Molecular cloning of Clock cDNA from the prawn, Macrobrachium rosenbergii

Jin-Shu Yang, Zhong-Min Dai, Fan Yang, Wei-Jun Yang

College of Life Sciences, Zhejiang University, 232 Wensan Road, Hangzhou, Zhejiang 310012, People’s Republic of China

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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.

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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-
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; Cahiill, 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 endogenous 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 Peneaus 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 rhythmic 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
the amino acid and mRNA sequences of CLOCK from the Chinese oak silkmoth, fruit fly, zebrafish, rainbow trout, African clawed frog, Japanese quail, chicken, house mouse, and human. The cDNAs were synthesized from thoracic ganglia total RNA of *M. rosenbergii* and used as a template for *Clock* amplification. A 1085-bp fragment (a in Fig. 1) was amplified, subcloned, and sequenced. The sequence was conceptually translated into a 361-amino acid residue sequence that exhibited 44–50% identity with other known CLOCKs. Based on the 1085-bp cDNA fragment, two pairs of nested gene-specific primers (ClkSF1, ClkSF2, ClkSR1, and ClkSR2 in Fig. 1 and Table 1) were designed and synthesized for 5′- and 3′-RACE. The 5′-RACE (r1) and 3′-RACE (fragment r2) were amplified and sequenced. These fragments, 1013- and 1605-bp in length, were overlapped with the initial PCR fragment (a in Fig. 1). The full-length cDNA was thus determined to be 2949-bp in length, based on PCR, 5′- and 3′-RACE (Fig. 1). Then, primers located near the 5′ and 3′ termini of the mRNA (ClkSAF and ClkSAR, Fig. 1 and Table 1) were designed and used for PCR amplification. The 2660-bp product (c in Fig. 1) was sequenced, confirming the sequence obtained from the partial cDNA fragments. The gene (termed *Mar-CLOCK*) was found to contain a 2115-bp open reading frame (ORF), 317-bp 5′-untranslated region, and 517-bp 3′-untranslated region with a polyadenylation signal (AATAAA) (Fig. 2). The ORF of *Mar-CLOCK* cDNA was conceptually translated into a 704-amino acid protein (termed *Mar-CLOCK*) with a calculated molecular mass of ~77 kDa. The basic helix–loop–helix (bHLH), PER-ARNT-SIM (PAS A and PAS B), and glutamine–rich domains were identified (Fig. 2). *Mar-CLOCK* exhibited highest identities with CLOCKs from the fruit fly (34.8%) and Chinese oak silkmoth (30.6%) (Fig. 3). Amino acid alignments of the bHLH and PAS domains of *Mar-CLOCK* to six other known CLOCKs (*Drosophila*, Chinese oak silkmoth, zebrafish, African clawed frog, chicken, and mouse) showed high similarities of >64%, >44%, and >80%, respectively (Fig. 4). The results indicated that *Mar-CLOCK* was a novel member of the bHLH-PAS superfamily of transcription factors.

### 2.2. Clock expression in various tissues

Northern blot analysis using 15 μg total RNA or 2–3 μg polyA+ RNA did not detect *Mar-Clock* expression (data not shown). Therefore, RT-PCR was used to characterize the tissue-specific expression of the *Mar-Clock* gene. The 147-bp *Mar-Clock* cDNA fragment was amplified by RT-PCR from heart, eyestalk, thoracic and abdominal ganglia, gut, muscle, stomach, subepidermal adipose tissue, hepatopancreas, gill, and ovary total RNA, using the primers ClkSF1 and ClkSR2 (Fig. 2). Southern blot analysis was performed to confirm the identity of the obtained bands. The results (Fig. 5) indicated that *Mar-Clock* was expressed in all tested tissues.

