IDENTIFICATION OF TWO DIFFERENT FORMS OF CRUSTACEAN HYPERGLYCEMIC HORMONE (CHH) IN SINUS GLANDS OF THE EURYHALINE CRAB *PACHYGRAPSUS MARMORATUS*

Céline Spanings-Pierrot[1,3], Jean-Yves Toullec[2], and David W. Towle[3]
[1]Université Montpellier II, UMR 5000, 34095 Montpellier cédex 5, France
[2]Université Paris VI, UMR 7079, 75252 Paris cédex 5, France
[3]Mount Desert Island Biological Laboratory, Salsbury Cove, ME 04672

Crustacean hyperglycemic hormone (CHH) is synthesized by neurosecretory cells which were first identified in the X-organ/sinus gland complex of the eyestalk of crustaceans and more recently in the pericardial organ (PO) and in the gut. In the green crab *Carcinus maenas*, two different CHH sequences have been determined in the neurohemal organs, one form found in the sinus gland (SG) and a second form in the PO (Dircksen et al., Biochem. J. 356: 159-170, 2001). In the crab *Pachygrapsus marmoratus*, we have observed similar results and cDNA sequencing indicated that a segment of 103 bp is spliced out of the SG-type CHH between nucleotides 412 and 515 in the 1439 nucleotides of the unspliced sequence of the PO-type CHH (Spanings-Pierrot and Toullec, unpublished data).

CHH is mainly known to be involved in hemolymph glucose regulation but it can also display overlapping activities (Van Herp, In "Recent Advances in Arthropod Endocrinology": 53-70, 1998). Interestingly, CHH seems to be involved in the control of osmoregulation in different decapod crustaceans. One sinus gland CHH isoform restores the ability to osmoregulate in destalked *Homarus americanus* at low salinity (Charmantier-Daures et al., Gen. Comp. Endocrinol. 94: 281-293, 1994). A CHH-like peptide released from gut endocrine cells regulates water and ion uptake at ecdysis in *C. maenas* (Chung et al., Proc. Nat. Acad. Sci. 96: 13103-13107, 1999). In the crab *P. marmoratus*, CHH purified from SG can directly stimulate Na^+ uptake in isolated posterior gills (Spanings-Pierrot et al., Gen. Comp. Endocrinol. 119: 340-350, 2000). Injection of CHH increases hemolymph osmolality and Na^+ concentration in destalked crayfish *Astacus leptodactylus* (Serrano et al., J. Exp. Biol. 206: 979-988, 2003). The involvement of CHH in the neuroendocrine mediation of osmoregulatory processes prompted us to initiate a molecular study on CHH of the crab *P. marmoratus* in order to examine whether CHH mRNA expression might change with salinity acclimation in this euryhaline crustacean. In this initial report, we examined the presence of CHH-related peptides and their expression in sinus glands of *P. marmoratus*, using reverse transcription and the polymerase chain reaction.

Sinus glands from crabs acclimated to seawater (SW, 36 ppt), diluted seawater (DSW, 10 ppt) and concentrated seawater (CSW, 45 ppt) were dissected in Montpellier. They were immediately placed in RNA*later*™ (Ambion) to preserve RNA for molecular analyses at MDIBL. Total RNA was first extracted with the RNAgents Total RNA Isolation System from Promega. Poly-A mRNA was reverse transcribed using oligo-dT and SuperScript II reverse transcriptase (Invitrogen). The resulting cDNAs were used as templates in polymerase chain reactions performed with specific forward and reverse primers designed according to the sequences of the SG-type CHH and the PO-type CHH in *P. marmoratus* (Table 1). The primer CHHP-PR was specifically designed to target the unspliced segment of the PO-CHH sequence. Expression of CHH mRNA was determined by means of real-time quantitative PCR performed

with a Stratagene MX4000 Multiplex PCR instrument using unlabeled primers and SYBR green fluorescence (Qiagen).

Table 1: Species-specific primers with 5'-3' sequences and expected product sizes for amplification of CHH cDNAs from sinus glands (SG, spliced) and pericardial organs (PO, unspliced) of the crab *Pachygrapsus marmoratus*.

Primers	Sequence (5'-3')	Expected size	
		Spliced SG-type	Unspliced PO-type
CHHP-XPF CHHP-XR	gct cct ctt agt gca aca ccc aat c aag gtt gct gta gca gtt ctc cct g	394 bp	489 bp
CHHP-XPF CHHP-PR	gct cct ctt agt gca aca ccc aat c gta cat ggc gtt atc gaa gca gtc g	none	400 bp

Amplification of CHH cDNA from SG by 40 cycles of conventional PCR revealed the presence of the two forms of CHH, i.e. the SG- but also the PO-type CHH, in the eyestalk neurohemal organ. Indeed, primers CHHP-XPF and CHHP-XR designed to produce both SG- and PO-type CHH amplified expected-size products with SG cDNA templates, and primers CHHP-XPF and CHH-PR designed to produce only PO-type CHH amplified a PO-like CHH in PO as template (not shown) but also with SG cDNA as template (Fig. 1). This result suggests that SG from *P. marmoratus* contains mRNAs for both forms of CHH. This hypothesis was confirmed by direct sequencing of the PCR products followed by a comparison with published sequences in GenBank with the BLAST algorithm (Altschul et al., Nucleic Acids Res. 25: 3389-3402, 1997). This observation is in agreement with similar results reported in the shore crab *C. maenas* (Townsend et al., Bull. Mt. Desert Island Biol. Lab. 41: 54-55, 2002).

