Circadian Rhythm and cDNA Cloning of the Clock Gene *period* in the Honeybee *Apis cerana japonica*

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ABSTRACT—Isolated individual foragers of *Apis cerana japonica* could be entrained under a light-dark cycle, and the predominant activity was concentrated to the later part of the photophase. Foragers showed circadian rhythm under conditions of constant light and constant dark with free-running periods of more and less than 24 hr, respectively. These observations indicated that *A. cerana* possesses a circadian clock controlling locomotor activity. To investigate the molecular mechanism underlying the circadian system we cloned cDNA for a homolog of the clock gene *period (per)* from the honeybee by a PCR-strategy. The cloned *per*-cDNAs consisted of two types, α and β, encoding a putative protein of 1124 amino acids and 1116 amino acids, respectively. The sequences of types α and β were identical except that the former possessed an additional 24 bp stretch corresponding to 8 amino acids in the conserved C2 block. These two types were assumed to be differentially spliced variants and found also in *per* cDNA of *A. mellifera*. In support of this idea, Southern blotting experiments showed that *per* of *A. cerana* is a single copy gene. RT-PCR analysis and subcloning of the products revealed that the both types α and β are expressed in the brain of the forager. A quantitative RT-PCR assay by which the level of *per* mRNA in one single brain can be detected was established. *Per* mRNA level showed daily oscillation under a light-dark cycle with a change of the ratio of type α to β.

INTRODUCTION

Many organisms including unicellular organisms have been shown to possess endogenous circadian clocks that provide temporal synchronization of life processes and adaptation of organisms to environmental cycles (Aschoff, 1960; Decoursey, 1983). Circadian rhythms driven by these clocks oscillate with a free-running period of about 24 hr under constant environmental condition (Pittendrigh, 1981; Saunders, 1982). The molecular mechanisms responsible for these circadian clocks are under extensive investigation using many organisms including insects (Giebultowicz, 1999) and several genes of the components constructing the clocks have been identified (Dunlap, 1999). Our knowledge about the mechanisms comes largely from genetic and biochemical studies in the fruitfly *Drosophila melanogaster*. The first clock gene, *period (per)*, an essential component of the circadian clock machinery, was originally cloned in *Drosophila* (Zehring *et al.*, 1984; Bargiello *et al.*, 1984). The *period* gene was extensively characterized, and the molecular mechanism of the *Drosophila* clock is generally thought to be based on negative feedback in which transcription of the *per* gene is regulated by its protein product PER (Dunlap, 1999). Rhythmic expression of the *per* gene caused by this feedback regulation has been proposed to be the main mechanism generating daily rhythmicity in *Drosophila*. In another insect, the moth *Antheraea pernyi, per* was also cloned and its function was analyzed (Reppert *et al.*, 1994; Levine *et al.*, 1995; Sauman *et al.*, 1996a, 1996b). The silkmoth *per* gene is also a necessary element of the circadian system (Sauman *et al.*, 1996b), although the molecular details of *per* expression and regulation are different between the silkmoth and fruitfly (Sauman *et al.*, 1996a). Recently, *per* genes were cloned and investigated in *Musca domestica* and *Lucilia cuprina*, dipteran species other than *Drosophila* (Piccin *et al.*, 2000; Warman *et al.*, 2000). Mammalian *per* genes have been cloned (Dunlap, 1999), and the gene products were shown to constitute a molecular circuit with other clock components such as Timeless, Clock and BMAL1, which are also common among fruitflies and mammals (Dunlap, 1999). Thus, isolation and analysis of the *per* gene has been critical to understanding the molecular mechanisms of circadian systems of insects and mammals.

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It was reported that honeybees (*Apis mellifera*) showed distinct free-running rhythms in activity at the level of individual and also at the colony level. The circadian activity rhythm of individual forager honeybees has been well investigated and the free-running period of less than 24 hr under constant darkness (DD) and greater than that under constant light (LL) have been observed (Spangler, 1973; Moore and Rankin, 1985). The circadian rhythm can be entrained to light-dark and temperature cycles (Moore and Rankin, 1985; 1993). In addition to these physical conditions, feeding cycles and social interaction also influence the circadian rhythms represented by colonies (Frisch and Aschoff, 1987; Moritz and Kryger, 1994; Moritz and Sakofski, 1991; Frisch and Koeniger, 1994). For example, introduction of a queen into a worker group that was entrained to a phase-shifted light/dark cycle caused a shift of the free-running phase under DD conditions (Moritz and Sakofski, 1991). The ontogenic development of circadian activity was reported in honeybee workers: the newly emerged bees had no circadian rhythm but the aged foragers showed a clear rhythm (Moore *et al.*, 1998; Sasaki, 1992). Further the endogenous circadian system of *Apis* is involved in sun compass and "time memory" which enables bees to remember the time of the day at which nectar sources are available (Saunders, 1982).

Though circadian phenomena of the honeybee have been well investigated from several points of view, the molecular mechanisms underlying the circadian rhythm have not been revealed. But recently Toma *et al.* (2000) reported the cloning of the *per* cDNA in *A. mellifera* (GenBank: AF159569) and the circadian change in *period* mRNA levels in the brain. In the present study we report the rhythms shown by individuals of *A. cerana japonica*, which is endemic in Japan and shows ecological characteristics different from *A. mellifera* (Sasaki, 1999). In order to investigate the molecular mechanism underlying the circadian system of *A. cerana*, we cloned cDNAs showing a homology to *per* gene sequences of other insects and examined the expression of *per* mRNA in the brain.

MATERIALS AND METHODS

Honeybees

A. cerana japonica was used in this study, unless otherwise stated. Honeybees (*A.cerana*) maintained at Tamagawa University were used to extract total RNA. The honeybees of the colonies, collected at Osaka Prefecture (Japan) and reared in the botanical garden of Kyoto University (Kyoto), were used in other experiments including the recording of locomotor rhythms.

One colony containing about 3000 workers and queen was maintained in an insect tron (Zootron, Amefrec, Co., Ltd. Tokyo) which was devised for rearing honeybees under indoor condition. The temperature (28°C) and relative humidity (60%) in the tron were kept constant throughout the experiments. The light-dark cycle was programmed to LD 12:12 with an intensity of about 3000 lux of photophase. Sugar water was supplied *ad libitum*. After the bees were entrained at least one week, the foragers visting the sugar dish were captured and used for the analysis of *per* mRNA expression.

Recording of individual locomotor rhythm

Foragers were captured at the entrance of the hives and single bees were kept in semi-transparent plastic boxes (6×9 cm). All experiments were carried out in biotrons (NC220, Nihon-Ika, Osaka, Japan) which were kept at 28°C and 60% RH. The captured bees were kept under a photoperiodic regime of light-dark cycle (LD) 12:12, or kept under constant darkness. Some were kept under constant light (LL) of 200−300 lux of light intensity and then transferred to constant darkness (DD). Food (50% sucrose solution) was available *ad libitum*. In the photoperiodic regime (LD 12:12) square-wave LD cycles were given by alternating the photophase of fluorescent illumination at 200−300 lux with a scotophase of complete darkness.

