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A new gene encoding a putative transcription factor regulated by the *Drosophila* circadian clock

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Circadian rhythms of locomotor activity and eclosion in *Drosophila* depend upon the reciprocal autoregulation of the *period* (*per*) and *timeless* (*tim*) genes. As part of this regulatory loop, *per* and *tim* mRNA levels oscillate in a circadian fashion. Other cycling transcripts may participate in this central pacemaker mechanism or represent outputs of the clock. In this paper, we report the isolation of *Crg-1*, a new circadianly regulated gene. Like *per* and *tim* transcript levels, *Crg-1* transcript levels oscillate with a 24 h period in light:dark (LD) conditions, with a maximal abundance at the beginning of the night. These oscillations persist in complete darkness and depend upon *per* and *tim* proteins. The putative CRG-1 proteins show some sequence similarity with the DNA-binding domain of the HNF3/fork head family of transcription factors. In the adult head, *in situ* hybridization analysis reveals that *per* and *Crg-1* have similar expression patterns in the eyes and optic lobes.

Keywords: circadian rhythm/*Drosophila*/optic lobes/*period* gene

Introduction

In *Drosophila melanogaster*, a circadian clock controls the eclosion of adults from pupae and the locomotor activity of the adult flies (see Hall, 1995; Rosbash *et al.*, 1996 for reviews). The circadian clock requires a proper expression of the *period* and *timeless* genes: *per* and *tim* null mutations completely abolish the eclosion and activity rhythms, whereas per^L and per^S mutations respectively lengthen and shorten the period of the pacemaker (Konopka and Benzer, 1971; Sehgal *et al.*, 1994). Modifications of the number of copies of the *per* gene produce more subtle changes of the circadian period (Smith and Konopka, 1982) and transient massive rises of *per* protein (PER) levels induce circadian time-dependent behavioral phase shifts (Edery *et al.*, 1994a).

The levels of *per* transcript oscillate with a 24 h period in wild-type flies (Hardin *et al.*, 1990; Hardin, 1994) and peak at the beginning of the night. *per* mRNA cycling is affected by the different *per* mutations in the same way that the circadian behavior is affected, indicating that PER influences the expression of its own gene through a feedback loop mechanism (Hardin *et al.*, 1990). This control appears to act mostly at the transcriptional level (Hardin *et al.*, 1992). Circadian fluctuations of PER protein abundance and phosphorylation occur in the fly head and lag behind mRNA oscillations by ~6 h (Siwicki *et al.*, 1988; Zerr *et al.*, 1990; Edery *et al.*, 1994b; Zeng *et al.*, 1994). PER protein enters the cell in the middle of the night, a few hours before the protein reaches its peak level (Curtin *et al.*, 1995).

The recently isolated *tim* gene (Gekakis *et al.*, 1995; Myers *et al.*, 1995), is a major *per* partner in the circadian feedback loop mechanism (Reppert and Sauman, 1995; Rosbash, 1995). *tim* mRNA abundance cycles with the same phase as *per* mRNA in fly heads, and *tim* as well as *per* mRNA oscillations depend upon both PER and *tim* protein (TIM) (Hardin *et al.*, 1990; Sehgal *et al.*, 1995). Like PER levels, TIM levels fluctuate with a 6 h lag behind *tim* transcript cycling (Hunter-Ensor *et al.*, 1996; Lee *et al.*, 1996; Myers *et al.*, 1996; Zeng *et al.*, 1996). In the absence of TIM, PER levels stay very low and PER does not cycle nor enter the cell nucleus (Gekakis *et al.*, 1995; Myers *et al.*, 1995; Price *et al.*, 1995). In per^0 flies, the TIM protein does not enter the nucleus but still cycles in LD as a result of its light-induced degradation (Hunter-Ensor *et al.*, 1996; Myers *et al.*, 1996; Zeng *et al.*, 1996). Such reciprocal effects on PER and TIM nuclear entry appear to result from the formation of a PER–TIM complex that would be responsible for the regulation of *per* and *tim* transcription (Gekakis *et al.*, 1995; Hunter-Ensor *et al.*, 1996; Lee *et al.*, 1996; Myers *et al.*, 1996; Saez and Young, 1996; Zeng *et al.*, 1996). However, it has been recently reported that in the pacemaker neurons of the silkmoth brain, PER and TIM are cytoplasmic and oscillate without delay compared with *per* and *tim* mRNAs (Sauman and Reppert, 1996). In addition, an antisense *per* RNA is found in the *per*-expressing cells of the silkmoth (Sauman and Reppert, 1996). These data suggest that another mechanism may be responsible for the circadian fluctuations of PER and TIM in the moth brain (Hall, 1996; Sauman and Reppert, 1996).

As the intimate *Drosophila* clock mechanism involves two proteins, PER and TIM, that can influence transcription, it is likely that transcriptional regulation participates in the transmission of the circadian information from the molecular clock to rhythmic outputs. Indeed, circadianly regulated genes have been isolated in several species (see Takahashi, 1993; Dunlap, 1996 for reviews) including cyanobacteria (Liu *et al.*, 1995), green plants (Millar and Kay, 1991) and vertebrates (Lavery and Schibler, 1993; Pierce *et al.*, 1993; Stehle *et al.*, 1993; Borjigin *et al.*, 1995; Falvey *et al.*, 1995; Fonjallaz *et al.*, 1996; Green

et al., 1996). Particularly relevant is the description of several clock-controlled genes in the mold *Neurospora* (Loros *et al.*, 1989; Loros and Dunlap, 1991; Arpaia *et al.*, 1993) in which the *frequency* (*frq*) gene is involved in a feedback loop mechanism that recalls the *Drosophila* clock (Aronson *et al.*, 1994). Recently, a set of diurnally regulated genes has been identified in *Drosophila* (Van Gelder *et al.*, 1995). One of them, *Dreg-5*, is under bona fide circadian control and shows oscillations of its transcript abundance that are abolished in per^0 flies (Van Gelder and Krasnow, 1996). The *per*-controlled *Dreg-5* gene encodes a 298-amino acid product of unknown function whose levels oscillate in fly head extracts with a phase similar to the mRNA phase (Van Gelder and Krasnow, 1996).

In order to identify new components of the *Drosophila* circadian system we have started a molecular screen for transcripts whose abundance undergoes daily fluctuations in the head. A subtracted head cDNA library has been made that is enriched for transcripts that are more abundant at Zeitgeber Time (ZT) 15 (3 h after light offset = ZT 12) than at ZT 3 (3 h after light onset = ZT 0), like *per* and *tim* mRNA. Here, we report the characterization of *Crg-1* (for Circadianly Regulated Gene), a gene whose transcript's abundance shows 24 h oscillations in LD conditions with the same phase as *per* and *tim* mRNAs. The mRNA cycling persists in complete darkness, and depends upon the PER and TIM proteins. In the *Drosophila* head, *Crg-1* is highly expressed in the eyes and optic lobes, similarly to *per* mRNA.