### Table 1 - Nucleotide sequences and positions of primers used in polymerase chain reactions

<table>
<thead>
<tr>
<th>Primer</th>
<th>Length (bp)</th>
<th>Position *</th>
<th>Direction</th>
<th>Sequence (5′-3′)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ClkF</td>
<td>20</td>
<td>375–394</td>
<td>F</td>
<td>GARAAGAARMGWMGAGATCA</td>
</tr>
<tr>
<td>ClkR</td>
<td>20</td>
<td>1440–1459</td>
<td>R</td>
<td>TCGGGYYYYBGAATTSMACTG</td>
</tr>
<tr>
<td>ClkSF1</td>
<td>19</td>
<td>867–885</td>
<td>F</td>
<td>ACAAAATTATGAGGGAGTGC</td>
</tr>
<tr>
<td>ClkSR1</td>
<td>19</td>
<td>1042–1060</td>
<td>R</td>
<td>TGACAGAGACCAAGACACAG</td>
</tr>
<tr>
<td>ClkSF2</td>
<td>21</td>
<td>1345–1365</td>
<td>F</td>
<td>TGAAGACTGGAAAGGGAACAT</td>
</tr>
<tr>
<td>ClkSR2</td>
<td>21</td>
<td>903–1013</td>
<td>R</td>
<td>GCCATTACACCGAAACGATTAC</td>
</tr>
<tr>
<td>ClkSAF</td>
<td>24</td>
<td>140–172</td>
<td>F</td>
<td>AAAAGGCTATTGCGAGTGCGAGTAC</td>
</tr>
<tr>
<td>ClkSAR</td>
<td>22</td>
<td>2787–2808</td>
<td>R</td>
<td>TTGCATCGACCCCATGAAACGAC</td>
</tr>
<tr>
<td>ActinF</td>
<td>21</td>
<td>–</td>
<td>F</td>
<td>TATGCAATCTCTGATGCATCAT</td>
</tr>
<tr>
<td>ActinR</td>
<td>19</td>
<td>–</td>
<td>R</td>
<td>AGGAGGGCCGATGGTGTAC</td>
</tr>
</tbody>
</table>

* Positions reflect nucleic acid locations in Fig. 2.
To investigate the light–dark (LD) cycle variations of Mar-Clock transcripts, semiquantitative RT-PCR was carried out as described in Experimental procedures. Gene expression in central nervous tissues (thoracic ganglia and eyestalk) and peripheral tissues (gill, ovary, hepatopancreas and muscle) was examined, and no significant temporal fluctuation was observed during the LD cycle (P > 0.05 by one-way ANOVA; Fig. 6).

Fig. 2 – Nucleotide and predicted amino acid sequences of the cDNA encoding CLOCK in M. rosenbergii (Mar-CLOCK). Deduced amino acid sequence of Mar-CLOCK is shown in the single letter representation above the respective codons. The asterisk indicates the stop codon. The consensus polyadenylation signal, AATAAA, is bolded. Nucleotide and amino acid sequences are numbered on the right of each line. Arrowheads represent primers. The bHLH (b), PAS A and PAS B (p) domains, and glutamine-rich region (QQQ) are marked, respectively.
Fig. 3 – ClustalX formatted alignment of Mar-CLOCK with dCLOCK (D. melanogaster) and apCLOCK (A. pernyi). Identical residues among ~50% of all CLOCKs are highlighted. Mar-CLOCK exhibited highest identities with dCLOCK (34.8%) and apCLOCK (30.6%).
We investigated whether expression of the Mar-CLOCK gene in several tissues was influenced by eyestalk ablation, constant light (LL) or constant dark (DD). After a 3-day (72 h) acclimation for each condition, total RNA was extracted from the central nervous system (brain, thoracic and abdominal ganglia; CNS), gill, heart, and muscle of six prawns, respectively, and semiquantitative RT-PCR was carried out as described in Experimental procedures. After eyestalk ablation or under DD, Mar-CLOCK gene expression increased in CNS (P < 0.01 by Student’s t test; Fig. 7). Likewise, an increase was observed in gill of eyestalk-ablated prawns (P < 0.01; Fig. 7). No significant expression change was found in heart and muscle, or any tested tissues under LL (P > 0.05; Fig. 7).

