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Research Report

Molecular cloning of *Clock* cDNA from the prawn, *Macrobrachium rosenbergii*☆

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ABSTRACT

CLOCK, which belongs to the basic helix–loop–helix (bHLH)/PER-ARNT-SIM (PAS) superfamily of transcription factors, is one of the most essential proteins involved in circadian systems of animals. *Clock* genes have been cloned from several species, including mammals, insects, birds, fish, and amphibians. In the present study, we successfully isolated a *Clock* homolog (termed *Mar-Clock*) from the giant prawn, *Macrobrachium rosenbergii*. The 2949-bp cDNA contained a 2115 bp open reading frame that encoded a putative CLOCK protein of 704 amino acids (termed Mar-CLOCK) exhibiting high identities with CLOCK homologs in other species (30–35%). This is the first report of a circadian clock gene from crustaceans. Mar-CLOCK possessed an exceptionally long glutamine-rich domain (140 amino acids) in its C-terminus, which usually ranges from 14 to 57 amino acids in other known CLOCKs and is supposed to function in transcriptional activation. Using RT-PCR, we observed that *Mar-Clock* was expressed in all tested tissues. Semiquantitative RT-PCR was performed to investigate the gene expression profile during the light–dark cycle. The results indicated that the expression of the *Mar-Clock* gene had no significant rhythmicity in central nervous tissues (thoracic ganglia and eyestalk) or peripheral tissues (gill, ovary, hepatopancreas, and muscle). Furthermore, gene expression tended to increase in the central nervous system (brain, thoracic, and abdominal ganglia) of eyestalk-ablated or constant dark (DD) prawns, and in the eyestalk-ablated gill. No expression change was found under constant light (LL) or in heart and muscle.

1. Introduction

Almost all organisms, from bacteria and fungi, to plants and animals, generate self-sustained oscillations of physiology, biochemistry, or behavior according to the 24-h periodicity of day and night, which is based on the circadian system (Young and Kay, 2001). The first circadian clock gene, *Period* was isolated in *Drosophila* (Reddy et al., 1984). In the following decade, another three related genes involved in the *Drosophila* circadian system, *Timeless* (Myers et al., 1995), *Clock* (Allada et al., 1998), and *Cycle* (Rutila et al., 1998) were identified. The molecular mechanisms of the *Drosophila* circadian clock have been extensively studied, and the complicated circadian system has thus been increasingly elucidated. To date, seven *Drosophila* circadian genes (*Period*, *Timeless*, *Double-time*, *Clock*, *Cycle*, *Vrille*, *Shaggy*, and *Cryptochrome*) have been identified, and their roles within the circadian mechanism have been assigned (see review; Young and Kay, 2001). Among them, two transcription factors, CLOCK and CYCLE, were found to be hetero-

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dimerized and translocated into the nucleus, and bound to so-called E-box sequences (see review; Kyriacou and Rosato, 2000) in the promoters of the *Period* and *Timeless* genes, thereby activating their transcription. PERIOD and TIMELESS accumulate in the cytoplasm and eventually enter the nucleus as a heterodimer, which represses transcription of their own genes by interfering with CLOCK and CYCLE, likely via direct binding of the PERIOD-TIMELESS dimer to the CLOCK-CYCLE dimer. This negative feedback loop is thought to be the basis of the *Drosophila* circadian system (Darlington et al., 1998). In mammals (see review; Okumura and Aida, 2001) and zebrafish (see review; Cahill, 2002), similar negative feedback loops have been reported, which indicates conservation of molecular circadian mechanisms among animals.

Clock gene was firstly identified in mice (Antoch et al., 1997; King et al., 1997). To date, *Clock* genes have been isolated from mammals (Abe et al., 1999; Antoch et al., 1997; Avivi et al., 2001; King et al., 1997; Steeves et al., 1999), insects (Allada et al., 1998; Bae et al., 1998; Chang et al., 2003; Darlington et al., 1998), fish (Mazurais et al., 2000; Whitmore et al., 1998), birds (Larkin et al., 1999; Yoshimura et al., 2000), and amphibians (Kim and Drysdale, unpublished), but not from crustaceans. As a member of the basic helix–loop–helix (bHLH)/PER-ARNT-SIM (PAS) superfamily of transcription factors, CLOCK has a basic DNA binding domain (bHLH) and protein dimerization domains (PAS A and PAS B), which show high conservation among species. The structure of the bHLH domain is well documented and has been described in many proteins (Atchley and Fitch, 1997), although the PAS domain is a more recent discovery and its known examples are now numerous (Ponting and Aravind, 1997). Expression of *Clock* gene varies among species. In *Drosophila*, *Clock* mRNA oscillates in a bimodal fashion (Darlington et al., 1998), peaking at mid-day (Zeitgeber Time 5 or ZT5) and late night (ZT23). In zebrafish (Whitmore et al., 1998) and chicken (Larkin et al., 1999), *Clock* transcripts reach maximum levels at or shortly after the transition from day to night (ZT12–ZT18). In mice, *Clock* expression rhythmicity has not been observed (Shearman et al., 1999). Based on these results, two hypothetical models were introduced (Reppert and Weaver, 1997). In insects, fish, and birds, rhythmically expressed *Clock* may form an essential autoregulatory factor for the circadian system, while in mammals, *Clock*'s transcriptional activity may be driven by more dynamically regulated CLOCK partners.