Locomotor activities of the individual isolated foragers were monitored using an infrared actograph. An infrared light beam was passed through the box and projected onto a photo-microsensor (EE-SPW321A, Omron Corp., Tokyo, Japan) which picked up any interruption in the light beam. The momentary signals were fed into a computer (NEC98VX, NEC, Tokyo, Japan) through an interface (48CH-MNT, Adocom Ele. Co. Ltd., Shiga, Japan), and these digitally recorded events were analyzed to estimate the free-running period shown in LL and DD conditions by χ^2 periodogram software provided by Dr. I. Ohshima.

cDNA cloning

Degenerate primers were designed on the basis of conserved regions of the insect *per* genes identified by the alignment of seven insect *per* sequences (Reppert *et al.*, 1994) and preferential codon usage for *Apis* was applied. Three primers were successfully used: two sense primers F1 (5'-TG[TC]GT[TAG]AT[TC][AT][GC][ACGT]-ATGCATGA[TC]GG-3') and F2 (5'-CTGG GATATCT [ACGT] CC[ACGT]CA[AG]GA-3'), and one antisense primer R1 (5'-ATT[AG]TA ATT [ACGT]A [AG]TTGATT-3').

The heads of workers were obtained and frozen immediately in liquid nitrogen. Total RNA was extracted from the heads by the acid guanidinium isothiocyanate phenol-chloroform (AGPC) method (Isogen, Nippon gene, Toyama, Japan). Reverse transcription of total RNA from the bee heads was performed by using 5 μg total RNA and 25 units of avian myeloblastosis virus reverse transcriptase (AMV reverse transcriptase first-strand cDNA synthesis kit; Life Sciences, inc., St. Petersburg, FL), in a 25 μl cDNA reaction mixture. One μl of the cDNA was amplified with 1.5 units of Taq DNA polymerase (Takara Shuzo, Kyoto, Japan) using primers F1 and R1 on a DNA thermal cycler model 9600 (Perkin Elmer). The PCR parameters were 30 sec template denaturation at 95°C, 1 min primer annealing at 42°C, 2 min primer extension at 72°C for 30 cycles. The products of this PCR were further amplified using a second set of oligonucleotide primers F2 and R1. The PCR parameters were the same as those of the first PCR. The final PCR products were electrophoresed on 0.7% agarose gels, purified by the glass powder method (AGC-001K DNA PREP, Diayatron, Tokyo, Japan), and were cloned into pGEM-T easy vector (Promega Co., Madison, WI) for sequencing. The sequencing reactions were carried out by the cycle sequencing method with an ABI-373 automatic DNA sequencer (Applied Biosystems) according to the manufacturer's protocol. All sequences were determined in both directions.

The 3'-terminal portion of *period* cDNA was cloned by the 3'-RACE (rapid amplification of cDNA ends) method. Seven μg of total RNA was reverse-transcribed (Superscript II RNase H⁻ Reverse Transcriptase, Life Technologies) using 3'-primer TAP1 (5'-GGCCAC-GCGTCGACTAGTACTTTTTTTTTTTTTTTTT-3'). The resulting cDNA was amplified using the anchor primer AP1 (5'-GGC CAC-GCGTCGACTAGTAC-3') and primer F3 (5'-AGGACCTCGCGT-CCTTCATG-3'). The PCR parameters were 30 sec template denaturation at 95°C, 1 min primer annealing at 55°C, 2 min primer extension at 72°C for 30 cycles. The products of this PCR were further amplified using a second set of primers TAP1 and F4 (5'-CACGACAG CGTGATGCTTGG-3'). The amplified 2.3 kb fragment was purified, and cloned into the pGEM-T easy vector.

The 5'-terminal portion of *period* cDNA was cloned by the 5'-

RACE method (5'RACE System for Rapid Amplification of cDNA Ends Version 2.0, Gibco BRL, Gaitherburg, MD) using primer GSP1 (5'-TGCTCAGTTGTACATGATACGCGA-3'), GSP2 (5'-ATATCAGGA TT-CGCTGGCCCTTTC-3') and nested GSP (5'-ATCCTGTGTTGAC-CTACGAC-3').

Finally, the complete cDNA coding sequences of *per* (consisting of two types α and β; see the materials and methods) were amplified from the bee head cDNA using two primers F5 (5'-AGCAGTG-TTGTGATTCAACGG-3'; -56 to -36, the first nucleotide A of the start codon was numbered +1) and R5 (5'-AA ACAAACGCTTCGAACTGG-3'; +3418 to +3437 [of type α]), which were based on the sequences obtained by 5'- and 3'-RACE, respectively.

Total RNA was prepared from the heads of *A. mellifera* foragers (Australian strain) and cloning of partial fragments of the *per* cDNA was performed according to the methods described above. To carry out PCR, two primers were designed on the basis of the decided sequence of *cerana*: one is sense primer R0 (5'-CTGGTGG-TTACTGCTCAACCTGTT-3') and antisense primer FS (5'-GTT-GTAATTTGAGTTGATTGTAA-3'), which are located in the C2 region (Reppert, 1994). PCR, cloning and sequencing were carried out as described above.

Southern blotting analysis of genomic DNA

The cDNA probe of 786 bp was generated with primer R0 (5'-CTGGTGGTTACTGCTCAACCTGTT-3') and the primer FS (5'-GTTGTAATTGAGTTGATTGTAAC-3'), which are located in C2 region (Reppert *et al.*, 1994), by PCR amplification using subcloned cDNA (type α) as the template. Probes were labeled with random primers and klenow fragment of *E. coli* DNA polymerase using a random prime labeling kit (Amersham Pharmacia Biotech, Piscataway, NJ) to obtain fluorescein-labeled DNA probes.

Total genomic DNA was isolated from muscles of *A. cerana* workers according to the standard method. DNA was digested with *Eco*RI and *Hin*dIII and aliquots of ~2 μg were separated on 1% agarose gels, transferred onto nylon membranes (Hybond-N+, Amersham Pharmacia Biotech), and immobilized by UV cross-linking. Hybridization and detection were performed according to the protocol supplied with the detection kit (RPN3510; Amersham Pharmacia Biotech) with slight modifications. Membrane-bound digested DNA was hybridized in rapid-hybridization buffer and the membranes were washed in 2× SSC for 20 min, 1× SSC for 15 min and 0.5× SSC for 15 min (each buffer contained 0.1% SDS). Following stringent washes, the membranes were incubated with blocking agent and then with anti-fluorescein-AP. The chemiluminescence of the hybridizing band was detected using an ECL instant camera (Amersham Pharmacia Biotech).

Isolation of mRNA and one step RT-PCR

Brains including optic lobes of five foragers were dissected out carefully at Zeitgeber time (ZT) 21:00 during the scotophase of LD 12:12 (Fig. 7), collected together and analysed immediately. mRNA was isolated from the brains using a mRNA purification kit (Amersham Pharmacia Biotech); the procedures included disruption by the guanidium isothiocyanate method and selective isolation of mRNA by oligo(dT)-cellulose chromatography. The polyadenylated mRNA was eluted with 200 μl of 10 mM Tris-HCl (pH7.5) containing 1mM EDTA. After DNAase treatment of the elution mRNA was precipitated by adding 70% ethanol, collected by centrifugation and finally redissolved in 40 μl of DEPC-treated water.