3. Discussion

One of the central goals of circadian biology is to identify and elucidate the molecular components of the circadian system. Here, we successfully isolated a novel gene from the prawn M. rosenbergii, Mar-CLOCK, which was 704 amino acids in length and showed impressive amino acid sequence identities with CLOCK proteins from Drosophila and oak silkmoth, suggesting...
Fig. 6 – Temporal expression analysis of Mar-Clock using semiquantitative RT-PCR. Gill, ovary, hepatopancreas, muscle, thoracic ganglia, and eyestalk were collected at the exact time points (ZT0, ZT6, ZT12, and ZT18). The 198-bp β-actin fragment was selected as an internal control for each tissue. Relative abundance refers to Mar-Clock transcript levels normalized to those of β-actin. In each case, peak values were set to 100 and the rest of the values normalized. The results are expressed as means ± SEM of independent experiments from separate RNA pools (n = 3). Representative gels for Mar-Clock and β-actin are shown above. Comparison of the expression levels did not reveal any significant rhythmicity in these tissues (one-way ANOVA; gill: P = 0.76; ovary: P = 0.60; hepatopancreas: P = 0.23; muscle: P = 0.87; thoracic ganglia: P = 0.61; eyestalk: P = 0.12).
a novel circadian member of the bHLH-PAS transcription factor superfamily. A BLASTP search using the putative full-length Mar-CLOCK sequence against the NCBI database revealed highest similarity with Drosophila CLOCK (34.8%). Therefore, based on their extensive homology, we named the new gene the circadian gene Clock. The complete mRNA sequence was submitted to the DDBJ/EMBL/GenBank databases (accession number AY842303).

The sequence identity is most impressive in the three domains that confer biological functions. The basic helix-loop-helix (bHLH) domain, spanning amino acids 11–58 of Mar-CLOCK, showed >64% identity among species. The PAS A and B domains, spanning amino acids 93–140 and 285–332, are also highly conserved among species and showed >44% and >80% identity, respectively. Because every CLOCK functions by forming a heterodimer with CYCLE (BMAL1 in mammals), we propose that Mar-CLOCK may possess a similar heterodimeric partner (Mar-CYCLE) that functions as a transcription factor by heterodimerization. The fact that all CLOCKs exhibit high overall identities and even higher identities in their functional domains may partially explain why various species in the animal kingdom share relatively conserved circadian systems (see review; Young and Kay, 2001).

Mar-CLOCK was found to have an exceptionally long glutamine (Q)-rich region (135 glutamines out of 140 amino acids), the longest of all CLOCK proteins identified to date (Table 2). The carboxyl-terminal Q-rich region, especially the polyglutamine repeats encoded by CAG or CAA triplets, is known to function in transcriptional activation of many transcription factors (Mitchell and Tjian, 1989). Removal of significant segments of the Q-rich activation domains gives rise to inactive or poorly active proteins (Allada et al., 1998;
In Drosophila, it was found that dCLOCK by itself could activate Period and Timeless promoters in vivo, and furthermore, a truncated dCLOCK lacking some of the Q-rich region only weakly activates Period and Timeless E-boxes (Darlington et al., 1998). Similarly, in the mouse circadian mutant Clock, in which part of the Q-rich domain is deleted, the mutant mCLOCK (CLOCKΔ19) and mBMAL1 still formed heterodimers that bound DNA but failed to activate transcription (Gekakis et al., 1998). Naturally, it can be inferred that, in Drosophila and mice, CLOCK functions as a transactivator, in which the Q-rich domain is the primary contributor to this activity. Recently, the reported absence of a Q-rich region in Antheraea pernyi CLOCK confounded the issue of which protein possesses activation function in the CLOCK:CYCLE heterodimer (Chang et al., 2003). We propose that, in Mar-CLOCK acts as a transcriptional activator owing to its Q-rich domain, even though it appears exceptionally long. It was previously reported that the transactivation activity of GAL4 factor increased with expanded Q stretches (Gerber et al., 1994). Based on this observation, the long Q-rich region of Mar-CLOCK may be indicative of its robust transactivation activity and high functional efficiency at relatively low doses.