Results

Subtractive cloning of the Crg-1 gene

In order to clone genes whose transcript abundance would cycle with a *per*-like phase, we entrained wild-type Canton S flies in LD 12:12 and sacrificed them at ZT 3 or ZT 15, when the *per* mRNA abundance is at its trough or peak respectively. *per* mRNA was used as an internal control for ZT 15-enriched transcripts during the subtraction procedure. Control transcripts that do not temporally fluctuate were also monitored (see Materials and methods). Reverse transcription and subtractive hybridization were performed as described in Pikielny *et al.* (1994) with minor modifications (see Materials and methods), using ZT 15 RNA as a source for tracer cDNA and ZT 3 RNA for driver cDNA. After three rounds of subtractive hybridization, the resulting (ZT 15 minus ZT 3) cDNA was enriched ~25-fold for *per* cDNA as shown by Southern blot analysis (data not shown). The subtracted cDNA was directionally cloned and replica filters of the library were differentially hybridized with the same subtracted (ZT 15 minus ZT 3) cDNA or a non-subtracted ZT 15 cDNA. Forty clones that gave signals of various intensity with the subtracted probe but no signal with the non-subtracted probe were tested by hybridization to Northern blots of ZT 3 and ZT 15 head total RNA. One of them (*Crg-1*) showed reproducible differences in mRNA abundance between ZT 3 and ZT 15 and was analyzed further.

Circadian and PER-dependent regulation of Crg-1

To analyze the daily oscillations of the *Crg-1* transcript, the cDNA insert was hybridized to a Northern blot containing head total RNAs from flies samples collected every 2 h during 24 h, in a LD 12:12 schedule (Figure 1A). The *Crg-1* cDNA probe (CRG-1 ABC) recognized at least three closely spaced transcripts ranging from 0.9 to 1.3 kb in size. Transcripts levels increased during the day to reach peak levels between ZT 12 and ZT 16 and then decreased. This waveform is very similar to that of the *per* mRNA, which reached a maximum level around ZT 14 (Hardin *et al.*, 1990), as shown by reprobing the same blot with a *per* cDNA. The overall *Crg-1* transcript level is comparable with *per* transcript level in fly head RNA, with the larger transcript being less abundant than the two others at any time. The larger transcript was individually monitored by using the CRG-1 A probe that is specific for the optional first intron of *Crg-1* (see Figure 4A). No clear phase or amplitude differences could be observed between the larger transcript and the three transcripts taken together (Figure 1A and B). Phosphorimager quantification indicated that the *Crg-1* transcripts abundance oscillated with a 3- to 4-fold peak-to-trough amplitude in LD 12:12, whereas the *per* mRNA levels oscillated with a 10- to 12-fold amplitude as expected (Figure 1B; Hardin *et al.*, 1990). When the same experiment was done after 3 days in constant darkness, *Crg-1* transcripts oscillations with comparable phase and amplitude were observed (Figure 1C). This indicated that *Crg-1* mRNAs oscillations persist without cycling light cues.

The *Crg-1* mRNA temporal expression was analyzed in per^0 flies entrained in LD 12:12 to determine whether *Crg-1* transcripts oscillations would be subject to PER control. Like *per* mRNA, the *Crg-1* mRNAs cycling was completely abolished in the arrhythmic per^0 mutant, even in the presence of LD cycles (Figure 2A and B). The control of the *Crg-1* transcripts oscillations by the PER protein was also indicated by the analysis of *Crg-1* RNA levels in per^S flies in complete darkness (Figure 2C). Like *per* transcript, *Crg-1* transcripts abundance oscillates with a period of ~20 h in per^S. As in wild-type flies, the amplitude of the *Crg-1* RNA oscillations was weaker than the amplitude of *per* RNA oscillations. These results show that the *Crg-1* and *per* mRNA oscillations are similarly driven by the PER protein, although *Crg-1* has a weaker amplitude. The *Crg-1* transcript levels in per^0 flies appeared to be closer to the ZT 15 levels than to the ZT 3 levels of wild-type flies (Figure 2B). This suggests that the *Crg-1* RNA oscillations are mostly due to a PER-induced decrease in levels. The per^0 result predicted that *Crg-1* mRNA cycling, like *per* mRNA cycling, would be suppressed in a tim^0 genotype (Sehgal *et al.*, 1994). Indeed, no *Crg-1* mRNA oscillations were observed in the tim^0 flies (Figure 3).

The Crg-1 gene encodes three differentially spliced transcripts

Crg-1 cDNA clones were obtained by probing a *Drosophila melanogaster* retina library with the 600 bp clone obtained from the subtracted library. The cDNA clones were characterized, and they defined three classes of transcripts. Their sequence was determined and compared with the sequence of genomic clones to draw a transcription map of the *Crg-1* gene (Figure 4). The length of the three types of cDNAs: 1296, 960 and 919 bp for types A, B and C respectively, are in good agreement with the size