One-step RT-PCR was performed using a kit purchased from QIAGEN (Chatsworth, CA) according to manufacturer's protocol. The reaction mixture (50 μl) contained reverse transcriptase, Taq DNA polymerase, dNTP mixtures (400 μM of each dNTP), 0.6 μM oligonucleotide primers, 2.5 mM MgCl$_2$, RNase inhibitor (10 units) and 1 μl of mRNA solution obtained by the method described above. First, reverse transcription reaction was performed at 50°C for 30 min, then the mixture was incubated at 95°C for 15 min to activate Taq DNA

polymerase, and PCR amplifications were carried out. The PCR parameters were 1 min template denaturation at 94°C, 1 min primer annealing at 50°C, 1 min primer extension at 72°C for 30 cycles.

Selective analysis of the expression of *per* (α) was performed with primers R0 and FI (5'-CTGTAACAAGGGATTGCGTGATCC-3') and that of total *per* (α+β) was with R0 and FD (5'-CTCGG-TTGACGATTTGCTGT-3'), respectively (Fig. 4). The sequence of FI is based on the additional 24 bp stretch in the type α cDNA, and R0 and FD are based on sequences common to both types. The amplified ca. 0.7 kb fragment was purified, cloned into the pGEM-T easy vector, and sequenced.

As an internal control, a partial DNA fragment (362 bp in length) of the *cerana* cytoskeletal actin sequence was amplified by RT-PCR in which primers 1S (5'-GAAATGGCAACTGCTGCATCATCCT-3') and 3A (5'-AGAATTGACCCACCAATCCATACG-3') based on the cytoskeletal actin sequence of *A. mellifera* (GenBank: AB023025; Ohashi *et al.*, 1996) were used. We cloned and sequenced the amplified DNA fragments and confirmed that they were cytoplasmic actin sequences: five nucleotide differences among the 362 bp sequence were found between the actin genes of *cerana and mellifera*.

Quantitative RT-PCR assay for *period* mRNA.

To detect the change in *per* mRNA level using a single brain of the honeybee, a quantitative RT-PCR analysis was established according to the methods reported previously (Nakayama *et al.*, 1992; Nakamura *et al.*, 1996) with slight modifications. Isolation of mRNA from the brain of foragers and RT-PCR method are performed as described above. After defined cycles of PCR for the analysis of *per* and cytoskeltal actin mRNA, 5 μl from a 50 μl reaction mixture was electrophoresed on a 1% agarose gel and amplified bands were detected by ethidium bromide staining. The intensity of the ethidium bromide fluorescence of each band was measured by the CCD imaging system (EDAS120, Kodak Digital Science, Eastman Kodak Company, New York). The PCR cycle-intensity curves are plotted on a semi-logarithmic graphs (Fig. 6). For estimation of the amount (*a*) of the template and the efficiency (*b*) of amplification in each PCR, regression line was fitted to the linear portion of the curve: the quantitative determination of target mRNA was performed before PCR products reached a plateau (Nakayama *et al.*, 1992). The amount of *per* mRNA was normalized with that of actin mRNA (Fig. 7).

RESULTS

Activity rhythms of individual foragers

Walking locomotor rhythms of the foragers were investigated to determine the existence of a circadian system in *A. cerana*. The locomotor activity of individual forager honeybees was monitored using infrared actographs. Honeybees were entrained under a LD 12:12 photoperiodic regime at constant temperature and a typical record is shown in Fig. 1A. Under LD 12:12, activitiy is concentrated to the later part of the photophase. The onsets of activity were found at the beginning of the photophase and clear offsets occurred at light-off on most days. Foragers could be entrained under LD 16:8 and LD 8:16, and they also showed same tendency of predominant activity in the later part of the photophase (data not shown).

Circadian free-running rhythm was observed under DD-condition with free-running period (τ) shorter than 24 hr (Fig.1B and C). Circadian periods were measured first under LL condition and then after transition to DD condition. Some bees showed a free-running rhythm under LL and DD, and typical record is shown in Fig. 1D. The free-running mode, which was recognized clearly by tracing the shifts of offset times, changed
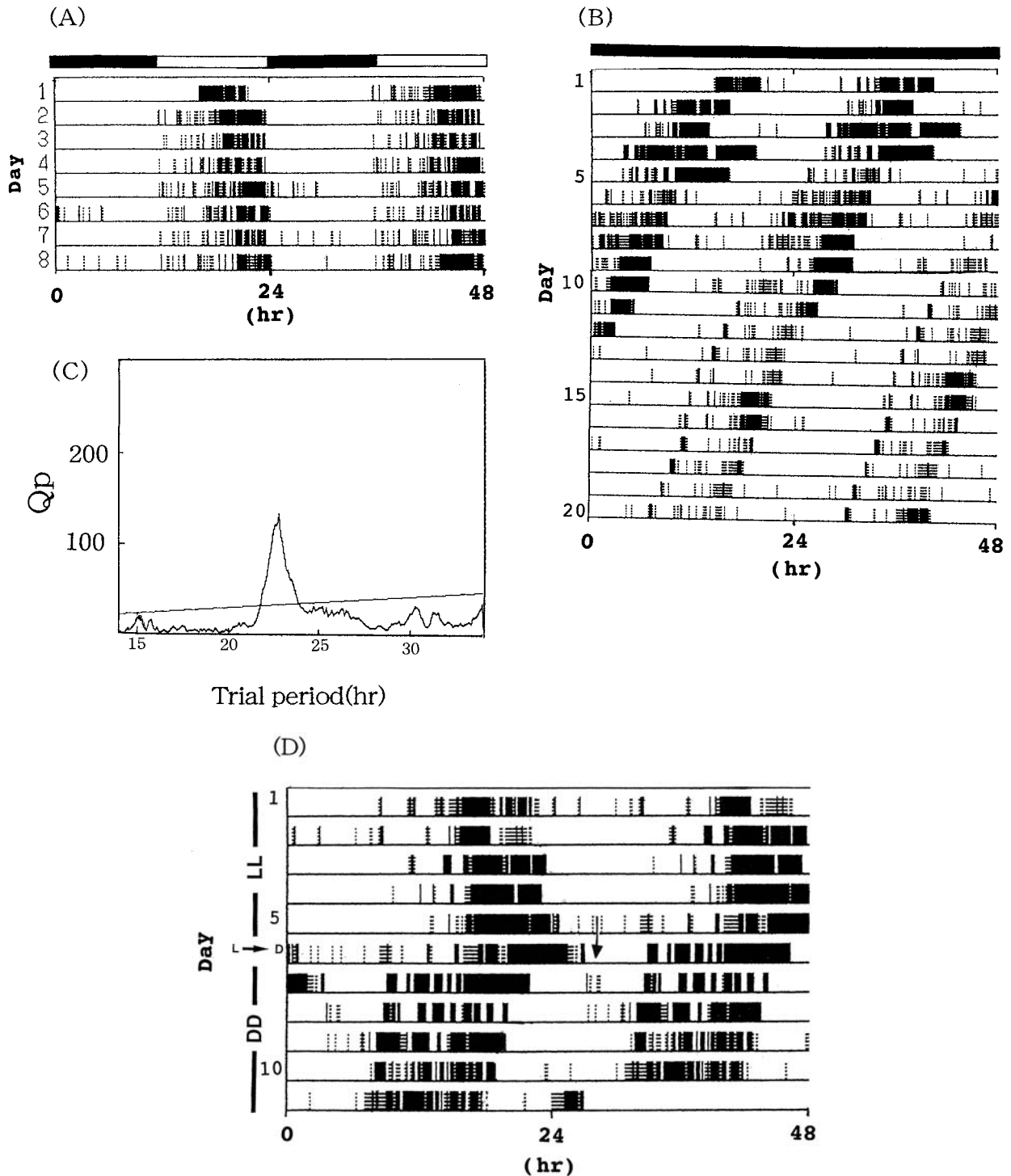
(A)