In the present study, RT-PCR analysis revealed that Mar-Clock was expressed in all tissues tested (heart, eyestalk, thoracic ganglia, abdominal ganglia, gut, stomach, muscle, subepidermal adipose tissue, hepatopancreas, gill, and ovary), which was consistent with the findings in mice (King et al., 1997), Drosophila (Lee et al., 1998), zebrafish (Whitmore et al., 1998), human (Steeves et al., 1999). These results, along with the fact that, in Drosophila, autonomous circadian oscillators are present throughout the body (see review; Stanewsky, 2002), suggest the co-existence of central and peripheral clocks in M. rosenbergii. However, the lack of detectable signal in all samples analyzed by Northern blotting indicates that the Mar-Clock mRNA levels were very low (data not shown). Considering the essential role of CLOCK in the circadian system, Mar-CLOCK may possess exceptional transactivation activity enhanced by its dramatic long glutamine-rich domain. In fact, an inverse relationship has been observed between glutamine repeat length and human androgen receptor (AR) expression levels (Brooks et al., 1997; Chaong et al., 1996).

Table 2 – The length polymorphism of glutamine(Q)-rich regions among all known CLOCKS

<table>
<thead>
<tr>
<th>Organism</th>
<th>Q-rich region/Qs (amino acids)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Macrobrachium rosenbergii</td>
<td>140/135</td>
</tr>
<tr>
<td>Drosophila melanogaster</td>
<td>57/47</td>
</tr>
<tr>
<td>Danio rerio</td>
<td>51/43</td>
</tr>
<tr>
<td>Gallus gallus</td>
<td>20/18</td>
</tr>
<tr>
<td>Mus musculus</td>
<td>19/17</td>
</tr>
<tr>
<td>Xenopus laevis</td>
<td>14/8</td>
</tr>
<tr>
<td>Antheraea pernyi</td>
<td>None</td>
</tr>
</tbody>
</table>

No significant oscillation in gene expression was observed in the present study in the tested tissues (gill, ovary, muscle, hepatopancreas, thoracic ganglia and eyestalk). In Drosophila, Clock mRNA oscillates in a bimodal fashion (Darlington et al., 1998), peaking at midday (ZTS) and late night (ZT23). In zebrafish and chicken, Clock transcripts reach maximum levels at or shortly after the transition from day to night (ZT12–ZT18) (Larkin et al., 1999; Whitmore et al., 1998). The daily oscillations of Clock transcripts are considered pacemakers for the whole circadian system (Reppert and Weaver, 1997). In mice, it was found that Clock expression was constitutive in SCN (Shearman et al., 1999) but rhythmic in liver (Lee et al., 2001). A separate study showed rhythmic expression of Clock in rat SCN (Abe et al., 1999). These reports indicate that rhythmicity is not a universal expression pattern for CLOCK in the circadian system. Many genes participate in the circadian system, but it is not necessary for all to be expressed rhythmically. Indeed, in Drosophila photoreceptors, PERIOD protein has been shown to cycle without cycling of Period mRNA (Cheng and Hardin, 1998), that is, circadian rhythmicity does not require cycling of the Period transcript. This model (see Fig. 2B of Reppert and Weaver, 1997) may explain the apparent discrepancy between the functional role of Mar-Clock and the absence of Mar-Clock mRNA rhythmicity. Clearly, failure of a transcript to cycle does not exclude a gene from candidacy as a clock gene.

