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Molecular characterization and distribution of CYCLE protein from *Athalia rosae*

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Abstract

cDNA encoding CYCLE (CYC) from the coleseed sawfly, *Athalia rosae* (Hymenoptera, Symphyta), was amplified by PCR. This is a first determination of hymenopteran CYC structure. *Ar*CYC had an overall identity of 66% with CYC of *Anopheles gambiae* and ca. 60% of *Drosophila melanogaster*. Structural investigation revealed that *Ar*CYC contained characteristic motifs of: bHLH, PAS A, PAS B, PAC and BCTR. Detailed analysis indicated high conservation of these regions among insects. Northern blot analysis showed that the mRNA of ca. 3 kb was transcribed both in the head and in the rest of the body. Southern blot analysis suggested the presence of a single copy of the gene in the genome. Western blot indicates that the quantity of CYC protein does not fluctuate under LD 12:12 in either the head or the rest of the body. Immunocytochemical examination revealed CYC-like antigen in the pars intercerebralis, dorsolateral protocerebrum, dorsal optic tract, tritocerebrum of the brain and the subesophageal ganglion.

Keywords: Sawfly; *Athalia rosae*; *cycle*; CYCLE (CYC); Circadian clock

1. Introduction

Organisms have adapted to the changing environment by shaping the circadian organization so that their physiological and behavioral events become rhythmically adjusted to light-dark conditions. The basic structure of circadian oscillation was formulated based on *Drosophila melanogaster*. The circadian rhythmicity is generated by a negative feedback loop to clock genes transcription by their protein products.

The first circadian clock gene discovered in *D. melanogaster* was *period* (*per*). Konopka and Benzer (1971) used chemical mutagenesis to isolate three mutant lines that differed in free-running period: per^0 (arrhythmic phenotype), per^S (19 h) and per^L (28 h). Cloning of *per* gene by Bargiello and Young (1984) and Reddy et al. (1984) was a milestone in the molecular view of circadian oscillation.

The discovery of the second clock gene, *timeless* (*tim*) led to an understanding of the negative feedback regulation of the clock genes by their own transcription (Myers et al., 1995, 1996). *D. melanogaster tim* mRNA and TIM protein are abundant in-phase with *per* transcript and PER protein abundance. PER and TIM form a heterodimer translocate to the nucleus (Lee et al., 1998). Light stimulates TIM degradation via Cryptochrome (CRY), a process that entrains oscillation with the light-dark cycle (Myers et al., 1996; Shirasu et al., 2003).

The next gene found was *doubletime* (*dbt*), encoding a homolog of mammalian casein-kinase Iε (CKIε), (Kloss et al., 1998). DBT causes PER phosphorylation and thereby PER clearance in *D. melanogaster*. DBT suppresses the level of PER, thus allowing TIM to accumulate in the cytoplasm during the night to dimerize with PER protein.

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When TIM level reaches a critical level, the activity of DBT is suppressed, thus making PER monomers available for TIM. The dimerization of PER and TIM subsequently allows the dimer to enter the nucleus late at night, where it represses *per* and *tim* gene transcription by inhibition of the transcriptional activator CLOCK (CLK)/CYCLE (CYC, also known as BMAL1 in mammals) heterodimer that binds to the E-box, an enhancer located in the transcription regulatory regions of *per* and *tim* (Hao et al., 1997; Kloss et al., 1998; Cyran et al., 2003). In *Drosophila clk* mRNA and CLK protein levels peak after dawn in antiphase to the mRNA peaks of *per* and *tim* which occur soon after dusk (Cyran et al., 2003). Blau and Young (1999) identified another clock gene, *vrille* (*vri*). *vri* mRNA oscillates in phase with *per* and *tim* mRNA. *vri* encodes a basic leucine zipper transcriptional factor, which targets CLK and CYC.

Cyran et al. (2003) proposed a new model based on two interactive feedback loops for the circadian clock protein–gene transcriptional regulation in *Drosophila.* The first loop consists of CLK and CYC, which activates transcription of *per* and *tim*. CLK/CYC function is inhibited by TIM/PER in the nucleus. In the second loop, CLK/CYC activates *vri* and *pdp1ε* transcription. *Clk* transcription is first repressed by VRI and then activated by PDP1ε. Degradation of PER in the early morning frees CLK/CYC to resume its function on the transcription of *per*, *tim*, *vri* and *pdp1ε* (Cyran et al., 2003).

