping gene, arginine kinase (Kotlyar et al., 2000), using primers specific to *P. marmoratus* arginine kinase cDNA (Table 1).

Statistics

Statistical analyses of time course data were performed by two-way ANOVA with Bonferroni *post-hoc* test of differences from zero time (data from crabs in 36‰ seawater). Statistical significance was accepted if $P<0.05$.

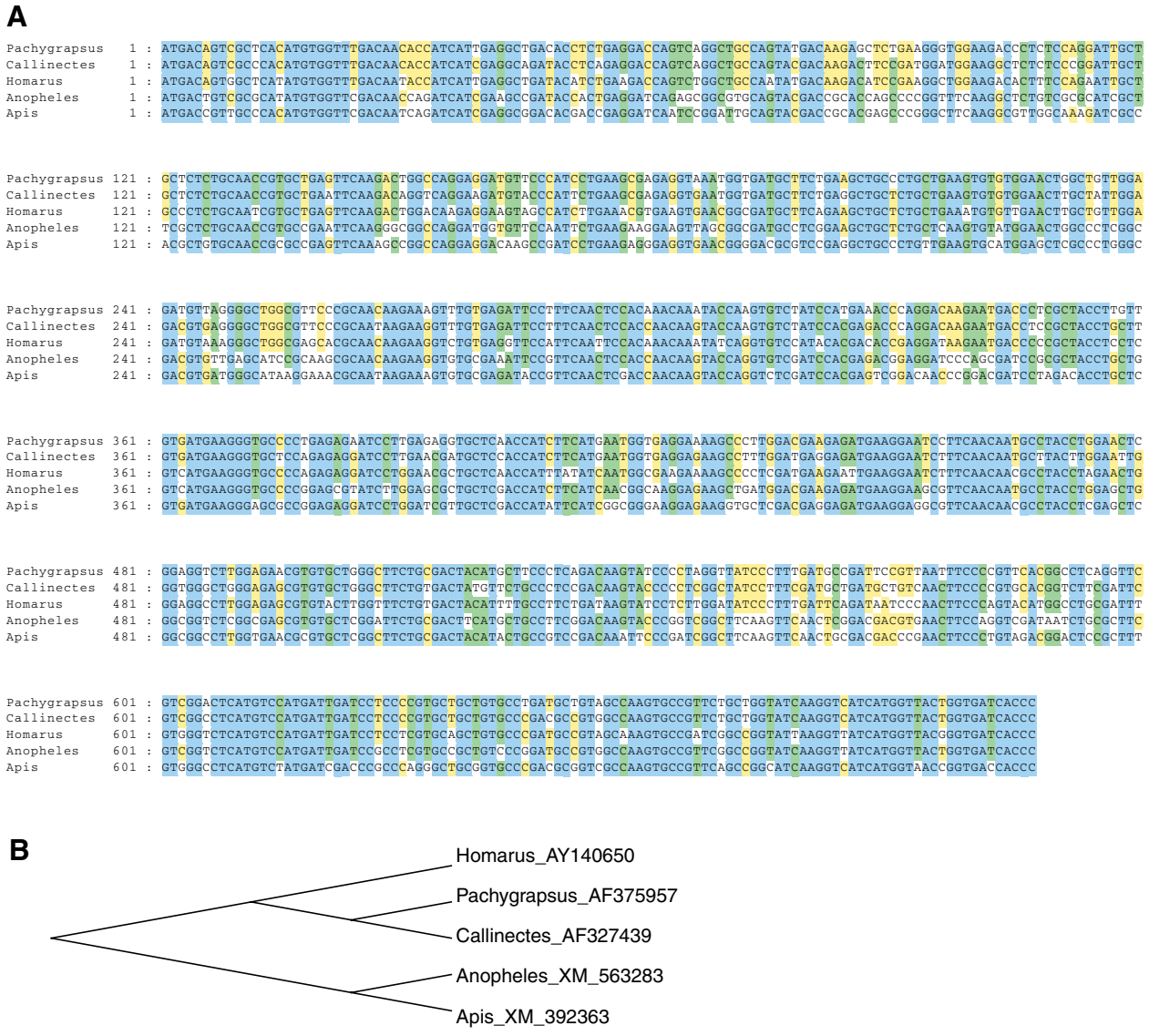

Fig. 1. Multiple alignment and phenogram of initial 707-nucleotide amplification product of Na^+/K^+-ATPase α-subunit from *Pachygrapsus marmoratus* (accession no. AF375957) with corresponding partial cDNA sequences from four other arthropods: blue crab *Callinectes sapidus* (acc. no. AF327439), American lobster *Homarus americanus* (acc. no. AY140650), mosquito *Anopheles gambiae* (acc. no. XM_563283), and honey bee *Apis mellifera* (acc. no. XM_392363). The alignment (A) and phenogram (B) were both generated using the Hein algorithm (Hein, 1990) in the MegAlign component of DNASTAR software and configured with GeneDoc (Nicholas and Nicholas, 1997). The blue background indicates 100% agreement between all five sequences, green 80%, and yellow 60%. Bootstrap values at each node of the phenogram=100.