We also investigated expression of the Mar-Clock gene under eyestalk ablation, constant light (LL), and constant dark (DD). The crustacean eyestalk is considered the center of neuroendocrine regulatory mechanisms involved in many aspects of physiology, including molting, vitellogenesis, pigment concentration and dispersion, carbohydrate metabolism, and growth (see review; De Kleijn and Van Herp, 1995). Ablation of the eyestalks can cause the stimulation of vitellogenesis in crustaceans, and so it is often used as a method for stimulating prawn reproduction. Eyestalk ablation has also been extensively used in crustacean circadian rhythm studies (Aréchiga and Rodríguez-Sosa, 1998; Barrera-Mera and Block, 1990; Kalmus, 1938), and constant light (LL) and constant dark (DD) conditions are often used to investigate how light affects the circadian system. In our studies, after eyestalk ablation, Mar-Clock gene expression tended to increase in CNS. This response may result from a lack of light stimulation because a similar increase was observed under the DD condition. We are currently conducting further studies to confirm our hypothesis.

All known circadian systems possess transcription-translation feedback loops (see review; Young and Kay, 2001), and thus, crustaceans are likely to do as well. Identification and characterization of the gene encoding Mar-CLOCK provide valuable information for elucidation of the crustacean circadian mechanism at the molecular level. Further research focusing on isolation of other clock genes and proteins, and their interactions with each other will help clarify the circadian system and determine the exact role that Mar-CLOCK plays in the circadian system.

4. Experimental procedures

4.1 Animals and RNA preparation

Giant prawns, M. rosenbergii, of both sexes, were obtained from a market in Hangzhou, China, and were reared in a 15-L glass aquarium with circulating freshwater at 25 ± 1 °C under a 12 h:12 h light:dark (LD) photoperiod. The prawns were acclimated for at least 2 weeks before experiments and were fed twice daily at ZT0 and ZT12. Animals used in this study were adults of similar weight (7.2 to 12 g).
Eyespot ablation: Six hard-shelled prawns were chosen for eyespot ablation. The prawns were placed in chilled water for 1–2 min until they were lightly anesthetized. The eyespots were ablated using a red-hot surgical scalpel and the prawns were then placed back into their container and monitored for recovery. Prawns were fed twice every day (ZT0 and ZT12) and a 3-day (72 h) acclimation was required before experiments.

Constant conditions: To investigate the effect of constant light (LL) and constant dark (DD), six prawns were transferred to LL and DD containers, respectively. Prawns were fed twice every day (ZT0 and ZT12), and a 3-day (72 h) acclimation was required before experiments.

Ten prawns were killed at each time point (ZT 0, 6, 12, or 18) by rapid emersion in chilled water and then decapitation. For eyespot-ablated, LL and DD prawns (six respectively), ZT6 was chosen for collection point. Tissues were collected quickly under liquid nitrogen. All tissues were homogenized in TRIzol® reagent (Invitrogen, CA, USA), and total RNA was prepared according to the manufacturer’s instructions.

4.2. Cloning and sequence analysis

First-strand cDNA was synthesized from 2 μg of total RNA from thoracic ganglia using the SuperScript™/First-Strand cDNA Synthesis Kit (Invitrogen, CA, USA), according to the manufacturer's set protocols. The first-strand cDNA from thoracic ganglia was used as the template, and the cDNA fragment encoding Mar-CLOCK was amplified by two rounds of PCR using the same pair of degenerate primers. Oligonucleotide primers for CLOCK were designed based on the highly conserved amino acid and mRNA sequences of the Chinese oak silkmoth (Antheraea pernyi; AAR14936 and AY330486), fruit fly (Drosophila melanogaster; T13071 and AF067207), zebrafish (Danio rerio; NP.840080 and NM.130957), rainbow trout (Oncorhynchus mykiss; AAF75783 and AF266745), African clawed frog (Xenopus laevis; AAF12827 and AF203107), Japanese quail (Coturnix japonica; BAB03454 and AB029889), chicken (Gallus gallus;AAD43283 and AF152531), house mouse (Mus musculus; AAC53200 and AF000998), and human (Homo sapiens; AAB83969 and NM.004898). In the first PCR, amplification was primed by pairs of degenerate oligonucleotides (ClkF and ClkR in Table 1), and the following program was used: 94 °C for 5 min, followed by 38 cycles of 94 °C for 30 s, 52 °C for 30 s, and 72 °C for 90 s. In the second PCR, the first-round PCR products were used as templates, and amplification was primed by the same pairs of primers (ClkF and ClkR in Table 1), and the program was: 94 °C for 5 min, followed by 35 cycles of 94 °C for 30 s, 54 °C for 30 s, and 72 °C for 1 min, with the addition of a final polymerization step at 72 °C for 10 min. The PCR product was subcloned into the PCR® 2.1-TOPO® vector using the TOPO TA Cloning® Kit (Invitrogen, CA, USA) for sequencing analysis.