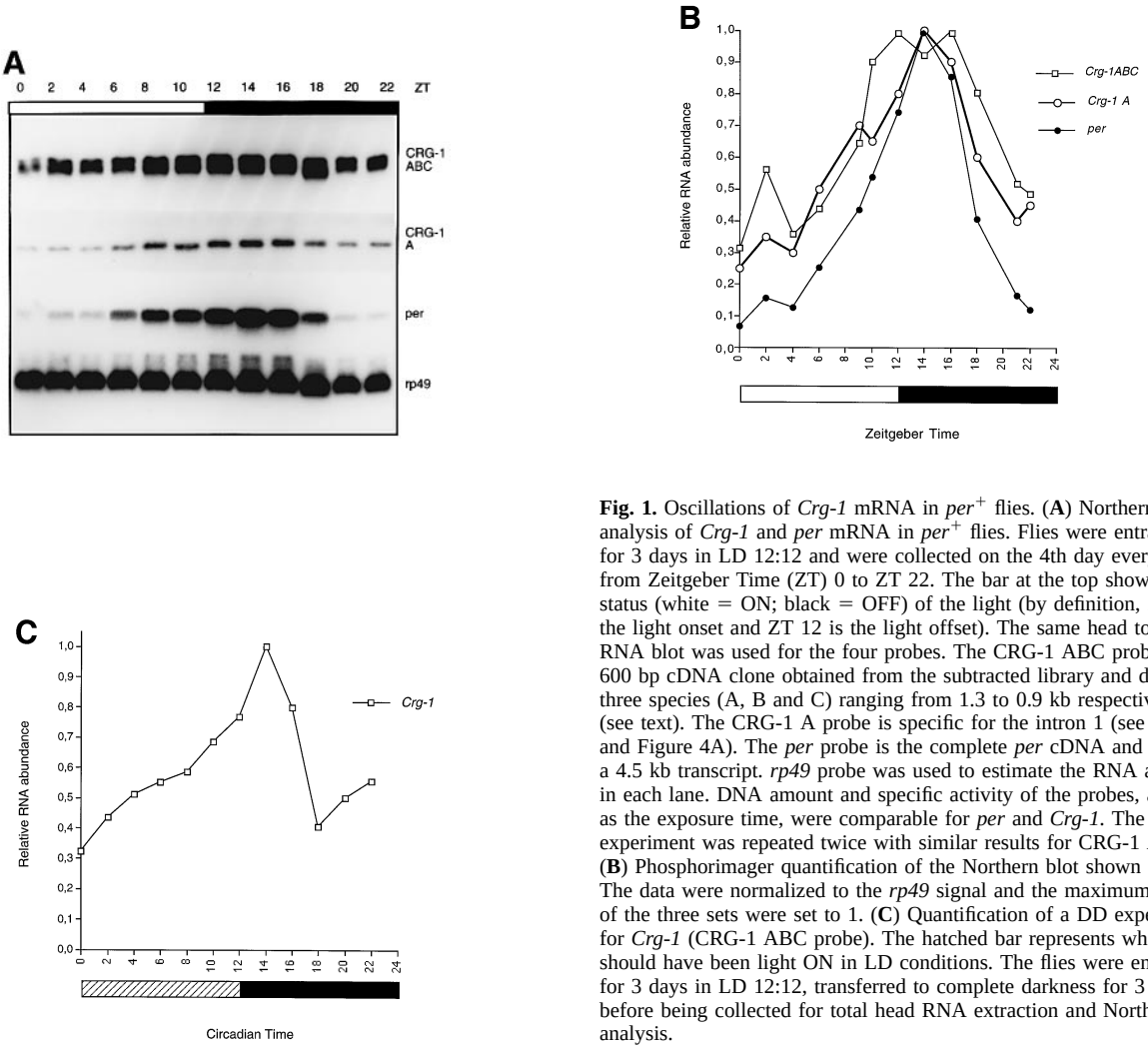

Fig. 1. Oscillations of *Crg-1* mRNA in *per*$^+$ flies. (**A**) Northern blot analysis of *Crg-1* and *per* mRNA in *per*$^+$ flies. Flies were entrained for 3 days in LD 12:12 and were collected on the 4th day every 2 h from Zeitgeber Time (ZT) 0 to ZT 22. The bar at the top shows the status (white = ON; black = OFF) of the light (by definition, ZT 0 is the light onset and ZT 12 is the light offset). The same head total RNA blot was used for the four probes. The CRG-1 ABC probe is the 600 bp cDNA clone obtained from the subtracted library and detects three species (A, B and C) ranging from 1.3 to 0.9 kb respectively (see text). The CRG-1 A probe is specific for the intron 1 (see text and Figure 4A). The *per* probe is the complete *per* cDNA and detects a 4.5 kb transcript. *rp49* probe was used to estimate the RNA amount in each lane. DNA amount and specific activity of the probes, as well as the exposure time, were comparable for *per* and *Crg-1*. The experiment was repeated twice with similar results for CRG-1 ABC. (**B**) Phosphorimager quantification of the Northern blot shown in (A). The data were normalized to the *rp49* signal and the maximum levels of the three sets were set to 1. (**C**) Quantification of a DD experiment for *Crg-1* (CRG-1 ABC probe). The hatched bar represents what should have been light ON in LD conditions. The flies were entrained for 3 days in LD 12:12, transferred to complete darkness for 3 days before being collected for total head RNA extraction and Northern analysis.

of the *Crg-1* transcripts as estimated from Northern blot analysis. RT–PCR were performed on head RNA using primers derived from the first and second exons and detected two cDNA products whose size and sequence was exactly the one predicted by the sequencing of the type B and C cDNA clones (data not shown). Because of the strong size bias toward small products introduced by the PCR reaction, RT–PCR products corresponding to the type A transcripts could not be detected with such primers, but they were detected with primers from the retained first intron and the second exon.

The three *Crg-1* transcripts result from differential splicing of the first 336 bp-long intron 1. Type A transcript retains intron 1 whereas types B and C transcripts result from the use of different donor sites for the splicing of intron 1. Type B uses a donor splice site at position 185 whereas the smaller type C transcript is generated by the use of an internal donor site at position 144. The three donor splice sites and the two acceptor splice sites of the gene fit with the GT/AG consensus. The 5′ ends were precisely defined by RACE (Rapid Amplification of cDNA Ends) using oligonucleotides from intron 1 and exon 2. Three classes of PCR products were obtained, which correspond to the three types of cDNAs. In each class, several clones were obtained that extend up to the same 5′-most G nucleotide, indicating that the three transcripts start at the same position. The two longest cDNA clones on the 3′ side ended at nucleotide 1296, and a putative polyadenylation site was found at position 1226, but we could not definitely identify this position as the 3′ end of the transcripts. We cannot exclude the possibility that the type A RNA represents a partially processed transcript, although its relative abundance compared with types B and C suggests that it is a mature species. Type A transcripts were also detected on poly(A)$^+$ RNA (data not shown), but the occurrence of several poly(A) stretches in the 3′ region (Figure 4B) could allow the co-purification of non-polyadenylated transcripts.

Conceptual translation of the three cDNAs identified open reading frames (ORFs) of 155, 127 and 118 residues in transcripts A, B and C respectively. The ORFs start in exon 1 for transcripts B and C, and within the retained intron 1 for transcript A. The putative proteins are out of frame in the 5′ part of the *Crg-1* transcripts (intron 1 for type A and exon 1 for types B and C), and share a common 100-amino-acid segment that starts at the beginning of exon 2 and ends within exon 3. In the type A sequence, an in-frame AUG codon is found at the end of intron 1 (position 480). Initiation at this AUG would add an amino-terminal segment of 14 residues to the 100-amino-acid core. Surprisingly, no AUG start codons could be found at the beginning of the ORFs derived from types B and