(B)

(C)

(D)

Fig. 1. Representative actogram recordings of an individual foragers under LD 12:12 cycle (A), DD (B), and first kept in LL and then transferred to DD at the time shown by the arrow (D). The one-day data were double plotted. The results of χ^2 periodogram analysis of DD (B) data are shown in (C), and the line indicates 95% confidence level (τ =23.1 hr). The white and dark bars represent light and dark condition, respectively.

discretely with no transitional phase after the LL to DD transition. The free-running periods (τ) were found to be 24.4±0.1 hr (mean±SD, n=8) in LL and 23.1±0.1 hr (n=5) in DD with small deviations, respectively. Moore and Rankin (1985) reported that τ-LL and τ-DD of locomotor activity of *A. mellifera* was 24.8 hr and 22.0 hr, respectively.

Cloning of *per* cDNA

To clone a *period* homolog from *A. cerana japonica*, we performed PCR with cDNA derived from the bee heads as the template. We made degenerate primers based on the conserved sequences revealed by the alignment of *period* amino acid sequences of several of insect species (Reppert *et al.*,

1994). Of a total of 18 primers examined, those corresponding to the peptide sequences CVISMHDG (residues 170–177 of the *A. pernyi period*), LGYLPQD (residues 335-341) and NQLNYN (residues 533–538) were successfully used for PCR amplification of cDNAs, which showed significant homology with *period* sequences of other insects.

The amplified DNA fragments consisted of two types, α (654 bp in length) and β (630 bp). Type β lacked 24 nucleotides (8 amino acids) from positions 526-549 of type α, whereas all other sequences were identical. A consensus sequence for the 3' splice site (acceptor site; Norton, 1994) was found (TCCCTTGTTACAG; a pyrimidine-rich region followed by dinucleotide AG) at the 3' terminal portion of the 24-bp region that was lacking in type β. However, no possible donor site (Norton, 1994) was found at the 5' terminal part of the stretch. These observations suggested that the two types of the *cerana per* sequence resulted from alternative splicing of the *per* mRNA precursor.

Using primers based on the nucleotide sequences common to both types of the cDNAs, we cloned the 5'- and 3'-portions of *per* cDNA by 5'- and 3'-RACE (Rapid Amplification of cDNA Ends) methods, respectively. To determine whether these RACE products were actually parts of a single transcript, PCR was performed using primers corresponding to the sequences of the 5'- and 3'-untranslated regions (UTR) obtained by RACE (see the materials and methods). We amplified two DNA fragments of 3506 bp (GenBank: AB-048825) and 3482 bp (AB048826), corresponding to types α and β, respectively. The full-length cDNAs of the types α and β contained open reading frames (ORFs) encoding putative proteins of 1124 and 1116 amino acids, respectively. The estimated molecular masses for the types α and β proteins were 124,479 and 123,613, respectively. The amino acid sequences of the two proteins were identical except for a 8 amino acid stretch, corresponding to the 24-bp region described above, present or absent in the type α and β proteins, respectively (Fig. 2).

The first methionine of the encoded proteins was followed by another methionine, and the surrounding sequences of the codons for both methionines showed Kozak consensus sequences for the initiation of translation (Kozak, 1987). A protein databases search with the deduced amino acid sequences of types α or β using the BLAST program revealed the highest homology scores with the overall identity of 33.0 or 33.2%, respectively, to Period protein from *Periplaneta americana*. Amino acid sequences of the conserved C2 domains of *A. cerana per* are aligned with those of other insects in Fig. 2. On the alignment, the *A. cerana* Per exhibited functionally important domains such as two PAS repeats (A and B in Fig. 2; Huang *et al.*, 1993), CLD (cytoplasmic localization domain; underlined in Fig. 2; Saez and Young, 1996) and short period domain (3 amino acid residues upstream and 16 residues downstream from the perS mutation site; Baylies *et al.*, 1992). Moreover, the *A. cerana* Per possessed the conserved amino acids to which the *per^L*, *per^S* and *per^01* mutations were mapped.

Comparison of *per* sequence of *A. cerana* with that of *A. mellifera*

Recently, the full-length cDNA sequence from *Apis mellifera*, a species closely related to *A. cerana*, was reported (Toma *et al.*, 2000). We have found that the *per* sequences were found to be very highly conserved between *A. cerana* and *A. mellifera*, at both nucleotide and amino-acid levels (Fig. 2), though the *cerana* cDNA has additional 18 nucleotides at just upstream of poly(A) sequence of 3'-end compared to *mellifera*. The reported *mellifera* sequence corresponds to type α: the 24 bp sequence that was found in the type α of *cerana* sequence was present at the corresponding position of the *mellifera* sequence. The identities of nucleotide and deduced amino acid sequences of the coding region were 95.7 and 99.2%, respectively, between the *per* sequences of *A. mellifera* and *A. cerana* (type α).

We examined whether *per* mRNA of type β is present in *A. mellifera* PCR was carried out with cDNA derived from heads of *mellifera* as template using primers (R0 and FS) by which two types (α and β) of fragments were amplified in *A. cerana*. As a result, we identified partial cDNA sequences of both types α and β also in *A. mellifera* (Fig. 2). In addition, one amino acid substitution from glycine (G) to glutamic acid (E) was found at position 481 in our *mellifera* sequence (Fig. 2) compared to that reported by Toma *et al.* (2000). Amino acid sequences of other insect per have glutamic acid (E) at this position (Fig. 2).

Southern blot analysis

A probe specific to the *per* cDNA sequence of *A. cerana* was used for genomic Southern blotting analysis to determine the copy number of the *per* gene. Digestion of the genomic DNA with *Eco*RI and *Hin*dIII was followed by hybridization with per cDNA-specific probe. Southern blotting analysis of digested genomic DNA revealed a single hybridization band in each lane (Fig. 3). This result indicated that the *per* gene of *A. cerana* is present as a single copy, and suggested that types α and β are expressed from the same locus. We found that digestion of genomic DNA of *mellifera* by *Hin*dIII followed by hybridization using the same probe also gave a single band of the same size as *cerana* (data not shown). This suggested that *per* genes of *cerana* and *mellifera* have a similar structure.

