ESTIMATION OF SODIUM/PROTON ANTIPORTER mRNA IN GILLS OF THE GREEN SHORE CRAB CARCINUS MAENAS BY QUANTITATIVE RT-PCR

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In the gills of euryhaline crustaceans, uptake of sodium ions from dilute salinities is accomplished by an apical amiloride-sensitive sodium/proton antiporter functioning in series with the basolateral sodium pump (Na^++K^+-ATPase). Na^++K^+-ATPase protein is expressed predominantly in mitochondria-rich epithelial cells lining the lamellae of posterior gills (Towle and Kays, J. Exp. Zool. 239:311-318, 1986). Whether the Na^+/H^+ antiporter is also differentially expressed in posterior gills, particularly at the level of transcription, was the subject of this study.

Cloning and sequencing the sodium/proton antiporter cDNA from gills of the green shore crab Carcinus maenas has revealed a 2,592-nucleotide sequence containing an open reading frame coding for a protein of 672 amino acids. Alignment with vertebrate isoforms has shown an overall 32-to-38% identity at the amino acid level and a substantially higher degree of similarity in putative transmembrane regions (Towle and Wu, Bull. Mt. Desert Isl. Biol. Lab. 33:122-123, 1994). In the current study, we utilized the antiporter sequence information to estimate the steady-state levels of antiporter mRNA in gills of Carcinus maenas, employing quantitative reverse transcription-polymerase chain reaction (RT-PCR).

Total RNA was isolated from the anterior five gills and the posterior three gills of Carcinus maenas kept in high (app. 33 parts per thousand) or low (10 parts per thousand) salinity for at least seven days. RNA was quantitated by UV absorption at 260 nm. Single-stranded cDNA was reverse transcribed from 10 μg of total RNA using oligo-dT as the primer. A measured quantity (up to 2 μl of the 50-μl reaction mixture) of the resulting cDNA product was used as the template for PCR. A pair of non-degenerate oligonucleotide primers was designed to allow amplification of a 784-nucleotide segment of the Carcinus sodium/proton antiporter cDNA. The primers were: 20F = 5'-TGCAAGTGGAGGAGGTGCTGTTTAT-3'; 21R = 5'-ATCAACCCAATCCACATCCCACTC-3', designed to hybridize to non-conserved regions of the antiporter sequence to reduce the likelihood of spurious products. Amplified DNA was biotinylated by replacing one-sixth of the dTTP in the PCR reaction mixture with biotin-dUTP. Amplification of the antiporter cDNA segment was carried out for 22, 24, and 26 cycles of 92°C for 30 sec, 60°C for 35 sec, and 72°C for 35 sec. Samples (10 μl) of the amplification products were electrophoresed on 0.8% agarose gels and transferred to Immobilon-S nylon membranes. Biotinylated RT-PCR products were visualized using the New England Biolabs Phototope method.

To achieve a quantitative estimate of mRNA expression using RT-PCR, production of the amplification product must be shown to be dependent on cDNA template abundance, which presumably reflects mRNA abundance. Furthermore, the amplification process itself must yield an approximate doubling of product with each cycle, placing a limit on the number of cycles which can be employed due to decreasing efficiency of amplification as the number of cycles increases. Using biotin-dUTP to label amplification products allowed us to decrease the number of

amplification cycles to a stage at which product appeared to be doubling with each cycle (Fig. 1). Moreover, we demonstrated clear dependence of product formation on template abundance.

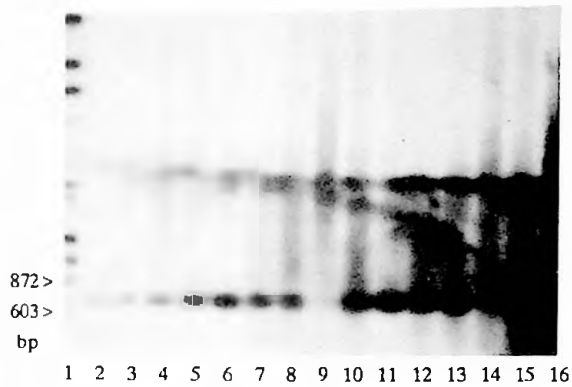

Figure 1. Demonstration of RT-PCR quantitation of antiporter cDNA derived from Carcinus gill mRNA. Increasing amounts of cDNA template (0.1, 0.3, 0.5, 0.7, 0.9 μl) amplified for 22 (lanes 2-6), 24 (lanes 7-11), and 26 cycles (lanes 12-16) revealed dependence of biotinylated product abundance on both template and cycle number. Lanes 9 and 14 contained failed amplification reactions. Biotinylated DNA standards are shown in lane 1.

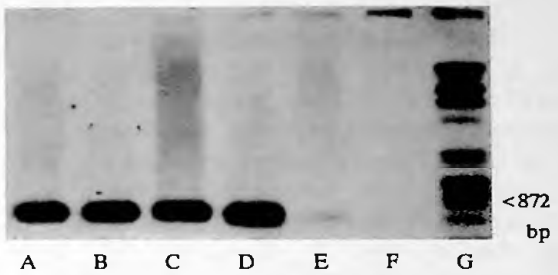

Figure 2. Quantitative RT-PCR estimation of antiporter mRNA abundance in anterior (A and C) and posterior (B and D) gills of Carcinus maenas acclimated to high (33 ppt, A and B) and low (10 ppt, C and D) salinity. Template cDNA was 2 μl of the 50-μl reverse transcription reaction mixture. Lane E was run with no reverse transcriptase and Lane F was the PCR control with no template. Amplification proceeded for 22 cycles. Lane G contained biotinylated DNA standards.

Application of quantitative RT-PCR to an estimation of Na^+/H^+-antiporter mRNA abundance in Carcinus gill revealed that posterior and anterior gills express antiporter mRNA at approximately equivalent levels (Fig. 2). Acclimation to reduced salinity, which is accompanied by increased uptake of sodium from the environment, does not appear to result in marked changes in antiporter mRNA abundance. Sodium/proton antiporters are multi-functional transporters, shown in mammals and fish to be important in pH regulation, volume regulation, and response to hormonal factors. Such functions may override the antiporter's role in transepithelial Na^+ transport in crustacean gill.

At least two Na^+/H^+-antiporters exist in crustacean tissues, an apical electrogenic 2 Na^+/1 H^+ antiporter (Shetlar and Towle, Am. J. Physiol. 257:R924-R931, 1989) and a basolateral electroneutral Na^+/H^+ antiporter (Ahearn et al., J. Exp. Biol. 196:319-335, 1994). We do not yet have evidence identifying the functionality of the antiporter cloned from Carcinus gill. A second isoform, not yet identified at the molecular level, may play a central role in transbranchial Na^+ movement in crustaceans.

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