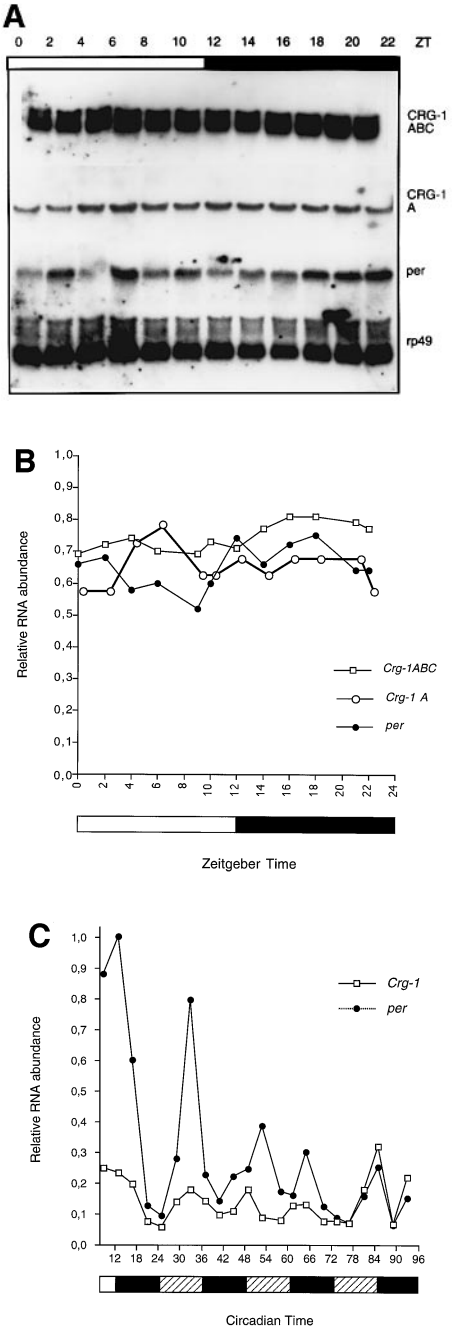

Fig. 2. Oscillations of *Crg-1* mRNA in *per* mutants. (**A**) Northern blot analysis of *Crg-1* and *per* mRNA in per^0 flies. Flies were entrained and collected as described for Figure 1A. The experiment was repeated twice with similar results for CRG-1 ABC and *per*. (**B**) Phosphorimager quantification of the data (average from two experiments for CRG-1 ABC). The relative RNA abundance values were obtained after normalization to the ones in Figure 1B, by hybridizing the probes to a LD and a DD blot together. Therefore the values for *per* and *Crg-1* can be compared between Figures 1B and 2B. (**C**) Quantification of a DD experiment with per^S flies. The flies were entrained for 3 days in LD 12:12 cycles and transferred to complete darkness for 3 days. Samples were collected every 4 h during the last LD day and the 3 h following DD days. The experiment was done with the CRG-1 ABC probe and repeated once with similar results.

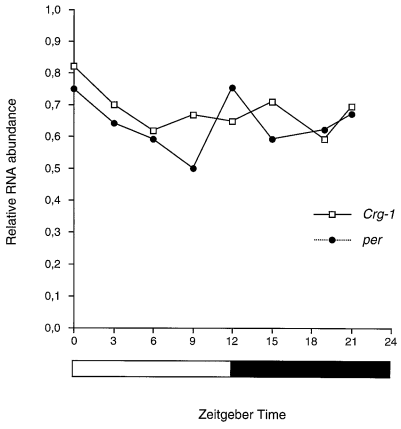

Fig. 3. Oscillations of *Crg-1* mRNA in tim^0 flies. Phosphorimager quantification of a Northern blot analysis of *Crg-1* (CRG-1 ABC probe) and *per* mRNA in tim^0 flies in LD 12:12. Flies were entrained and collected as described for Figure 1A.

C. We therefore looked for possible non-AUG initiation codons (see Discussion), downstream of the first codon of the *Crg-1* B and C ORFs. In the B transcript, one ACG codon is found at position 135 and two GUG codons are found within the 41 bp segment (at positions 159 and 183) that is present in transcript B but not in C. These putative initiators would generate translation products of 117, 109 or 101 residues respectively, from type B transcripts. No putative initiation codons were found in frame for type C transcripts. One CUG codon occurs at position 369 in transcript A (upstream of the AUG) and would initiate a 151-amino-acid protein from this transcript. The –4 to –1 upstream sequences of both the AUG and the non-AUG putative initiation codons do not fit to the CANN consensus defined by Cavener and Ray, (1991).

Preliminary results from the molecular cloning of the *Crg-1* genomic DNA from *Drosophila virilis* indicate that the sequence of exon 1 is not conserved between these two *Drosophila* species whose divergence is estimated to be about 50 million years old (Beverley and Wilson, 1984). This suggests that the exon 1-encoded part of the putative proteins is not functionally important. However, we have found in the *D.virilis* DNA a 31 bp sequence that is strictly identical to the first 31 nucleotides of the exon 1 of the *D.melanogaster* gene, suggesting some conserved function in the transcriptional or post-transcriptional circadian regulation of the gene or in the translational initiation (see Discussion).

CRG-1 ORFs encode a motif related to the HNF3/fork head DNA-binding domain

The putative translation products encoded by the *Crg-1* A, B and C transcripts were compared with protein sequence databases, and a short segment of similarity with the DNA-binding domain of the HNF3/fork head family of transcription factors was identified. Figure 5A shows the alignment of the CRG-1 protein sequence with the DNA-binding domain sequences of four proteins from this family. The similarity between CRG-1 and all the other HNF3/fork head proteins is much weaker than within the HNF3/fork head group. CRG-1 and the HNF3/fork head DNA-binding domain sequences can be aligned within a region of ~30 residues that is encoded by the exon 2 of the *Crg-1* gene. This region corresponds to the highly conserved K/RPP hook and α-helices 1 and 2 of the