Transcription of *per* in the brain

To investigate transcription of the *per* gene, RT-PCR analysis was performed using mRNA isolated from brains of foragers. When we used primers R0 and FI which is designed based on the additional 24-bp stretch of type α (Fig. 4), amplification of ca. 0.7-kb fragment was detected (Fig. 5: lane 2). We cloned and sequenced the amplified DNA fragments and confirmed that they were type α of 681 base in length. Primers RO and FD, based on common sequence in types α and β, similar but slightly larger size of fragments were amplified (lane 3). Cloning and sequencing of the fragments revealed that these consisted of two types of α (753 base) and β (729 base) fragment (Fig. 4). Transcription of the cytoplasmic actin

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                         perL    A
A.cerana         -------FCAVISMHDGLVLYTTPSICTALGYLKDAWIGRSFIDYVHPKDKATLADQIKN 164-216
A.mellifera      -------FCAVISMHDGLVLYTTPSICTALGYLKDAWIGRSFIDYVHPKDKATLADQIKN 164-216
P.americana      -------FCVVVSMQDGVVVFTTPSITDVVGFPKDMWLGRSFIDFVHPRDRTAFANHITS 230-282
D.melanogaster   ERVKEDSFCCVISMHDGIVLYTTPSITDVLGYPRDMWLGRSFIDFVHLKDRATFASQITT 233-292
M.domestica      EKLKEDSFCCVISMHDGIVLYTTPSITDVLGFPRDMWLGRSFIDFVHPKDRATFASQITT 174-233
A.pernyi         -------FSCVISMHDGVVLYATASLTSTLGFPRDMWVGRSFIDFVHPRDRNTFASQITN 168-220
L.cuprina        DKFKEDSFCCVISMHDGIVLYTTPSITDVLGFPRDMWLGRSFIDFVHTKDRATFASQITT 176-235
                 *  * ** ** *  * *      *    * * ****** **   *    * *

                 A
A.cerana         GIVSPQEE-RPKGINGRRASLFCGLRKYTRSFA--HQSINKEARSNLYLPFHLTLSFRDF 217-273
A.mellifera      GIVSPQEE-RPKGINGRRASLFCGLQKYTRSFA--HQSINKEARSNLYLPFHLTLSFRDF 217-273
P.americana      GVITPLSNSNPKGGSHPGKNSFYCCLRRYRGLK--STGYGVTEKEVSYLPFQLNMTFREL 283-340
D.melanogaster   GIPIAESR---GSVPKDAKSTFCVMLRRYRGLK--SGGFGVIGRPVSYEPFRLGLTFREA 293-347
M.domestica      GIPIAESR---SSIPKDARSSCCVMLRRYRGLK--SGGYGVIGRSVNYEPFRLGLTFREA 234-288
A.pernyi         ELAIPKIVSLTEETDQTMENPGSTMVCRIRRYRGLSCGFSVKNTTTAYLPFLLKFKFKNV 221-280
L.cuprina        GIPIAESR---SSMPKDARSTFCVMLRRYRGLK--SGGYGVIGRSVNYEPFRLGLTFREA 236-290
                          *                     *     *** *     *

                                                                    B
A.cerana         RDRTTEQQHK-------------AMFLVVTAQPVHSAYKAPEETIIS--SVFTTRHNATC 274-318
A.mellifera      RDRTTEQQHK-------------AMFLVVTAQPVHSAYKAPEETIIS--SVFTTRHNATC 274-318
A.mellifera*                           HSAYKAPEETIIS--SVFTTRHNATC -
P.americana      LPHSNPLEVEGNTSPESVPGGCNSMFLVITAKLICSAYKHAGETCAS--PKFVTRHLATC 341-398
D.melanogaster   PEEARPDNYMVSNG--------TNMLLVICATPIKSSYKVPDEILSQKSPKFAIRHTATG 348-399
M.domestica      PEEARSDNSLPSG---------TNMLLVICATPIKSAYKVCDELLSRKTPKFAIRHTKTG 289-339
A.pernyi         NEDKGNVIYL-------------VIQAVPFFSAFKTSNEVLAKT-VSFVIRHSADG 281-322
L.cuprina        PEEARSDNYLPSG---------TNMLLVICATPIKSAYKVSDELLSHKSAKFTIRHTATG 291-341
                                       *    *   *    *         * **

                 B                                                   C
A.cerana         YLSHVDPDVVQYFGYLPQDMVGRSLFDFYHPEDLPFIKDIYETVIK---LEGASFRSKPY 319-375
A.mellifera      YLSHVDPDVVQYFGYLPQDMVGRSLFDFYHPEDLPFIKDIYETVIK---LEGASFRSKPY 319-375
A.mellifera*     YLSHVDPDVVQYFGYLPQDMVGRSLFDFYHPEDLPFIKDIYETVIK---LEGASFRSKPY -
P.americana      KLNYVDPECMPYLGYLPHEMLGNSVLDFYHPEDLPFLKEVYQIVMQ---ENGAPFRSKPY 399-455
D.melanogaster   ISHVDSAAVSALGYLPQDLIGRSIMDFYHHEDLSVMKETYETVMKKGQTAGASFCSKPY 400-459
M.domestica      IISTVDSGAVSALGYLPQDLIGRSILDFYHHEDLTVLKEIYETVMKKGQTAGASFCSKPY 340-399
A.pernyi         NLEYIDAESVPYLGYLPQDITNRDALLLYHPGDLGYLQEIYGSLVK----EGNVTRSKTY 323-378
L.cuprina        IISHVDSAAVSALGYLPQDLIGRSILDFYHHEDLIVLKEIYETVMKKGQTAGASFCSKPY 342-401
                 *   ****       ** **     *              * ** *

                 C perO1
A.cerana         RFGIQNGDYVVLETEWSSFINPWTKKLEFVVGQHRILKGPANPDIFRVSCTTEHSQLTNI 376-435
A.mellifera      RFGIQNGDYVVLETEWSSFINPWTKKLEFVVGQHRILKGPANPDIFRVSCATEHSQLTNI 376-435
A.mellifera*     RFGIQNGDYVVLETEWSSFINPWTKKLEFVVGQHRILKGPANPDIFRVSCATEHSQLTNI -
P.americana      RFRSENGGYILLETEWSSFVNPWSKKLEFVVGQHRVLKGPENADVFMAPVEDDTLQ---I 456-512
D.melanogaster   RFLIDNGCYVLLETEWTSFVNPWSRKLEFVVGHHRVFQGPKQCNVFEAAPTCKLK----I 460-515
M.domestica      RFLVQNGCYVLLDTEWTSFVNPWSRKLEFVIGHHRVFQGPKNLNVFDPPPSNKPK----L 400-455
A.pernyi         RMMTQNGHYMKVETEWSAFINPWSKKLEFVTGKHYIIEGPANPDVFQNPENVLKL----- 379-433
L.cuprina        RFLIDNGCYALLETEWTSFVNPWSRKLEFVIGHHRVFQGPKNVHVFEPPPNTKPK----L 402-457
                 *  **  *   *** * ***  ***** **   **    *

A.cerana         SEEVLKEAKIIQEEIRTLLDESIQRKSDITELDVSKRCKDLASFMENLLQETRTPGFGKD 436-495
A.mellifera      SEEVLKEAKIIQEEIRTLLDESIQRKSDITELDVSKRCKDLASFMGNLLQETRTPGFGKD 436-495
A.mellifera*     SEEVLKEAKIIQEEIRTLLDESIQRKSDITELDVSKRCKDLASFMENLLQETRTPGFGKD -
P.americana      SEEVLKESKIIQEEIRSLLSEMVKNNGHLEKQQMSKRCRDLATFMESLMDDITKP-DLKL 513-571
D.melanogaster   SEEAQSRNTRIKEDIVKRLAETVSRPSDTVKQEVSRRCQALASFMETLMDEVSRADLKLE 516-575
M.domestica      SEEAMQRITRIKEDILKLLSETISRPSDTVKQEVSRRCQALASFMEPLMNEVARQDLKLE 456-515
A.pernyi         TEEQKNQAKMYRDSIIRIMKDVLTKPAEIAKQQMSKRCQDLAHFMEMLIEEQPKPVDDLR 434-493
L.cuprina        SEDSQARIARIKEDIVKLLAETVSRPSDTVKQEVSRRCQALASFMETLMDEVSRTDLKLE 458-517
                 *        *          *       * **  ** **  *