Results

The initial amplification product of 707 nucleotides revealed a close match with Na^+/K^+-ATPase α-subunit cDNA sequences from other arthropod species upon BLAST analysis and multiple alignment (Fig. 1). The partial nucleotide sequence obtained from *Pachygrapsus marmoratus* most closely resembled the Na^+/K^+-ATPase α-subunit cDNA sequence of another euryhaline, brachyuran crab *Callinectes sapidus* (accession no. AF327439) (Towle et al., 2001) and was somewhat less similar to the corresponding sequence of a more stenohaline decapod crustacean *Homarus americanus* (acc. no. AY140650). The two selected insect α-subunit sequences were grouped separately by the alignment protocol.

We completed the sequencing of the α-subunit cDNA using 3′- and 5′-RACE techniques and primers based on the sequence of the initial amplification product. An incomplete sequence of 1554 nucleotides was initially submitted to NCBI (Accession No. AF375957). Finishing out the 3′ end proved to be straightforward, requiring only the design of additional primers for primer walking. However, completion of the 5′ end was more problematic, producing two amplification products in situations where one was expected. We became convinced that in fact there are two α-subunit cDNA sequences in *P. marmoratus* gills, differing only in the inclusion of 81 nucleotides in the so-called 'D' form that are absent in the 'C' form. The additional 81 nucleotides in the longer D form reside close to the probable translation start site. The two mRNA sequences are predicted to lead to the production of two different α-subunit proteins varying in size by 27 amino acids (Fig. 2).

Analysis of the two α-subunit protein sequences indicate that both contain the same ten transmembrane domains predicted by transmembrane hidden Markov modeling (TMHMM) (Sonnhammer et al., 1998), consistent with topologies based on experimental and computational analyses (Kaplan, 2002) (Fig. 2). Prosite analysis (release 20.3) revealed the amino acid sequence DKTGTLT at position 390-396 containing the trademark P-type ATPase motif including the aspartate that becomes phosphorylated during the reaction cycle (Kaplan, 2002). A single cAMP- and cGMP-dependent protein kinase phosphorylation site was also identified at position 954-957. An inspection of the two amino acid sequences using the eukaryote linear motif (ELM) resource for functional sites in proteins (Puntervoll et al., 2003) revealed a most interesting distinction between the C and D forms of the α-subunit. In the extended D form, the sequence of 27 additional amino acids contains the motif RTDSYR, identified by ELM as a putative binding site for the regulatory protein 14-3-3 (Mackintosh, 2004) (Fig. 2).

A BLAST search (Altschul et al., 1997) of the 'non-redundant' and 'other EST' databases of GenBank revealed cDNAs or expressed sequence tags from other crustacean species corresponding to both C and D forms of the α-subunit (Fig. 3). The amino acid sequence encoded by an expressed sequence tag from the shrimp *Litopenaeus vannamei* (O'Leary et al., 2006) matched the C form of *P. marmoratus*, whereas a second EST independently obtained from *Fenneropenaeus chinensis* (Wang et al., 2006) matched the D form. In the only crustacean genome currently being sequenced, that of *Daphnia pulex*, five α-subunit encoding genes are predicted by the semi-HMM-based nucleic acid parser (SNAP) (Korf, 2004; Colbourne et al., 2005). However, none of these appear to encode an N terminus similar to that found in the *P. marmoratus* D form. By contrast, α-subunit N termini containing a putative binding site for 14-3-3 are found in many other species, including the honeybee *Apis mellifera* (acc. no. XP_623072), sea urchin *Strongylocentrotus purpuratus* (acc. no. XP_795226), and zebrafish *Danio rerio* (acc. no. AAG27058) (Rajarao et al., 2001).

Analysis of α-subunit mRNA expression by quantitative PCR first used primers NAKPMF1 and NAKPMR1 (Table 1) that amplified C and D forms simultaneously. Such analysis showed a complex response to salinity change that was gill specific and also salinity specific. Following transfer from 36 to 10‰ salinity, α-subunit mRNA levels in all of the tested gills increased during the experimental period, with gill 7 responding within 2 h and other gills showing a delayed response (Fig. 4A). By 24 h, all gills exhibited significantly enhanced expression of α-subunit mRNA ($P<0.001$). The basic pattern was repeatable in a completely independent set of gill preparations made the following year (Fig. 4B) and demonstrated a gill-specific time course of transcript changes following short-term exposure to dilute salinity.