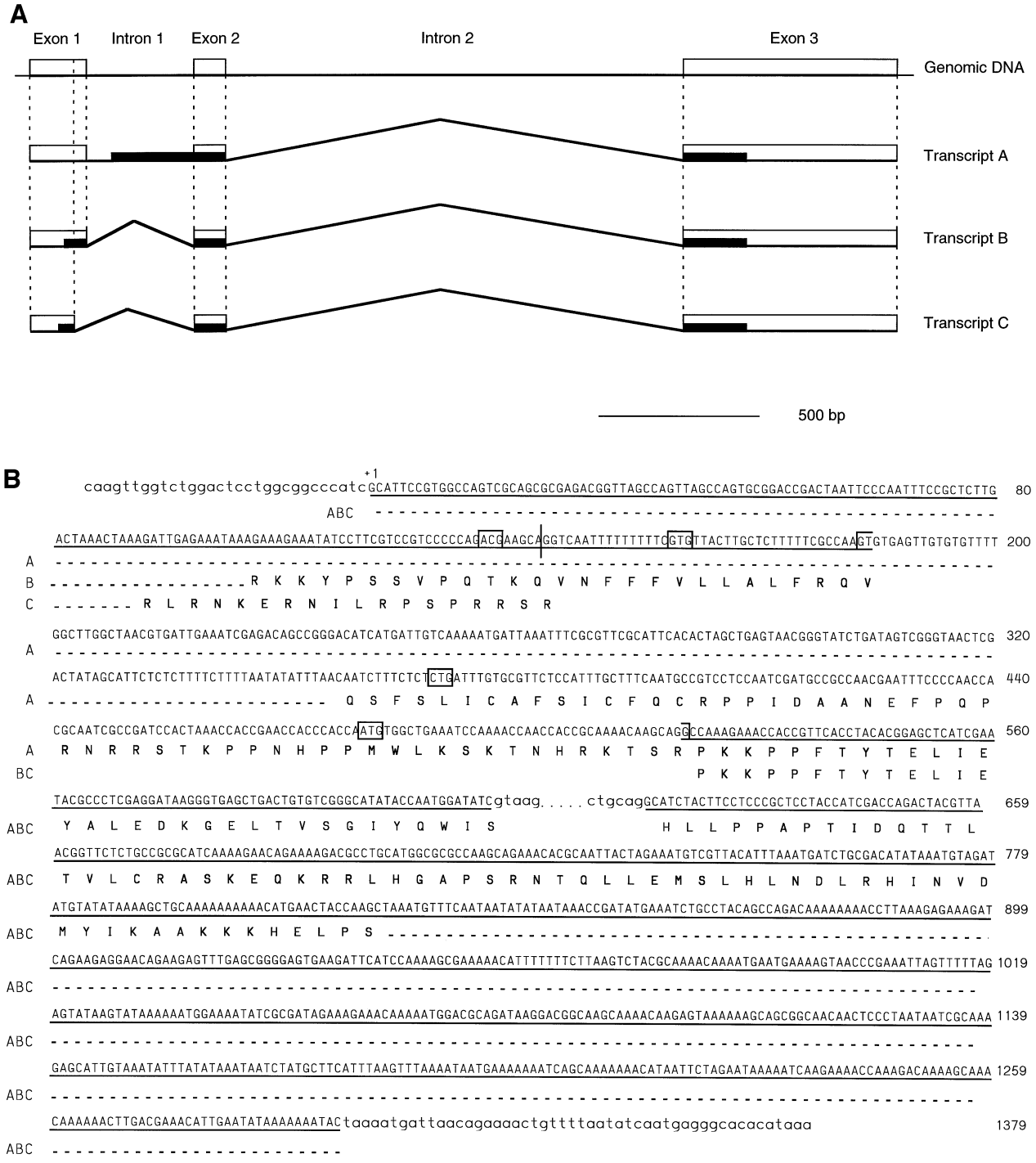

Fig. 4. *Crg-1* encoded transcripts and conceptual proteins. (**A**) Transcription map of the *Crg-1* gene. Transcribed sequences are drawn as thin bars, exons as open boxes, and ORFs as black boxes. (**B**) Transcripts cDNA sequences and conceptual translation. Lower case letters are used for 5′ and 3′ untranscribed sequences as well as intron 2 junction sequences. Upper case letters are used for exons (underlined) and retained intron 1 sequences. The vertical bar indicates the donor splice site used for type C transcripts. Putative translation initiation codons are boxed. On the left side, A, B and C letters show transcript types that correspond to the displayed ORFs. The ORFs are represented with single letter codes for residues; untranslated regions (UTRs) are represented as dashed lines. The numbering starts at the transcription start and is based on the longest transcript (type A). The 3′ end of the transcripts is uncertain. The end of intron 2 and the beginning of exon 3 show some sequence similarity with the αβ-repeats, a class of repeated sequences that have been found in transcribed tandem arrays at the 87C heat-shock locus, whereas non-transcribed units are dispersed through the *Drosophila* genome (Lis *et al.*, 1981).

winged helix DNA-binding domain, which were defined by the HNF3γ-binding site co-crystal structure (Clark *et al.*, 1993). A gap of two residues had to be inserted between helices 1 and 2 to keep the sequences aligned. This inter-helix region was diverged between the HNF3/fork head proteins (see Kaufmann and Knöchel, 1996). Another conserved motif is the stretch of basic residues located at the very end of the HNF3/fork head DNA-binding domain. The HNF3/fork head DNA-binding domain represents a variation of the helix-turn-helix (HTH) motif (Brennan,