                                  perS
A.cerana         VLATDERSFSGSRNPLLQEHDSVMLGEISPHHEYYDSKSSTETPPSYNQLNYNENIERFF 496-555
A.mellifera      VLATDERSFSGSRNPLLQEHDSVMLGEISPHHEYYDSKSSTETPPSYNQLNYNENIERFF 496-555
A.mellifera*     VLATDERSFSGSRNPLLQEHDSVMLGEISPHHEYYDSKSSTETPP                -
P.americana      ELPQEEHSFS--------EHDSVMLGEISPHHDYYDSKSSTETPPSYNQLNYNDNIQRFF 572-623
D.melanogaster   LPHENELTVS--------ERDSVMLGEISPHHDYYDSKSSTETPPSYNQLNYNENLLRFF 576-627
M.domestica      LPNENELTVS--------ERDSVMLGEISPHHDYYDSKSSIETPPSYNQLNYNENLQRFF 516-567
A.pernyi         LEIQDADHSY--------YERDSVILGGISPHHEYD-SKSSTETPLSYNQLNYNDNLQRYF 494-545
L.cuprina        LPNENELTVS--------ERDSVMLGEISPHHDYYDSKSSTETPPSYNQLNYNENLQRFF 518-569
                 *  ***  ** *****  *   **** ***  ******** *  *  *

A.cerana         KSKPPVATMYGSDEEIINS-----SNDEGGK--------TSPNSAVRKCMSPIN------ 556-596
A.mellifera      KSKPPVATMYGSDEEIINS-----SNDEGGK--------TSPNSAVRKCMSPIN------ 556-596
P.americana      ESKPKTTLSDESGESKIEANRSLMSTDEEGKSGPAAD--SSLGSSNRKCCSPVN------ 624-675
D.melanogaster   NSKPVTAPAELDPPKTEPPEPRGTCVSGASGPMSPVHEGSGGSGSSGNFTTASNIHMSSV 628-687
M.domestica      NSKPVTAPVETDPIKMEQSYSTPANTGSNLSPMQCFE-DSGGGSSSRNCTSGSNLNMGSV 568-626
A.pernyi         NSHQSNAFVDNNLLPSRNPLYLSAPHFSESIKN--------VPSAMEYSGDVIDL----- 546-592
L.cuprina        NSKPVTAPVEFDPIKMEQTYNAPADERSTLSPVQCFE-GSGGGSGSSGNFTSGSNLNMGSV 570-628
                      *

A.cerana         -----
A.mellifera      -----
P.americana      -----
D.melanogaster   TNTSI 688-692
M.domestica      TNTSN 627-631
A.pernyi         -----
L.cuprina        TNTSN 629-633
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Fig. 2. Alignment of the C2 region of the Period proteins from *A. cerana* determined in this study, *A. mellifera* (AF159569), *P. americana* (U12772), *D. melanogaster* (AF033029), *M. domestica* (AH007818), *A. pernyi* (U12769) and *L. cuprina* (Y19108). A partial sequence (AB050744) of *A. mellifera* obtained in this study is also included as *A. mellifera**. Identical amino acids are indicated by asterisks. The PAS domains are enclosed by the square brackets. The PAS-A (A) and PAS-B (B) repeats, and the PAC (C; Ponting and Aravind, 1997) domain are boxed in gray. The CLD is underlined. The sites of the *perL*, *perO1* and *perS* mutations in *Drosophila* and corresponding amino acids of other insects are shown in reverse contrast. The 8-amino acid peptide regions of *A. cerana* and *A. mellifera* determined in this study, which are lacking in type β cDNA, are boxed.
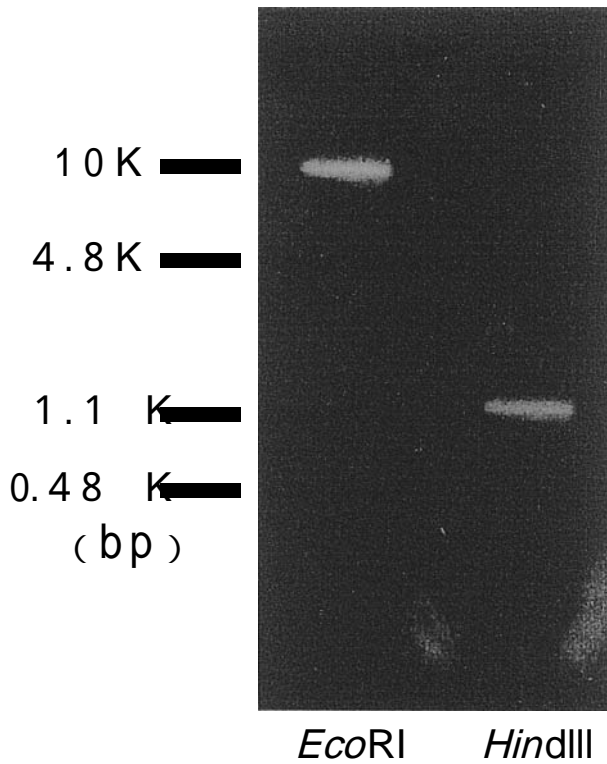
Fig. 3. Southern blotting analysis of the *per* gene of *A. cerana*. The *cerana* genomic DNA digested with *Eco*RI and *Hind*III was electrophoresed and hybridized with *A. cerana per* specific probe. The sizes of markers are indicated at the left.
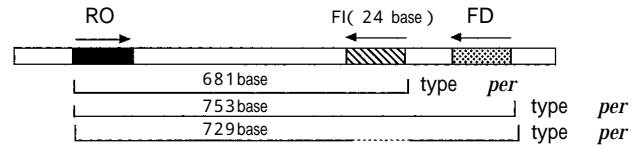