When crabs were transferred from 36 to 45‰, a condition in which the animals hypoosmoregulate their hemolymph, we observed that α-subunit expression remained low in all of the gills except for gill 7, which showed a marked increase in α-subunit mRNA within 6 h after the transfer, an increase that was maintained through the 48-hour experimental period (Fig. 4C). This surprising result was confirmed in a subsequent independent experiment in the following year (Fig. 4D). Our data indicate that α-subunit gene transcription in gill 7 is particularly responsive to hypersaline conditions.

In many invertebrates, arginine kinase is an important

Fig. 2. Complete nucleotide and predicted amino acid sequence of the D form (accession no. DQ173924) of Na^+/K^+-ATPase α-subunit cDNA amplified from gills of *Pachygrapsus marmoratus*. The C form (acc. no. DQ173925) lacks the 81-nucleotide segment and 27-amino-acid segment indicated in blue. A consensus 14-3-3 binding site RTDSYR (Aitken, 2002), indicated by a rectangle, is found near the N terminus of the D form but not the C form. In-frame stop codons are indicated in red. Ten transmembrane domains predicted by transmembrane hidden Markov modeling (TMHMM) (Sonnhammer et al., 1998) are underlined. Prosite (release 20.3) analysis identified the amino acid sequence DKTGTLT at position 390-396 (bold typeface) as a characteristic P-type ATPase motif including the aspartate that becomes phosphorylated during the transport cycle (Kaplan, 2002). A cAMP- and cGMP-dependent protein kinase phosphorylation site (RRNS) is predicted at position 954-957 (bold typeface). Brackets enclose the 707-nucleotide section initially amplified and aligned in Fig. 1 with corresponding segments from other species.

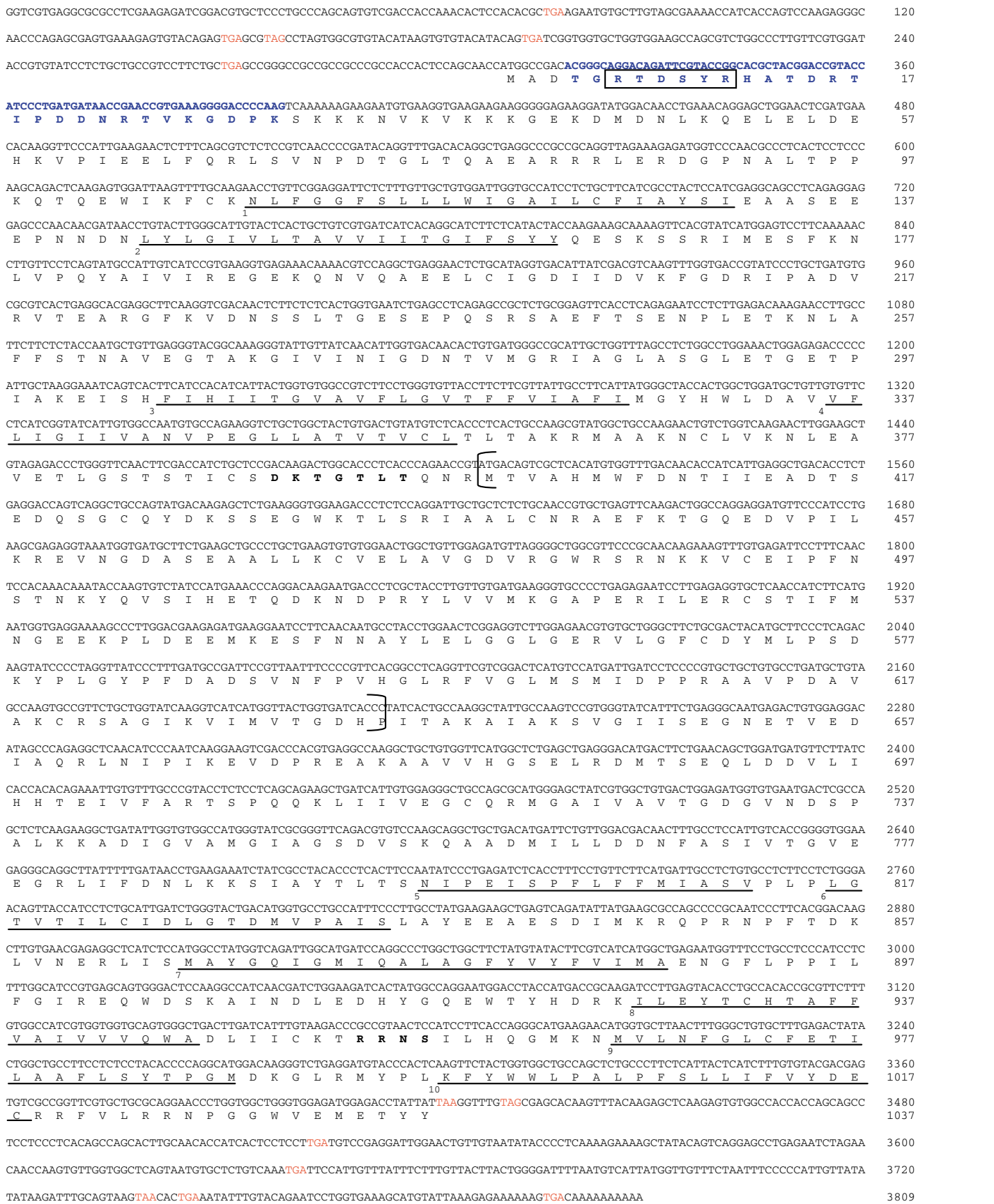

Fig. 2. See previous page for legend.