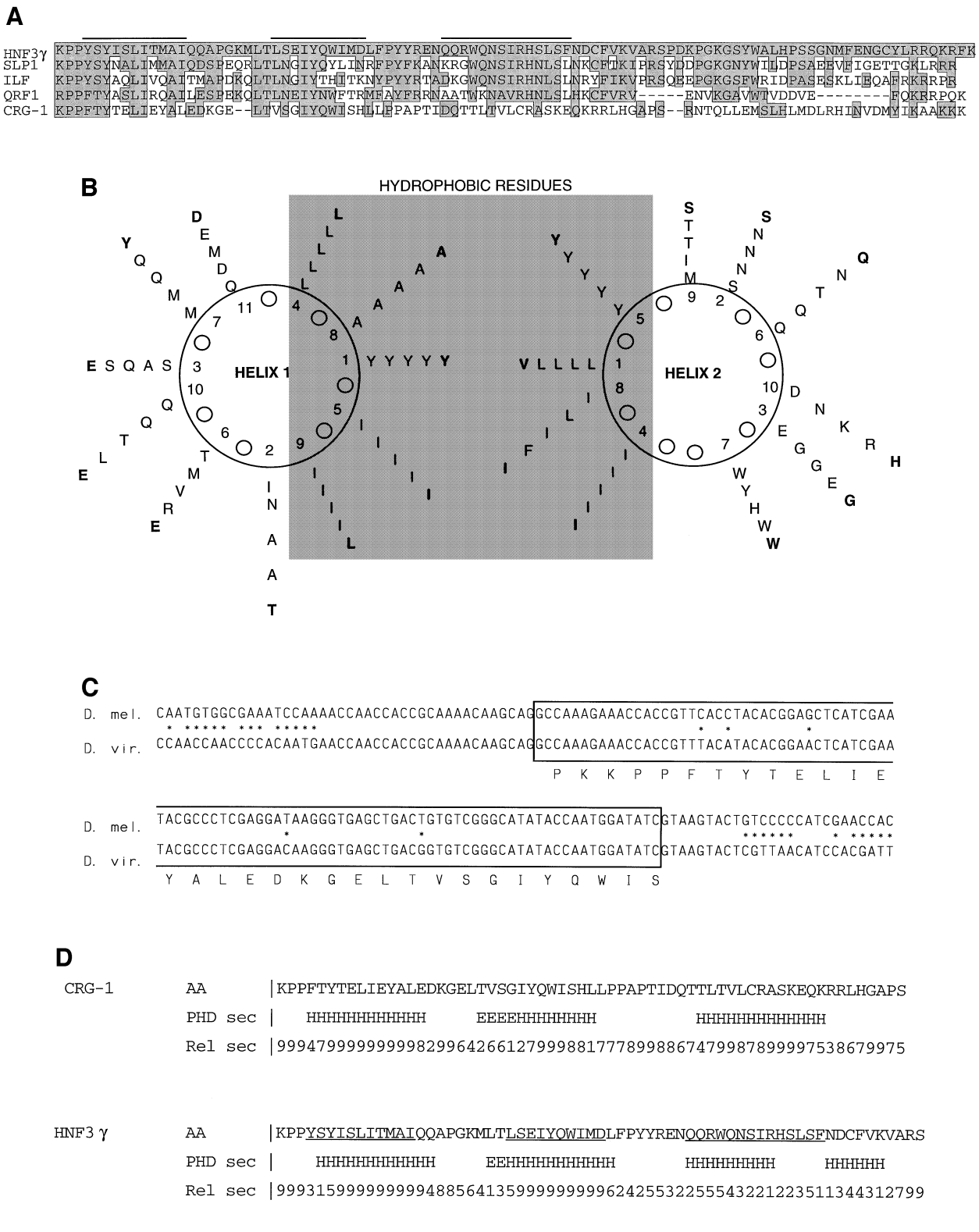

Fig. 5. Sequence similarity between CRG-1 and the HNF3/fork head DNA-binding domain. (**A**) Alignment between the amino acids sequence of CRG-1 and the DNA-binding domains of four members of the HNF3/fork head family: HNF3γ, SLP-1, ILF and QRF1 (modified from Kaufmann and Knöchel, 1996). The residues that are structurally related to the corresponding HNF3γ residues in the alignment are shaded. The bars above the HNF3γ sequence show the position of the three α-helices determined by crystallography (Clark *et al.*, 1993). (**B**) Helical wheel representation of the amino acid sequences in the α-helices 1 (11 residues) and 2 (10 residues) of HNF3γ, SLP1, ILF, QRF1 and CRG-1 (in bold characters), according to the HNF3γ structure (Clark *et al.*, 1993). The shaded area shows the hydrophobic sides of the two helices. (**C**) Alignment of the nucleotide sequence of the genomic DNA encoding the *Crg-1* exon 2 from *D.melanogaster* to the corresponding region from *D.virilis*. Sequences corresponding to the exon 2 of *D.melanogaster* are boxed and their conceptual translation is indicated below (see Figure 4B). Asterisks represent nucleotide differences between the two species. (**D**) Output of the Predict protein program (Rost and Sander, 1996) indicating the position of the predicted helices in the CRG-1 and HNF3γ sequences. H letters indicate α-helices, E indicate β-sheets and numbers below give the corresponding reliability indexes. Underlined residues in the HNF3γ sequence show the positions of the α-helices observed in the crystal structure (Clark *et al.*, 1993).

1993). The three helices form the core of the structure, with helix 3 (the recognition helix) embedded into the major groove of the DNA (Clark *et al.*, 1993). The replacement of the HNF3γ amino acids of helices 1 and 2 by the corresponding CRG-1 residues in a helical wheel representation of the sequence shows a very strong conservation of the structure, which keeps the hydrophobic residues on one side of the two α-helices (Figure 5B).

In *D.virilis* genomic DNA, only five silent nucleotide substitutions were found within the 97 bp of the sequence corresponding to the *D.melanogaster* second exon (Figure 5C). Therefore, the putative CRG-1 proteins from these two *Drosophila* species have identical primary sequences within the HNF3/fork-head-like domain, indicating that this is the correct protein sequence of the second exon and that it is under functional constraint.

The similarity between the CRG-1 protein and the HNF3/fork head DNA-binding domain totally disappears at the end of helix 2. However, the analysis of the CRG-1 sequence with algorithms used to predict protein secondary structures (Rost and Sander, 1994) indicates a high probability of helix structure in the region of CRG-1 corresponding to helix 3 in the HNF3 sequence, in addition to the expected helices 1 and 2 (Figure 5D). Proline residues flank helices 1 and 2 in both the HNF3 sequence and in the CRG-1 sequence, which further underscores the structural similarity.

Crg-1 and per show similar expression patterns in the Drosophila head

Northern blot analysis showed that *Crg-1* is mostly expressed in the head with a strong contribution from the eyes (data not shown). In order to define precisely *Crg-1* expression sites in the adult head, *in situ* hybridization was performed with *Crg-1* and *per* probes on frozen head sections of wild-type flies collected at ZT 15, when transcripts levels are the highest (Figure 6).

Expression in the retina was observed in photoreceptors R1–R6, R7 and R8 on horizontal (Figure 6, panel A) and frontal (panel C) sections. In the optic lobes, *Crg-1* expression was detected in the regions between neuropils. Strong staining was seen in the distal lamina and the region between lamina and medulla (panels A and C), whereas a weaker signal was observed in the proximal lamina (panel C, double arrows) and between the medulla and the lobula complex (panels A and D, double arrowheads). The same subsets of cells were recognized by a *per* probe (panel B; also Rachidi *et al.*, 1997). The positive cells represent only a fraction of the cells located in these regions, and very few cells are labeled in the cortical region surrounding the central brain (panels A and B). Previous studies using anti-PER antibodies (Siwicki *et al.*, 1988; Zerr *et al.*, 1990; Ewer *et al.*, 1992) or flies carrying *per* β-galactosidase transgenes (Liu *et al.*, 1988; Ewer *et al.*, 1992) have described comparable PER expression patterns in the eyes and optic lobes. Although we cannot exclude that different cells express *per* and *Crg-1* in these regions, it seems rather likely that the two probes label the same cells.

Neuronal *per* expression has been reported in a small set of large cells located between the medulla and the protocerebral neuropil, the so-called lateral neurons (LNs) (Siwicki *et al.*, 1988; Zerr *et al.*, 1990; Ewer *et al.*, 1992; Helfrich-Förster, 1995), and in the dorsal neurons (DNs) above the calyces of the mushroom bodies (Ewer *et al.*, 1992). The lateral neurons could not be clearly identified by *in situ* hybridization with a *Crg-1* probe (nor with a *per* probe; see Rachidi *et al.*, 1997). It is possible that the LNs contribute to the signal detected at the border between the medulla and the central brain (Figure 6, panels A and B). However, *Crg-1* labeling was clearly observed in the area corresponding to the PER-expressing dorsal neurons (panel E). In the central brain, numerous cell bodies were detected by the CRG-1 probe at the border of the optic lobes while some others are seen in the regions surrounding neuropils within the central brain, as observed for PER (Siwicki *et al.*, 1988; Ewer *et al.*, 1992).

The *Crg-1* gene was mapped to cytological position 3E by *in situ* hybridization to polytene chromosomes from larval salivary glands. A deletion mapping of *Crg-1* was done by testing for the presence of one or two copies of