The full-length Mar-Clock cDNA was obtained by using the rapid amplification of cDNA ends (RACE) method. The cDNA for 5′-RACE was synthesized from gill total RNA using the FirstChoice™ RLM-RACE Kit (Ambion, TX, USA) according to the manufacturer’s protocols. The gene-specific primers (ClkSR1 and ClkSR2 in Table 1) for 5′-RACE were designed based on the nucleotide sequences of the Mar-Clock cDNA fragment amplified by reverse transcription (RT)-PCR. The cDNA fragment encoding the 5′-region of Mar-Clock was amplified by two rounds of PCR. The first round of PCR was performed using a gene-specific primer (ClkSR1 in Table 2) and the 5′-RACE adapter outer primer included in the RACE kit. The program for PCR amplification was 94 °C for 5 min, followed by 38 cycles of 30 s at 94 °C, 30 s at 55 °C, 2 min at 72 °C, and a final step of 72 °C for 10 min. In the nested PCR, the first-round PCR products were used as templates, and amplification was primed by the nested gene-specific primer (ClkSR2 in Table 2) and the 5′-RACE adapter inner primer included in the RACE kit. The program for nested PCR amplification was 94 °C for 5 min, followed by 35 cycles of 30 s at 94 °C, 30 s at 60 °C, 1 min at 72 °C, and a final step of 72 °C for 10 min. The amplified fragment was purified, subcloned, and sequenced.

The cDNA for 3′-RACE was also synthesized from gill total RNA using the FirstChoice™ RLM-RACE Kit according to the manufacturer’s protocol and used as a template for PCR. The first round of PCR was primed by a gene-specific primer (ClkSF1 in Table 1) and the 3′-RACE adapter outer primer provided in the kit, using LA Taq™ DNA polymerase (TaKaRa, Shiga, Japan). The program for PCR amplification was 94 °C for 5 min, followed by 35 cycles of 30 s at 94 °C, 30 s at 55 °C, 5 min at 72 °C, and a final step of 72 °C for 10 min. Nested PCR was performed using the nested gene-specific primer (ClkSF2 in Table 1) and the 3′-RACE adapter inner primer provided in the kit. The program for nested PCR amplification was 94 °C for 5 min, followed by 35 cycles of 30 s at 94 °C, 30 s at 62 °C, 90 s at 72 °C, and a final step of 72 °C for 10 min. The amplified fragment was purified, subcloned, and sequenced.

Purified recombinant plasmids were sequenced using the ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, USA), diluted 1:1 in halfBD™ Sequencing Reagent (GenPak, Stony Brook, USA), according to the manufacturer’s instructions. The DNA was sequenced from both strands on an ABI PRISM 377 Genetic Analyzer (Applied Biosystems). Sequences were edited and analyzed using Vector NTI Version 9.0 (InforMax, Frederick, USA).