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                                *        20         *        40         *        60
Artemia_Alpha1      : ---------------------------------MAKGKQKKGK---DLNELKKELDIDFHKIPI :   28
Artemia_Alpha2      : -----------------------------------MGKKQGK---QLSDLKKELELDQHKIPL :   25
Pachygrapsus_C      : --------------------------MADSKKKNVKVK-KKGEK--DMDNLKQELELDEHKVPI :   35
Litopenaeus_EST     : --------------------------MADSKKKPQKAKGKKGDM--DLNDLKQELELDEHKVPI :   36
Pachygrapsus_D      : MADTGRTDSYRHATDRTIPDDNRTVKGDPKSKKKNVKVK-KKGEK--DMDNLKQELELDEHKVPI :   62
Fenneropenaeus_EST  : MADTGRTDSYRHATDRTIPDDNRTVKGDPKSKKKPQKAKGKKGDK--DLNDLKQELELDEHKVPI :   63
Callinectes         : MADTGRTDSYRHATDRNIPDDNRTVKGDPKSKKKNVKGK-RKGEKEKDMDNLKQELELDEHKVPI :   64
Homarus             : MADTGRTDSYRHATDRTIPDDNRTVKGDPKSTKKMPKAKAKKGEK--DMDNLKRELELDEHKVPI :   63

                             *        80         *       100         *       120         *
Artemia_Alpha1      : EECYQRLGSNPETGLTNAQARSNIERDGPNCLTPPKTTPEWIKFCKNLFGGFALLLWTGAILCFL :   93
Artemia_Alpha2      : EELCRRLGTNTETGLTSSQAKSHLEKYGPNALTPPRTTPEWIKFCKQLFGGFQMLLWIGSILCFI :   90
Pachygrapsus_C      : EELFQRLSVNPDTGLTQAEARRRLERDGPNALTPPKQTQEWIKFCKNLFGGFSLLLWIGAILCFI :  100
Litopenaeus_EST     : EELFQRLTVNPDTGLSQSEAKRRIERDGPNALTPPKQTPEWVKFCKNLFGGFSLLLWIGAILCFI :  101
Pachygrapsus_D      : EELFQRLSVNPDTGLTQAEARRRLERDGPNALTPPKQTQEWIKFCKNLFGGFSLLLWIGAILCFI :  127
Fenneropenaeus_EST  : EELFQRLTVNPDTGLSQSEAKRRIERDGPNALTPPKQTPEWVKFCENLFGGFSLLLWIGAIL--- :  125
Callinectes         : EELFQRLSVNPDTGLTQAEARRRLERDGPNALTPPKQTPEWVKFCKNLFGGFSLLLWIGAILCFI :  129
Homarus             : EELFQRLSVNPDSGLTQAEALRRTERDGPNALTPPKQTPEWVKFCKNLFGGFSLLLWIGAILCFI :  128
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Fig. 3. Multiple alignment of N-terminal amino acids of eight crustacean Na^+/K^+-ATPase α-subunits illustrating two groups, one composed of sequences containing a 14-3-3 protein binding site (RTDSY) close to the N terminus and the other group truncated and lacking this binding site. Accession numbers are given in parentheses: *Artemia franciscana* Alpha1 (X56650) (Macías et al., 1991), *Artemia franciscana* Alpha2 (Y07513) (Baxter-Lowe et al., 1989), *Pachygrapsus marmoratus* C form (DQ173925) (present study), *Litopenaeus vannamei* EST (CK572083) (O'Leary et al., 2006), *P. marmoratus* D form (DQ173924) (present study), *Fenneropenaeus chinensis* EST (BM303114) (Wang et al., 2006), *Callinectes sapidus* (AF327439) (Towle et al., 2001), *Homarus americanus* (AY140650) (Parrie and D.W.T., unpublished). The alignment was produced with Multalin version 5.4.1 (Corpet, 1988) and processed with GeneDoc (Nicholas and Nicholas, 1997). Blue background indicates 100% amino acid agreement between the eight sequences, green indicates 75–99%, yellow 50–74%.