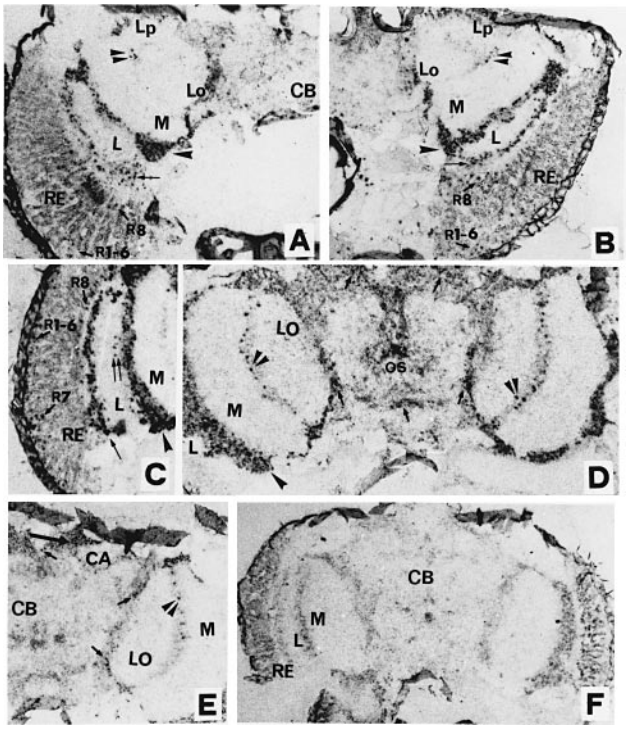

Fig. 6. *In situ* hybridization analysis of *Crg-1* expression in the adult wild-type head. (A and B) Horizontal sections through the eye and optic lobe. In the retina, *Crg-1* (**A**) and *per* (**B**) staining is observed in cells whose location correspond to R1–R6 and R8 photoreceptors cell bodies (small arrows). In the optic lobe the two probes label cells of the distal (thin arrow) lamina border, cells between lamina and medulla (arrowheads), and cells between medulla and lobula complex (at the junction between the lobula plate and the lobula, double arrowheads). (**C**) Frontal section through the eye and optic lobe. *Crg-1* staining is detected in the photoreceptors of the eye (small arrows). In the optic lobe, *Crg-1* stains a cell layer at the distal lamina (thin arrow), a few cells scattered in the proximal lamina (double thin arrows), and cells between lamina and medulla (arrowhead). (**D** and **E**) Frontal section through optic lobes and central brain. *Crg-1* staining is seen in the same inter-neuropils regions of the optic lobes (single and double arrowheads), as well as scattered central brain cell bodies, mostly at the periphery (small arrows). Positive cells are also detected on the top of the calyces of the mushroom bodies (large arrow). (**F**) *Df(1)64j4/Df(1)TEM-*202 *per*$^{-}$ female section showing no staining with a *per* probe. Abbreviations: CA, calyces; CB, central brain; L, lamina; LO, lobula complex; Lo, lobula; Lp, lobula plate; M, medulla; OS, esophagus; RE, retina.

the gene in females heterozygous for deficiencies localized to this region of the X chromosome (see Materials and methods). This allowed us to map *Crg-1* to the 3E–3F border region, distally to the *echinus* (*ec*) locus (FlyBase, 1996). To our knowledge, no mutations altering circadian rhythmicity have been identified in this region.

Discussion

In this paper, we have identified a new gene whose transcript levels undergo daily fluctuations in the *Drosophila* head. *Crg-1* RNA oscillations do not depend upon external light cues and persist with a 24 h period in complete darkness. The results define the circadian nature of the *Crg-1* transcript oscillations in the fly head. The *Crg-1* RNA rhythm requires the presence of PER and TIM as demonstrated by its complete suppression in per^0 and tim^0 flies. Furthermore, *Crg-1* RNA cycling in wild-type and per^S genotypes indicates that the phase and the period of the RNA oscillations are locked to those of *per* RNA. *per*-dependent RNA oscillations with a similar phase in wild-type flies were reported for *Dreg-5*, a transcript of unknown function that was recently isolated on the basis of its daily RNA fluctuations in the *Drosophila* head (Van Gelder and Krasnow, 1996).

The decrease of *per* and *tim* RNA levels is temporally correlated with the nuclear entry of the PER–TIM complex (Curtin *et al.*, 1995; Hunter-Ensor *et al.*, 1996; Zeng *et al.*, 1996), and overexpression of PER abolishes *per* RNA cycling by repressing its levels (Zeng *et al.*, 1994). *Crg-1* RNA levels in per^0 flies appear to be closer to peak rather than trough levels of wild-type flies. This supports the idea that *Crg-1* RNA cycling is driven by repression, although the lower amplitude of *Crg-1* RNA oscillations makes the distinction between peak and half-peak levels less certain than for *per* mRNA. High RNA levels in per^0 flies were also reported for *Dreg-5* (Van Gelder and Krasnow, 1996). *per*-promoter-driven RNA oscillations are mostly regulated at the transcriptional level (Hardin *et al.*, 1992). The phase similarity between *Crg-1* and *per* RNA cycling is consistent with the finding that both oscillations could depend on the same transcriptional mechanism, although it cannot be excluded that a PER–TIM-dependent post-transcriptional process regulates the stability of *Crg-1* transcripts. In this context, high A/U content (70%) of the 485 nucleotides 3′ UTR might predict rather unstable CRG-1 transcripts. A post-transcriptional contribution to *per* RNA cycling has been suggested by the finding of *per* transcript oscillations in flies carrying a promoterless *per* transgene (Frisch *et al.*, 1994).

Crg-1 RNA cycles with a lower amplitude (3- to 4-fold) than *per* (10-fold) in heads of wild-type flies. The amplitude of *Dreg-5* RNA cycling was also reported to be lower than that of *per* (Van Gelder and Krasnow, 1996), whereas *tim* RNA amplitude is similar or even greater than that of *per* RNA (Sehgal *et al.*, 1995). This could be due to the existence of *Crg-1*-expressing cells that are not under clock control; their constitutive expression would mask the overall cycling amplitude, as demonstrated for *per* in female bodies (Hardin, 1994). On the other hand, a lower amplitude may reflect differences in the mechanisms responsible for the circadian regulation of *per* and *tim* on one hand and *Crg-1* (and possibly *Dreg-5*) on the other. In the vertebrate liver, for example, the transcription factor DBP shows dramatic (>100-fold) circadian oscillations of its RNA and protein levels (Wuarin and Schibler, 1990; Fonjallaz *et al.*, 1996), but the amplitude of its target gene C7α-hydroxylase is only 5- to 10-fold (Lavery and Schibler, 1993).

The *Crg-1* expression pattern in the adult head closely resembles the PER expression pattern. The one possible exception is our failure to detect the PER-expressing lateral neurons. This could indicate that *Crg-1* is not expressed in these cells, suggesting that its cycling might be an output activity of a subset of pacemaker cells. However, the difficulty in identifying the LNs with a *per* probe (see Rachidi *et al.*, 1997) makes this a particularly weak negative result. In the eyes, and optic lobes, and region containing dorsal neurons, the signals obtained with *per* and *Crg-1* probes are virtually indistinguishable at the level of resolution obtained with *in situ* hybridization (Figure 6; also Rachidi *et al.*, 1997). The glial nature of the *per*-expressing cells in the optic lobes is strongly supported by the absence of labeling with an antibody directed against the neuronal marker *elav* (Ewer *et al.*, 1992). Taken together, these results suggest that *Crg-1* expression is also glial in the optic lobe lamina and medulla. The PER-expressing glial cells in the distal medulla appear to be in contact with the tangential arborization of the lateral neurons (Helfrich-Förster, 1995). It has been suggested that they could modulate the activity of the lamina interneurons L1 and L2, which show circadian variations of axonal size in *Musca* (Helfrich-Förster, 1995; Pyza and Meinertzhagen, 1995).

All three *Crg-1* transcripts detected in the adult head by Northern blot hybridization undergo circadian cycling of level with comparable phase and amplitude. Conceptual translation revealed three ORFs with two unusual features: (i) the first codon in each of the three transcripts ORFs is not in frame with the others, although the three ORFs are identical within the second and third exons; and (ii) the ORFs derived from the B and C transcripts do not contain putative AUG initiation codons. The only in-frame AUG codon is located at the end of intron 1 in the type A transcript; we cannot exclude that this is the only *Crg-1* transcript that is translated. However, this unique in-frame AUG is not in a good sequence context and is preceded within the first intron by four out-of-frame AUG. If transcripts B and C are translated, initiation would have to occur within exon 1, on a different non-AUG codon for each transcript. Directed mutagenesis and *in vitro* translation experiments have suggested that a GUG codon is used as initiator for the *Drosophila* choline acetyltransferase gene (Sugihara *et al.*, 1990) and that a CUG codon is used for the *erect wing* gene (DeSimone and White, 1993). Studies with *Drosophila* cultured cells have shown that a CUG codon is the main initiator for the *E74A* gene (Boyd and Thummel, 1993).