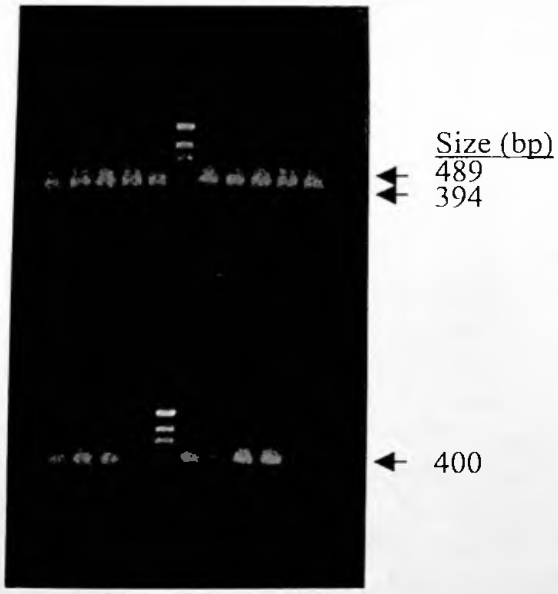

Fig. 1: Amplification of CHH cDNAs from sinus glands (SG) of the crab *Pachygrapsus marmoratus* as shown by an ethidium-bromide-stained electrophoretic gel. Left side of DNA ladder: SG from DSW-acclimated crabs. Right side of DNA ladder: SG from CSW-acclimated crabs. Primer combinations were CHHP-XPF/CHHP-XR (upper gel) or CHHP-XPF/CHHP-PR (lower gel). Note two products with CHHP-XR (spliced and unspliced) and one product with CHHP-PR (unspliced) (arrows).

Preliminary results from the real-time quantitative PCR demonstrated that the mRNA coding for the SG-type CHH was highly expressed compared to the PO-type CHH in SG from

SW-acclimated crabs. The detection of amplification of the PO-type CHH started only after 35 cycles indicating a lower amount of mRNA coding for the unspliced form (PO-type CHH) compared to the characteristic spliced form in SG (Fig. 2).

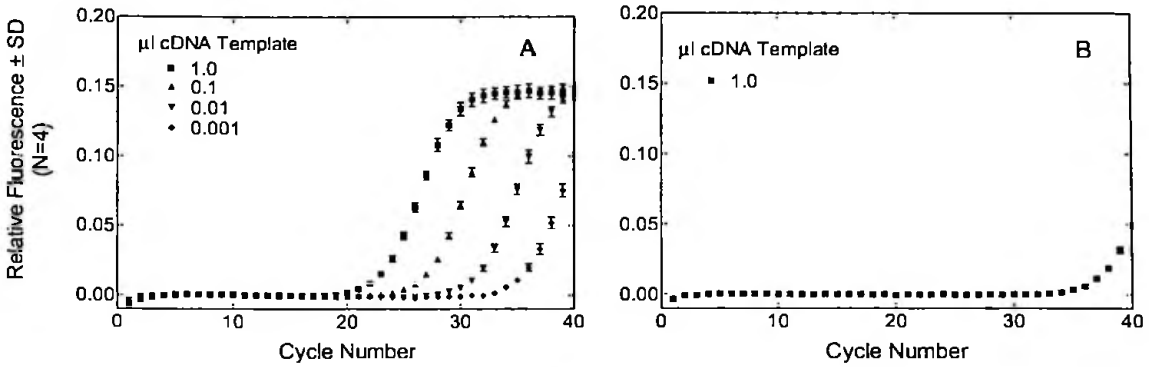

Fig. 2: Real-time quantitative PCR performed with a dilution series (in quadruplicate measurements) of SG cDNA from SW-acclimated crabs as template. The spliced SG-type CHH was targeted in A, using specific primers CHHP-XPF/CHHP-XR and the unspliced PO-type CHH was targeted in B, using specific primers CHHP-XPF/CHHP-PR. Amplification is detected as a function of cycle number by increased fluorescence of SYBR green upon binding to the double-stranded DNA products. The cycle number at which fluorescence is initially detectable is inversely proportional to the logarithm of the starting template concentration. In B, no fluorescence signals for 0.1, 0.01, or 0.001 µl template were recorded within the 40-cycle amplification.

CHH-related peptide purified from the SG of the crab *P. marmoratus* can stimulate Na^+ uptake in perfused gills (Spanings-Pierrot et al., 2000). In gills from other crab species, PO extracts stimulate Na^+/K^+-ATPase activity and Na^+ absorption (Sommer and Mantel, J. Exp. Zool. 248: 272-277, 1988; Kamemoto, Zool. Sci. 8: 827-833, 1991). Moreover, in *C. maenas*, the PO-type CHH appears to regulate neither hemolymph glucose nor Y-organ ecdysteroid synthesis (Dircksen et al., 2001) but it has been demonstrated that expression of PO-type CHH mRNA increases after transfer to low salinity (Townsend et al., Amer. Zool. 41: 1608-1609, 2001). The increased CHH mRNA would presumably lead to higher circulating levels of the CHH peptide, which would in turn activate Na^+ uptake. These observations and suggestions, combined with the present results, suggest that PO-type CHH may specifically regulate osmoregulatory processes while SG-type CHH may be involved in other physiological regulations.

This research was supported by a MDIBL New Investigator Award to C.S.-P. and by National Science Foundation grants (IBN-0196423 and DBI-0100394) to D.W.T.