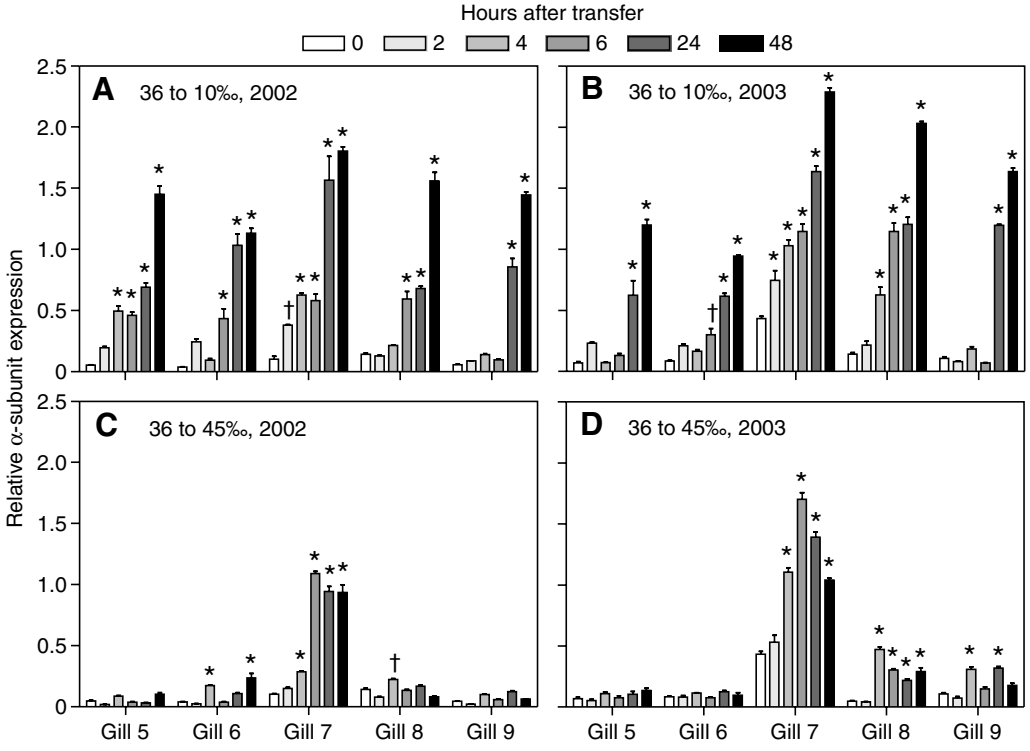

Fig. 4. Quantitative PCR analysis of Na^+/K^+-ATPase α-subunit mRNA abundance in individual gill preparations from *Pachygrapsus marmoratus* following transfer for various time periods (2–48 h) from 36‰ seawater to 10‰ (A,B) or to 45‰ (C,D). Values are means ± s.d. (N=3 or 4 measurements at each time point using gills pooled from three or four animals) of data obtained with primers NAKPMF1 and NAKPMR1 designed to detect both isoforms simultaneously (Table 1). Two independent experiments from 2002 (A,C) and 2003 (B,D) are presented. Relative expression levels are indicated in relation to a standard reference (one sample of gill 6 at 48 h after transfer to 10‰). Significant differences from zero time values (crabs in 36‰ seawater) are indicated: *P<0.001; †P<0.01.

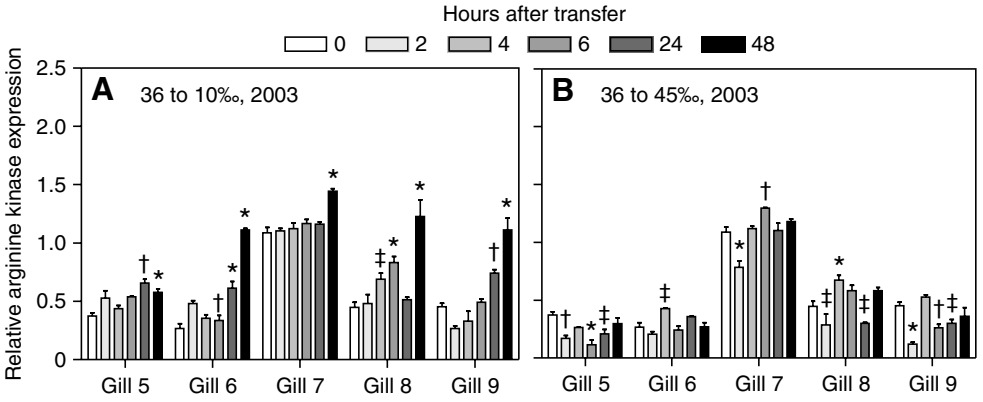

Fig. 5. Quantitative PCR analysis of arginine kinase mRNA abundance in individual gills of *Pachygrapsus marmoratus* transferred from 36‰ to 10‰ (A) or to 45‰ (B) seawater for time periods indicated above the figure. Mean ± s.d. (N=3 or 4 measurements at each time point using gills pooled from three or four animals). Relative expression levels are indicated in relation to a standard reference (one sample of gill 6 at 48 h after transfer to 10‰). Significant differences from zero time values (36‰ seawater) are indicated: *P<0.001; †P<0.01; ‡P<0.05.