Fig. 4. Schematic drawing of selective PCR for the amplification of type α and total ($\alpha+\beta$) of *per*. To amplify the type α and total ($\alpha+\beta$), a set of primers R0 and FI, and that of R0 and FD was used, respectively. See the text in detail.
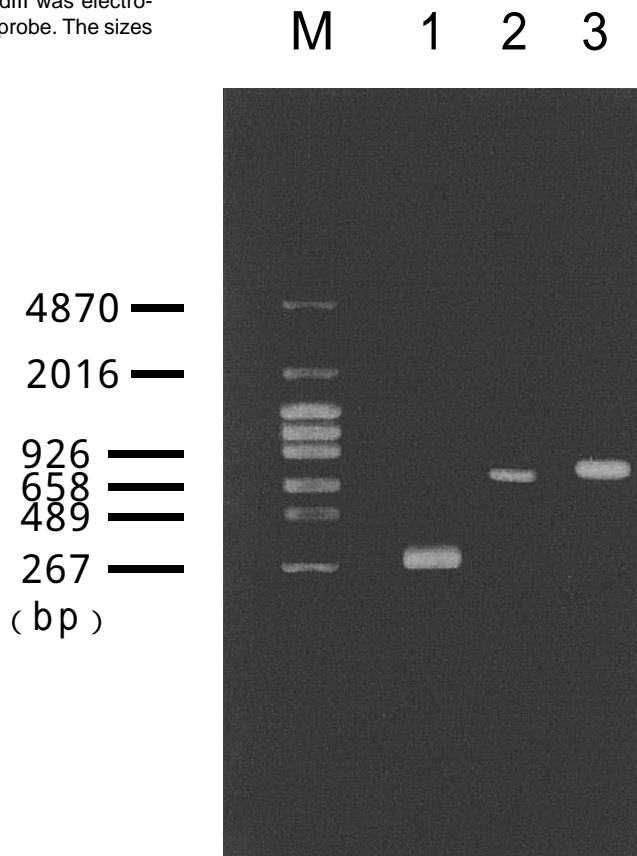
Fig. 5. RT-PCR of *per* and actin fragments using mRNA isolated from brains of *A. cerana*. PCR products were separated on 1% agarose gel. The gel was stained by ethidium bromide. Lane 1 (actin); lane 2 (*per*, type α: primers RO-FI); lane 3 (total *per*, types α and β: primers RO-FD). The sizes of markers (lane M) are indicated at the left.
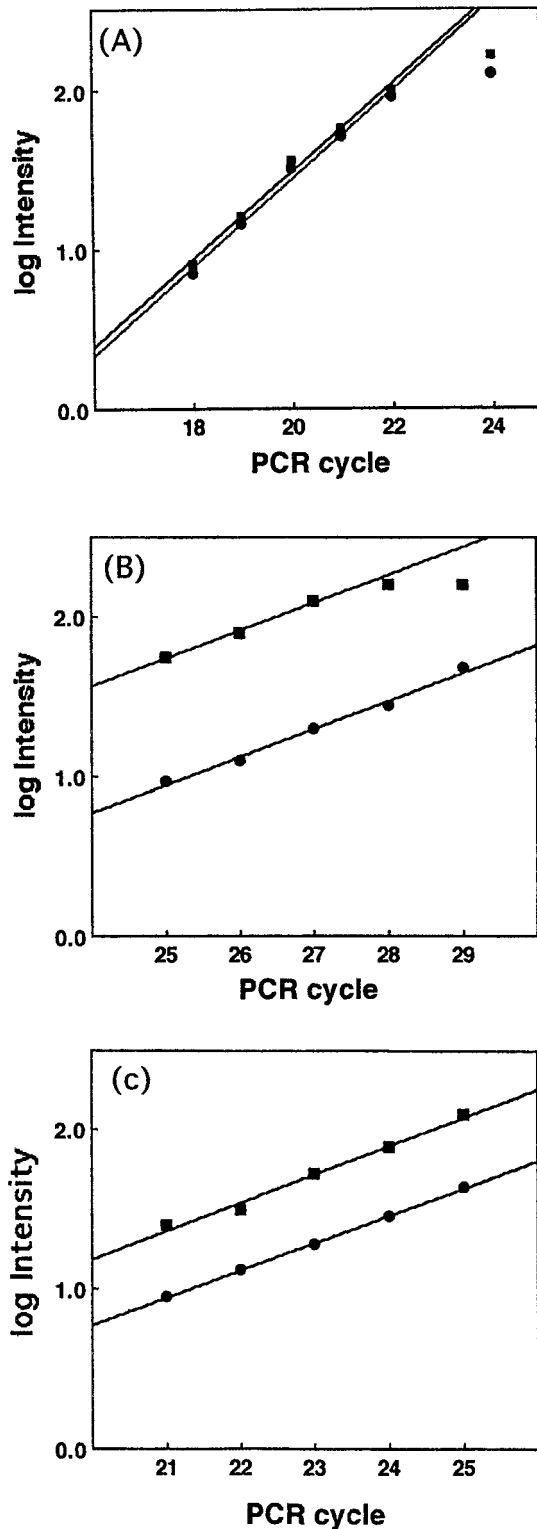
Fig. 6. The PCR cycle-product intensity curves used for quantification of mRNA. The curves were plotted on a semi-logarithmic graphs. Representative data of quantitative RT-PCR experiments using mRNA isolated from single brain of the forager captured during photophase at ZT 9:00 (circle symbols) and scotophase at ZT 21:00 (square symbols) were indicated. The mRNA levels of actin (A), *per* type α (B), and total *per* (α and β) (C) were measured. Oblique lines show the regression lines determined by the linear portions of the curves, and amounts of the templates were caluculated as described in text.

gene was also examined to check the efficiency of mRNA isolation and cDNA synthesis (lane 1). The detected bands were derived from cDNA and not from contaminating genomic DNA because no band was detected when reverse transcription (RT) was omitted. These observations indicated that the both types α and β of *per* mRNA are expressed in the brain of *A. cerana*.

Daily oscillation of *per* mRNA level in the brain of foragers

To examine the temporal change of *per* mRNA level in the brain, we performed a quantitative RT-PCR assay by which expression of type α or total (α+β) transcripts is selectively detected using different pairs of primers (Fig. 4), and the *per* mRNA level in one single brain can be quantified.

Honeybees were entrained under LD12:12 at colony level and the forager's brains were collected over 24 hr at 3 hr intervals (Fig. 7). Representative data used for the quantification of targeted mRNA by the method are shown in Fig. 6. The results show that the levels of cytoplasmic actin mRNA were almost the same between the samples collected at ZT 9 and ZT 21, whereas the mRNA levels of type α and total (α+β) were high at ZT 21 compared to ZT 9. The efficiencies of amplification in the PCR for type α and total (α+β) were found to be almost same (1.49–1.51); this allowed the comparison between the levels of type α and total (α+β).

Fig. 7 shows the daily changes of *per* mRNA levels in the brain of the bee kept under LD 12:12 photoperiodic regime. The levels of *per* (α) and total (α+β) were high during the dark-period compared to the light-period. They increased steeply
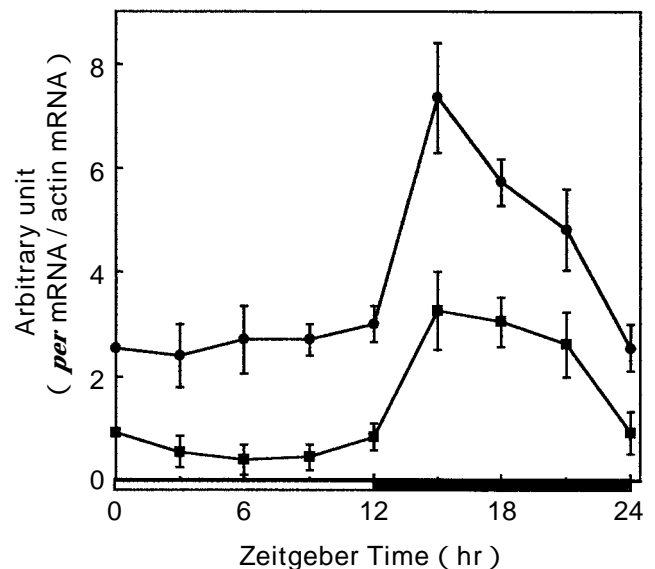

Fig. 7. Daily oscillation of *per* mRNA levels examined at 3 hr intervals (square symbols: type α , circle symbols: total *per*). RNA levels of *per* and actin were determined by the quantitative RT-PCR assay, and the ratio of amount of *per* mRNA over that of actin was expressed as the relative arbitrary unit. The horizontal bar at the bottom represents the LD 12:12 lighting cycle: open bar and closed bar indicate the photophase and the scotophase, respectively. Vertical bars represent the SEM (n=5).