Recent data suggests that a negative feedback system constitutes the core mechanism for circadian oscillation in other groups of organisms including insects. However, even though different groups of organisms possess the same set of genes, the components may be assembled in unique ways or may serve different functions. Differences might be due to post-translational modifications, subcellular localization and specific roles for the protein within the circadian system.

CYC and CLK are two transcriptional factors of particular interest. Recent reports suggest that mouse CLK is not required for circadian oscillation (DeBruyne et al., 2006). However, *Drosophila Clk* null mutants are arrhythmic at the behavioral and molecular levels, presumably due to the fact that CLK encompasses transactivation domain (Allada et al., 1998; Chang et al., 2003). dCYC does not have the transactivation domain in the C-terminus, unlike most insects such as *Antheraea pernyi* where CYC loses its activity when that domain is truncated (Chang et al., 2003). This is where diversities reside among insects. Therefore, cloning of CYC will help provide a clue to the evolution of clock genes in insects.

To help understand the diversity of circadian clock at the molecular level in insects we chose *Athalia rosae* (Hymenoptera, Symphyta) as a novel model system to study circadian and photoperiodic clocks in insects. This paper reports the gene cloning, determination of the primary structure and cellular distribution of the CYC-like protein within the central nervous system of *A. rosae*.

2. Materials and methods

2.1. Animals and sample preparation

The founder population of *A. rosae* was collected during the spring of 2006 from Hyogo Prefecture in Japan (35°N, 135°E). Cultures of *A. rosae* were maintained under LD 16:8 at 20 °C according to Sawa et al. (1989). Larvae were fed fresh leaves of Daikon radish (*Raphanus sativus*, Cruciferea, Brassicaceae), and adults were supplied with 10% honey solution. Adult males were collected from stock colonies, entrained for 1 week in LD 12:12 at 25 °C, and used for experiments. Insects were anesthetized by CO_2 and decapitated in phosphate-buffered saline (PBS) during the light phase (ZT 6), where ZT stands for Zeitgeber Time and the onset of light is defined as ZT 0. Samples were immediately frozen in liquid nitrogen and stored at −80 °C until the extraction of total RNA.

2.2. Construction of an adaptor-ligated single and double-stranded cDNA

Total RNA was isolated from the heads (HDs) using Isogen (Nippon Gene, Tokyo, Japan) according to the supplier's instructions. Poly(A)$^+$ RNA was purified using a Gene Elute mRNA Miniprep Kit (Sigma, St. Louis, MO). Poly(A)$^+$ RNA was used to construct first single stranded cDNA (ss cDNA) followed by synthesis of an adapter-ligated double stranded cDNA (ds cDNA) using a SMART PCR cDNA Synthesis Kit (Clontech, Palo Alto, CA). For ds cDNA synthesis the MMLV reverse transcriptase (PowerScript Reverse Transcriptase, Clontech, Palo Alto, CA) and Advantage 2 PCR Enzyme System (Clontech, Palo Alto, CA) were used according to the suppliers instructions.

2.3. Partial characterization of CYC from A. rosae

A set of primers derived from conserved regions of RDKMN(T/S), (5′-MGN GAY AAR ATG AAY AC-3′) and those corresponding to the peptide sequence of FFCR(M/I)K, (5′-TTC ATN CGR CAR AAR AA-3′) was used. The PCR conditions employed were: 94 °C for 2 min for denaturing followed by 94 °C for 30 s and 55–45 °C for 30 s and 68 °C for 30 s for 35 cycles in a Gene Amp PCR 9600 System. Amplified fragment was inserted into pBlue-Script II vector (Stratagene, La Jolla, CA), and the sequences of several independent clones were determined. The sequences contained an ORF that extended beyond the boundary of sequence homologies. Clones from separate amplifications were compared to rule out PCR errors. To clone the cDNA encoding the remaining parts of CYC protein, gene-specific primers were designed for RACE PCR.

2.4. Isolation of 5′- and 3′-ends of the cDNAs by RACE PCR

To amplify the 5′- and 3′-end portions of *cyc* cDNA, RACE PCR strategy was employed using gene-specific