component in energy metabolism, catalyzing the phosphorylation of ADP at the expense of phosphoarginine, and it is strongly expressed in crustacean gills (Kotlyar et al., 2000). In *P. marmoratus*, arginine kinase mRNA levels were influenced by environmental salinity, but in a minor fashion compared with α-subunit (Fig. 5). Transfer of crabs from 36 to 10‰ salinity elicited minor but statistically significant changes in arginine kinase mRNA levels by 48 h after the transfer, but the pattern and extent of induction did not resemble that of the Na^+/K^+-ATPase α-subunit, suggesting that the substantial changes in α-subunit mRNA expression were in specific response to the salinity challenge. Transfer of crabs from 36 to 45‰ similarly lead to some statistically significant changes in arginine kinase mRNA but there was no consistent pattern, even within gills, and the changes were quite small relative to the changes observed in α-subunit expression. Interestingly, in comparison to other gills, gill 7 consistently showed the highest level of arginine kinase mRNA, perhaps contributing to the apparently robust response of that gill to salinity stress.

When we examined the differential expression of the two α-subunit isoforms with respect to salinity and time course, we found more apparent variability among the samples than when we analyzed total α-subunit expression. Transfer of crabs from 36 to 10‰ resulted in a generalized increase in transcripts encoding both isoforms, consistent with our observations of total expression. All tested gills showed a trend toward increased transcript levels with longer exposure to dilute salinity, with a generally greater expression of the short isoform, C (Fig. 6A,B). However, in crabs transferred from 36 to 45‰, high expression levels of isoforms C and D were observed primarily in gill 7 (Fig. 6C,D), in agreement with experiments measuring expression of both isoforms simultaneously (Fig. 4C,D).

Sequencing genomic DNA upstream of the transcription start site of the α-subunit gene using genome walking (Fors et al., 1990) revealed a 462-nucleotide sequence adjoining the first exon that contained four putative binding sites for the transcription factor SP1, two each for ATF/CREB, AP1, and HSF, and one for MYOD (Fig. 7). Predicted CAAT and TATA box-like regions were also identified. Sequencing genomic DNA downstream of the transcription start site confirmed the existence of the short C isoform at the genomic level, but repeated attempts to demonstrate the genomic equivalent of the longer D isoform were unsuccessful. Amplifications of genomic DNA using primers that targeted potential genomic regions upstream and downstream of the predicted 81-nucleotide D-isoform inclusion consistently produced only a C-isoform amplicon. Primers targeted specifically to the 81-nucleotide inclusion itself failed to produce any product when genomic DNA was employed as template.

Discussion

In this study, we demonstrated the occurrence of two Na^+/K^+-ATPase α-subunit-encoding transcripts in gills from the hyper–hypoosmoregulating crab *Pachygrapsus marmoratus*, differing only in the inclusion of an 81-nucleotide sequence near the translation start site in the D form. Differential exon splicing in the sea urchin *Hemicentrotus pulcherrimus* is known to produce Na^+/K^+-ATPase α-subunit transcript variants differing only in the 5′ leader region (Yamazaki et al., 1997). We anticipated that the α-subunit cDNA variants found in *P. marmoratus* were, similarly, the result of differential exon splicing, since the only difference between the two variants that we have clearly identified lies in the 5′ region of the sequence. We thus expected to find the D isoform in the genome, with the C isoform arising by alternative splicing. However, exhaustive analysis of genomic DNA by PCR with many combinations of primers and amplification conditions demonstrated the existence of only the C isoform at the genomic level. This surprising finding suggested to us that the 81-nucleotide addition characteristic of the D isoform might have arisen by trans-splicing, a process by which nascent pre-mRNA receives a ribonucleotide segment