Table 1. Temporal change in the ratio between type α and β transcript

	Arbitary unit of *per*			Number of subclone			
	Type α	:	Total (α+β)	Type α	:	Type β	Total
ZT 6:00	0.40	:	2.7	1	:	19	(20)*
ZT 9:00	0.45	:	2.7	3	:	21	(24)*
ZT 18:00	3.1	:	5.7	10	:	6	(16)**
ZT 21:00	2.6	:	4.9	13	:	9	(22)**

The levels of *per* transcripts at ZT 6:00, 9:00, 18:00 and 21:00 in Fig. 7 were represented as arbitary unit. *–**: significantly different (Fisher's exact probability test: $p < 0.01$).

at the beginning of the dark period, and then decreased gradually. The level of total *per* transcript was higher than that of type α at any time, and the ratio between the levels of type α and total (α+β) changed by time (Fig. 7 and Table 1). In addition the peak-to-trough ratios in the two graphs are different. These observations suggest that the ratio between the abundance of the two types changed by time in LD cycle.

To confirm the change of the ratio in *per* transcripts directly, we cloned and sequenced the products (corresponding to the band of lane 3 in Fig. 5) of RT-PCR using primers R0 and FD. The results show that the ratio of type α clones to type β clones was significantly different between the light- and dark-period (Table 1). The ratio of type α clones to total (α+β) clones was low (5–13%) at the light-period (ZT 6 and ZT 9), and it increased to 57–63% at the dark-period (ZT 18 and ZT 21). These observations coincided with the results of RT-PCR assay (Table 1).

DISCUSSIONS

The foragers of *A. cerana* exhibit circadian locomotor rhythm which is entrained by light-dark cycles with activity largely concentrated to the late photophase (Fig. 1A). This daily rhythm pattern resembles that reported in *A. mellifera* (Moore and Rankin, 1993). Isolated *A. cerana* showed a short life-span as described for *A. mellifera* (Moore and Rankin, 1985), and almost all died within two weeks or so. During the experimental period in this study, however, the honeybees showed overt rhythms with free-running periods that were longer than 24 hr in LL and shorter than 24 hr in DD. The free-running period changed discretely without any transitional phase after LL to DD transition (Fig. 1D). These are the first observations indicating that *A. cerana* possesses a circadian clock as well as *A. mellifera*. As a first step to investigate the molecular mechanism underlying the biological clock of the honeybees, we cloned the *period* cDNA from *A. cerana*.

The *cerana per* cDNA sequence showed the highest degree of conservation to *mellifera per*. Furthermore, we demonstrated that the *per* cDNAs consist of two types (α and β) in *A. cerana*, and that this is also the case in *A. mellifera*. The type α and β were different only in a 24-bp nucleotide sequence present only in type α. A consensus motif for the 3' splice site was found in this 24-bp nucleotide sequence, although no possible 5' splice site was found, indicating that the two types of cDNA resulted from alternative splicing and

not from reverse transcription of a premature transcript. Use of another 3' splice site, which is predicted to be located immediately upstream of the 24 bp region on the genome, probably results in the production of type α. On the other hand, use of the 3' splice site found in the 24-bp region should give rise to the type β by skipping the 24-bp region. The type α cDNA encodes a protein with an additional 8-amino acid stretch just upstream of the highly conserved "short period domain", to which many mutations affecting the period length were mapped (Baylies *et al.*, 1992). This 8-amino acid stretch could not be aligned with the *per* sequences from any other insect species (Fig. 2).

Colot *et al.* (1988) identified 6 interspersed blocks of conserved regions C1 to C6 by comparison of the *per* sequences of three species of *Drosophila*. The *cerana per* protein sequence also showed significant conservation in the C1, C2 and C3 blocks (alignment of C2 blocks is shown in Fig. 2). Identity of the amino acid sequences in the C1, C2 and C3 blocks between *A. cerana* (type β) and *Drosophila melanogaster* were 38.0, 44.8 and 50.0%, respectively. On the other hand, C4, C5 and C6 blocks are less well conserved in *A. cerana*. We could not align these C-terminal regions between *cerana* and any *Drosophila* species, except for a few short stretches of 10–15 amino acids. This overall pattern of conservation, higher conservation in C1-3 and lower in the C-terminal half is also observed in other nondipteran insects such as *A. pernyi* (Reppert *et al.*, 1994) and *P. americana* (data not shown; GenBank: U12772). In addition, we found no significant conservation in the C-terminal region among the *Apis, Antheraea*, and *Periplaneta* sequences. Such variation in the C-terminal region among various insects suggests that this region may not be critical in the central clock mechanism. In contrast to the N-terminal half regions, the functions of the C-terminal half blocks of *per* protein are not well understood. It is tempting to speculate that the diverged C-terminal sequences of *per* might be involved in the temporal regulation of behaviors characteristic of each insect group.

In the N-terminal half of the *cerana per* sequence, we detected motifs and domains thought to be important for the central clock mechanisms with high identity to *Drosophila* sequences. Especially, the PAS, CLD and short period domains show significant identity with other insect sequences (Fig. 2). The PAS and CLD domains are functionally important in the regulated nuclear entry of Period/Timeless heterodimer (Saez and Young, 1996), which is followed by self-inhibition of *per* gene expression by Per protein. These are assumed to be critical regulatory steps in the generation of self-sustaining daily oscillations. Thus, the higher degrees of conservation found in these domains indicated that the *cerana* period is a true structural and likely a functional ortholog of the *Drosophila period* gene.

The RT-PCR followed by sequencing the DNA fragments, which were amplified using primers of R0 and FD, indicated that both types α and β of *per* mRNA are expressed in the brains of *A. cerana*. Moreover, the level of *per* mRNA in the brain showed robust daily change under a LD cycle as re-

ported in other insects (Hardin *et al.*, 1990; Sauman and Reppert, 1996). Phase relationships of the daily change of *per* (type α and total) mRNA abundance are similar to that reported in *A. mellifera* (Toma *et al.*, 2000). These observations, together with the sequence data, supports the idea that the *cerana per* is a functional ortholog of the clock gene *period.*

We found that the ratio between the abundance of two types α and β changed by time in LD cycle (Fig. 7 and Table 1). In *Drosophila*, the *per* gene generates two types of transcripts by alternative splicing (Cheng *et al.*, 1998). These two types differ only in the presence (type A) or absence (type B') of an alternative intron in the 3' untranslated region. Splicing of this intron was enhanced at low temperatures, and a mechanism that allows the preferential daytime activity of flies on cold days was proposed (Majercak *et al.*, 1999). It is interesting to examine whether environmental and ontogenetic factors affect the levels of the two types transcripts in *A.cerana.* Further investigation of the *per* in *A. cerana* is currently underway in our laboratory to reveal the molecular mechanism underlying the circadian rhythm in this species.

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