In crustaceans, especially in crayfish, features of the circadian system were extensively investigated for decades. The crayfish is a nocturnal crustacean that displays a variety of circadian rhythms controlled by periodic function of the nervous system (Fanjul-Moles and Prieto-Sagredo, 2003). The circadian rhythm of locomotor activity in crayfish has been known for a long time, and shows entrainment by environmental factors such as light and food (Fernández de Miguel and Aréchiga, 1994). It was reported that removal of the eyestalk of crayfish *Potamobius* resulted in an increase in activity as well as an apparent loss of circadian activity rhythm (Kalmus, 1938). Kalmus (1938) concluded that the eyestalk neurosecretory system was the source of control of locomotor rhythm. Furthermore, some other behavioral and physiological circadian rhythms such as retinal shielding pigment migration, electroretinogram (ERG) amplitude, heart rate, as well as metabolic and endocrine functions have been reported (see review; Fanjul-Moles and Prieto-Sagredo, 2003). Analysis of self-sustaining ERG rhythms in isolated eyestalk or protocerebrum tissue demonstrated that endogenetic circadian pacemakers exist in either or both eyestalk and protocerebrum (Aréchiga and Rodríguez-Sosa, 1998; Barrera-Mera and Block, 1990). Recently, two important clock proteins, PERIOD (PER) (Aréchiga and Rodríguez-Sosa, 1998) and cryptochrome (CRY) (Fanjul-Moles et al., 2004), were detected in the eyestalk and brain of the crayfish, *Procambarus clarkii*, by using immunocytochemical techniques. Interestingly, the CRY level in brain showed significant daily variation, which suggested its possible role in the circadian system. Although some of these rhythms are well described, there is scant information about the molecular mechanisms involved in the generation and synchronization of circadian rhythms.

Like crayfish, the prawn, *Macrobrachium rosenbergii*, is nocturnal and displays a great variety of circadian rhythms such as food intake, molting, mating, and oviposition (Wetzel, 2001). Eyestalk ablation was found to induce stimulation of ovarian maturation and shortening of molt interval in *M. rosenbergii* (Okamura et al., 2002), and an increase in hemolymph glucose levels in *Penaeus japonicus* (Yang et al., 1997). Several CHH family neuropeptides (molt-inhibiting hormone, MIH; vitellogenesis-inhibiting hormone, VIH; crustacean hyperglycemic hormone, CHH) in the eyestalk contribute to these effects (Okamura et al., 2002). Kallen reported that CHH showed apparent daily rhythmicity in crayfish hemolymph (Kallen et al., 1990). All these findings suggest a possible role for the circadian system within crustacean eyestalk endocrine mechanisms.

Here, we isolated and characterized the *Clock* gene in *M. rosenbergii* (termed *Mar-Clock*). This is the first report of a circadian clock gene from crustaceans. *Mar-Clock* encoded a putative protein of 704 amino acids (termed Mar-CLOCK) exhibiting high identities (30–35%) with other known CLOCKs. Using RT-PCR, we found that the *Mar-Clock* gene was expressed in all tested tissues (heart, eyestalk, thoracic and abdominal ganglia, gut, muscle, stomach, subepidermal adipose tissue, hepatopancreas, gill, and ovary). No significant rhythmical expression was detected in central nervous tissues (thoracic ganglia and eyestalk) or peripheral tissues (gill, ovary, hepatopancreas, and muscle). After eyestalk ablation or under constant dark (DD), the *Mar-Clock* gene expression was shown to increase in the central nervous system (brain, thoracic and abdominal ganglia) and in gill of eyestalk-ablated prawns. No significant expression change was observed in constant-light (LL) prawns or in heart and muscle. These findings provide useful information for elucidation of the crustacean circadian system.

2. Results

2.1. *Identification of the Mar-Clock gene*

Two degenerate oligonucleotide primers, ClkF and ClkR (Fig. 1 and Table 1), were designed based on the conserved regions of