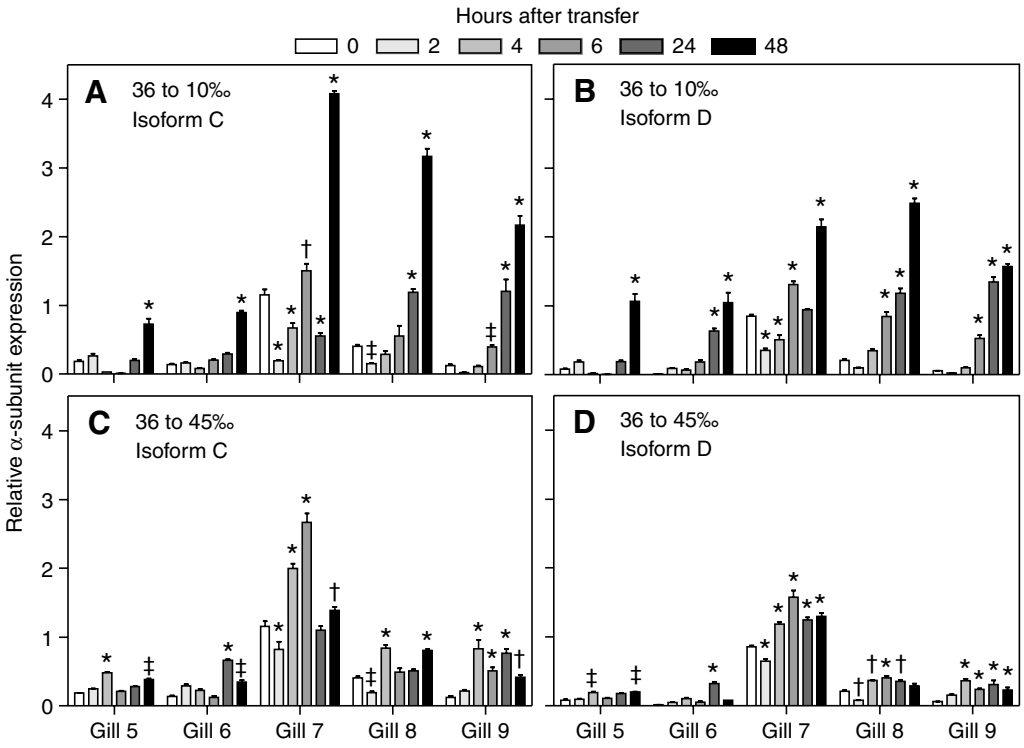

Fig. 6. Quantitative PCR analysis of mRNA encoding C and D forms of Na^+/K^+-ATPase α-subunit in individual gills of *Pachygrapsus marmoratus* transferred from 36‰ seawater to 10‰ (A,B) or to 45‰ (C,D) seawater. Values are mean ± s.d. (N=3 measurements at each time point using gills pooled from three or four animals). Relative expression levels are indicated in relation to a standard reference (one sample of gill 6 at 48 h after transfer to 10‰). Significant differences from zero time values (animals in 36‰ seawater) are indicated: *P<0.001; †P<0.01; ‡P<0.05.

encoded by genomic DNA not associated with the gene in question (Maniatis and Tasic, 2002). However, the presence in several non-crustacean species of genomic sequences encoding similar N termini led us to conclude that the lack of success in amplifying the D form from genomic DNA of *P. marmoratus* was more likely the result of a failure to discover the proper PCR amplification conditions rather than its actual absence.

The discovery of a predicted 14-3-3 protein binding site near the N-terminus of the α-subunit D form of *P. marmoratus* and other species suggests that the Na^+/K^+-ATPase may be responsive to this regulatory protein *in vivo*. The 14-3-3 protein family binds to phosphoserine or phosphothreonine on target proteins and regulates their translocation between cytoplasmic or endoplasmic reticulum sites and the plasma membrane (Dougherty and Morrison, 2004; Mackintosh, 2004). In the case of mammalian Na^+/K^+-ATPase, 14-3-3 was shown to be essential for the dopamine-induced endocytosis of α-subunit protein in opossum kidney, apparently by binding directly to the N terminus of the protein (Efendiev et al., 2005). In gills of euryhaline crabs, such a mechanism may be important in the short-term minute-by-minute regulation of Na^+/K^+-ATPase function at the plasma membrane.

Longer term (hours to days) regulation of Na^+/K^+-ATPase function in gills would depend, at least in part, on the availability of α-subunit mRNA and protein. We observed significant changes in α-subunit mRNA abundance following transfer from seawater to either dilute or concentrated media, the former eliciting

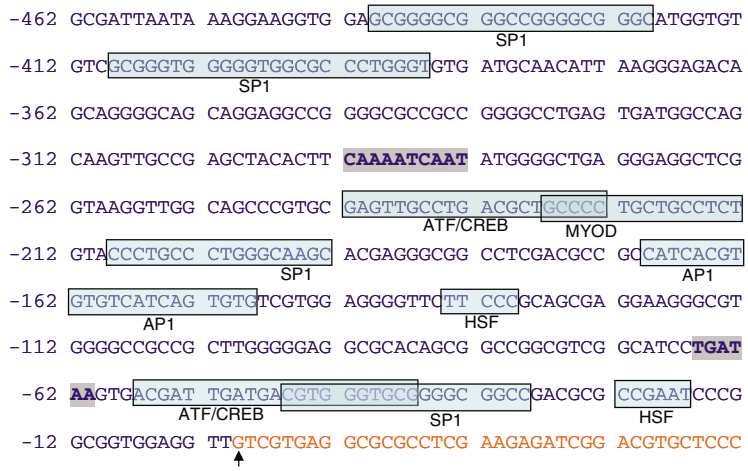

Fig. 7. Genomic DNA sequence of *Pachygrapsus marmoratus* Na^+/K^+-ATPase α-subunit promoter region including the transcription start site (arrow) and 462 additional upstream nucleotides determined by genome walking. CAAT and TATA box regions are shaded, and predicted binding sites for transcription factors are indicated by labeled rectangles.