4.3. Reverse transcription-polymerase chain reaction

RT-PCR was carried out to investigate whether or not Mar-Clock was expressed in heart, eyestalk, thoracic and abdominal ganglia, gut, stomach, muscle, subepidermal adipose tissue, hepatopancreas, gill, and ovary. The first-strand cDNA was synthesized as described above. PCR was performed with primers ClkSF1 and ClkSR2 (Table 1) and the program was 94 °C for 5 min, followed by 38 cycles of 30 s at 94 °C, 30 s at 52 °C, 20 s at 72 °C, and a final step of 72 °C for 10 min. The PCR products were separated on a 1.5% agarose gel, transferred to a nylon membrane (Hybond-N, Amersham Pharmacia Biotech, Uppsala, Sweden), and ultraviolet cross-linked. The 147-bp Mar-Clock cDNA fragment was amplified by using primers ClkSF1 and ClkSR2 and purified using the QIAquick PCR Purification Kit (QIAGEN, Hilden, Germany). The fragment was labeled with digoxigenin–deoxyuridine triphosphate at 37 °C overnight using the DIG High Prime Labeling Kit, and hybridization was performed overnight at 42 °C using the DIG Easy Hyb System (Roche Molecular Biochemicals, Mannheim, Germany) according to the manufacturer’s instructions. The blots were washed at room temperature, twice at low stringency (2× standard saline citrate [SSC], 0.1% sodium dodecyl sulfate [SDS]) for 5 min and then at 55 °C twice at high stringency (0.1× SSC, 0.1% SDS) for 15 min. After washing, hybridized probes were detected by alkaline phosphatase-conjugated anti-DIG antibody and the CSPD chemiluminescent detection system.

4.4. Semiquantitative reverse transcription-polymerase chain reaction analysis

We performed semiquantitative RT-PCR experiments to measure expression levels of Mar-Clock mRNA in gill, ovary, muscle, hepatopancreas, thoracic ganglia, and eyestalk during light–dark cycles. At the same time, to investigate whether the gene expression was influenced by eyestalk ablation, LL or DD, the central nervous system (brain, thoracic and abdominal ganglia; CNS), gill, heart, and muscle tissues were examined. Total RNA extraction and RT were performed as described above. The first-strand cDNA fragments were amplified using primer set ClkSF1 and ClkSR2 (Table 1) for the 147-bp Mar-Clock fragment. As an internal control, a 198-bp fragment of the constitutively expressed β-actin gene (Accession # AF221096) was amplified using primers actinF
and actinR (Table 1). Aliquots (10 μl) of the PCR reaction were collected after cycles 22–38 to determine the linear range of the reaction. We chose 32 cycles for Mar-Clock and 24 cycles for α-actin as endpoints for monitoring RNA levels. The following program was used in these reactions: 94 °C for 5 min, followed by indicated cycles of 94 °C for 30 s, 54 °C for 30 s, 72 °C for 30 s, and a final extension step at 72 °C for 10 min. The bands were quantitated with Dolphin 1D Version 1.1.0 (Wealtec Corp., Sparks, USA). Relative abundances were expressed as Mar-Clock transcripts levels to those of α-actin. All data were given as means ± SEM of independent experiments from separate RNA pools (n = 3). Statistical analyses were performed using one-way ANOVA (light–dark cycle) and Student’s t test (eyestalk ablation, LL or DD). The differences were considered significant for P < 0.05.

4.5. Protein alignment

To identify proteins homologous to previously identified CLOCKS, we aligned both the whole protein and conserved domains using ClustalX Version 1.81 (Thompson et al., 1997). The comparison sequence data were obtained from NCBI (GenBank): fruit fly (Drosophila melanogaster, dCLOCK, T13071), limulus (Antheraea pernyi, apCLOCK, AAR14936), zebrabass (Danio rerio, zCLOCK, NP_840080), clawed frog (Xenopus laevis, AAF12827), quail (Coturnix japonica, BAB03454), chicken (Gallus gallus, cCLOCK, AAD43283), owl (Tyto alba, AA006119), mouse (Mus musculus, mCLOCK, AAC53200), rat (Rattus norvegicus, BAA81819), mole (Spalax galili, CAC85403) and human (Homo sapiens, hCLOCK, AAB83969).

4.6. Nucleotide sequence accession number

The nucleotide sequence presented in this manuscript has been submitted to the DDBJ/EMBL/GenBank databases and assigned the accession number AY842303.

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