hyperosmoregulation of the hemolymph and the latter hypoosmoregulation. Moreover, gills responded differently both in terms of the time course and the degree of response, indicating a gill-specific pattern of transcriptional regulation. All gills examined responded to diluted seawater, albeit with different time courses (posterior gills generally responding more rapidly), but only gill 7 responded to concentrated seawater. This finding by itself suggests that all gills that we examined may participate in ion uptake from dilute media but only gill 7 may participate in ion excretion into concentrated media. The specificity of the response to a concentrated medium indicates distinct osmoregulatory roles among the gills, and that the increased Na^+/K^+-ATPase mRNA expression is not simply part of a cellular osmoregulatory mechanism, since the cells of all of the gills were exposed to the same salinity stress but only gill 7 responded transcriptionally to concentrated seawater.

Such specificity of gill function in euryhaline crustaceans has been suggested previously by studies of transport physiology and enzyme activity. In the green shore crab *Carcinus maenas*, for example, individual gills show different levels of Na^+/K^+-ATPase specific activity, the posterior gills generally being higher than the anterior gills but nearly all responding to salinity change (Siebers et al., 1982). In the mangrove crab *Ucides cordatus*, gill 5 (7 in total) is more permeable to sodium than gill 6; flux ratio analysis showed that gill 5 is associated with active ion uptake from dilute seawater whereas gill 6 is associated with active ion extrusion into concentrated seawater (Martinez et al., 1998). Our gene expression results for *P. marmoratus* support the conclusion that specificity of function does indeed reside in individual gills and that gill 7 (of 9) responds to concentrated seawater in a fashion that suggests it may be specifically involved in active ion extrusion, thus facilitating hypoosmoregulation of the hemolymph.

A related study with the South American varunid crab *Chasmagnathus granulatus* in which Na^+/K^+-ATPase α-subunit expression was assessed in pooled posterior (6–8) and anterior (3–5) gills showed massive induction of transcript abundance, mainly in posterior gills, within 24 h following transfer of crabs from 30 to 2‰ salinity and by 96 h following transfer from 30 to 45‰ (Luquet et al., 2005). In the portunid crab *Callinectes sapidus*, statistically significant increases in α-subunit transcript abundance appeared in a posterior gill (7) by 96 h following transfer from 32 to 10‰ salinity (Lovett et al., 2006), a delayed response compared with *P. marmoratus*.

The apparent complexity of transcriptional regulation of α-subunit mRNA levels in *P. marmoratus* suggests a corresponding complexity of promoter structure. Indeed, we show here that the promoter region of the α-subunit gene of *P. marmoratus* contains at least five different transcription factor binding sites. (Because we were successful in identifying only the C isoform of the α-subunit at the genomic level, our description of the promoter region refers only to that isoform.) The predicted promoter region contains CAAT and TATA-like boxes plus a high G+C content (67%), indications that it is indeed intimately involved in transcriptional regulation of the α-subunit gene. Unlike the Na^+/K^+-ATPase α1-subunit gene of the branchiopod crustacean *Artemia franciscana* (Garcia-Sáez et al., 1997), the promoter region in *P. marmoratus* is contiguous with the predicted transcription start site and thus the first exon.

Within the *P. marmoratus* α-subunit promoter are four predicted binding sites for stimulating protein 1 (Sp1), a common zinc-finger-like transcription factor that interacts with CpG islands during transcriptional regulation. Two binding sites are predicted for activator protein 1 (AP1). These transcription factor binding sites are also found in the promoter region of the α1-subunit gene of *A. franciscana* (Garcia-Sáez et al., 1997) as well as in mammalian α-subunit genes (Keryanov and Gardner, 2002). At positions –36 and –222, binding sites for activating transcription factor/cyclic-AMP response element binding protein (ATF/CREB) are predicted for the *P. marmoratus* α-subunit gene. Proteins of the ATF/CREB family are known to regulate gene expression during cellular responses to environmental stress (Wilkinson et al., 1996; Fawcett et al., 1999) and binding sites for them have been reported previously in α-subunit promoter regions of several vertebrate species (Suzuki-Yagawa et al., 1992; Yu et al., 1996). In combination with other transcription factor binding sites predicted for the *P. marmoratus* α-subunit gene (Fig. 7), it is apparent that the transcriptional regulation of this gene may offer a number of alternative choices, allowing for the complexity of mRNA response that we observed in gills when crabs were transferred from seawater to dilute or concentrated environments.

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