Such in-frame codons have been found in the first exon of *Crg-1*, but their use would require an internal guiding of the ribosome to initiate translation at the proper site for each of the *Crg-1* transcripts. It is possible that the sequence of the non-coding part of exon 1 prevents ribosome scanning of the transcripts and directs initiation to internal sites (see Oh *et al.*, 1992). The choice of the initiator codon could then depend on the exon

1-downstream sequences, which are specific for each of the *Crg-1* transcripts. A dramatic influence of downstream sequences on internal initiation has been shown for the *E74A* mRNA (Boyd and Thummel, 1993). The comparison between the products derived from *in vitro* translated *Crg-1* mRNAs and the proteins recognized by anti-CRG-1 sera in *Drosophila* extracts should help to understand how the *Crg-1* transcripts are translated.

The CRG-1 putative proteins show some similarity with the DNA-binding domain of the HNF3/fork head family of transcription factors. These proteins share a domain of ~100 amino acids, the so-called winged helix domain that is sufficient to bind DNA in a sequence-specific manner (reviewed by Kaufmann and Knöchel, 1996). Interestingly, the region of similarity between CRG-1 and the winged helix domain corresponds exactly to exon 2 of the *Crg-1* gene, supporting a modular construction of the protein. The comparison of CRG-1 with the HNF3/fork head DNA-binding proteins suggests two very unusual features of CRG-1. First, the sequence similarity is limited to a segment of ~30 amino acids, which contains the first two α-helices of the winged helix domain, and to the basic residues at the end of this domain. Interestingly, the N-terminal segment of the HNF3/fork head DNA-binding domain, highly conserved in CRG-1, has been shown to contain the nuclear localization signal for HNF3β (Qian and Costa, 1995). Second, the predicted size of the CRG-1 proteins does not exceed the DNA-binding domain itself. If this short region reflects a functional relationship to the HNF3/fork head domain, CRG-1 may represent a new distantly related member of this family. To our knowledge, all the HNF3/fork head members are much larger than the ±100-amino-acid winged helix domain, and transcriptional activation functions have been mapped beyond that domain (Pani *et al.*, 1992). The size of CRG-1 suggests that it does not contain an activation domain and that it is more likely a transcriptional repressor. Along this line, it should be noted that the overproduction of a truncated HNF3β protein with only the DNA-binding domain inhibits the expression of HNF3 target genes in cultured hepatoma cells (Vallet *et al.*, 1995). If CRG-1 acts as a transcriptional repressor, the *per* and *tim* clock genes as well as the putative downstream gene *Dreg-5* will have to be tested as possible targets.

All of these data suggest that *Crg-1* encodes a transcription factor which functions in many if not most *per*-expressing cells. *Crg-1* may be an output gene; its mRNA cycling is driven by the PER–TIM pacemaker and CRG-1 affects the transcription of downstream genes. Alternatively, CRG-1 may participate in the clock mechanism itself, by contributing to the PER–TIM feedback loop. Alterations of the *Crg-1* gene product or its level and analysis of their consequences on the *per* and *tim* cycle as well as on locomotor activity rhythms should distinguish between the two possibilities.

Materials and methods

RNA extraction and analysis

Flies (3–5 days old) were entrained in LD 12:12 cycles for 3 days and collected on dry ice, or eventually kept in complete darkness for 3–4 days before collection. All experiments were done at 25°C. Between one and four bottles, each containing 100–200 flies were used for each time point. Heads were separated from bodies and head total RNA was extracted according to the GHCl extraction protocol of the methods book from G.Rubin's laboratory. 15–30 μg of total RNA samples were loaded on formaldehyde gels that were transferred onto nylon membranes according to Sambrook *et al.* (1989). Random priming labeling of the DNA probes, hybridization at 42°C and washing at 65°C were done as described in Sambrook *et al.* (1989). The hybridization signals were quantified with a PhosphorImager (Molecular Dynamics).

Construction of the subtracted (ZT 15–ZT 3) cDNA library

Poly(A)$^+$ RNA was isolated from total head RNA with magnetic oligo(dT) beads (Promega). About 1 μg of poly(A)$^+$ RNA made from flies collected at ZT 3 and ZT 15 was used to produce the tracer and driver cDNAs. Partial hydrolysis of the RNA, reverse transcription, G-tailing and PCR were performed as described by Pikielny *et al.* (1994). The ZT 3 and ZT 15 cDNAs were tested for their relative abundance of *per*, rare transcript control *elav* (Robinow and White, 1988) and abundant transcript control *rp49* (O'Connell and Rosbash, 1984), by hybridization of the corresponding gene-specific probes to Southern blots of the cDNAs. ZT 15/ZT 3 ratios of 10, 1 and 1 were obtained for *per*, *elav* and *rp49*, respectively. Three subtractive hybridization rounds were performed as described in Pikielny *et al.* (1994), except that non-subtracted ZT 3 cDNA was used as driver for the three rounds of subtraction. During the third round, only ~50% of the tracer cDNA was removed by the subtractive hybridization. The subtracted (ZT 15–ZT 3) cDNA was tested for its content in the different control transcripts: *rp49* showed a 10-fold decrease in abundance, whereas *elav* and *per* were enriched 3- and 25-fold, respectively. This enrichment in *per* transcript allowed the detection of control clones containing *per* DNA when using the subtracted (ZT 15–ZT 3) cDNA as a probe, whereas they were not detected when probed with the non-subtracted ZT 15 cDNA.

Cloning and sequencing

The subtracted (ZT 15–ZT 3) cDNA was digested with *Hin*dIII and *Sal*I and directionally cloned into the M13 mp18 vector. The original mp18 clone was used to screen a retina cDNA library (generous gift from C.Zuker). The retinal cDNAs were sequenced using the sequenase kit and RACE 5′ extensions were done with a 5′-AmpliFinder RACE kit (Clontech). Genomic clones were obtained by screening a *D.melanogaster* genomic library from R.Blackman.

In situ hybridization to head sections

In situ hybridizations to adult head sections were performed as described in Rachidi *et al.* (1997). *per* and *Crg-1* ABC cDNA probes were labeled with a DIG random priming kit (Boehringer).

Mapping using deficiencies

Crg-1 gene copy number was determined by quantification of the hybridization signal from Southern blots of several deficiencies that cover the 3E region of the X chromosome and comparison with wild-type male (one copy) and female (two copies) controls. One copy of *Crg-1* was estimated in females *Df(1)cho3* (3C;3F), *Df(1)cho5* (3D;4A) and *Df(1)GA102* (3D4-5;3F7-8). *Crg-1* was mapped proximal to *slc* in 3E by the estimation of two copies in females *Df(1)N-8* (3C2-3;3E3-4) and *Df(1)dm75e19* (3C11;3E4), and to 3E7-8 by the estimation of one copy in females *Df(1)HC244* (3E8;4F11-12) and one copy in males *Tp(1;2)w-ec* (3C1-2;3E7-8;37D). *Crg-1* was mapped distal to *ec* in 3E-F by the two copies estimated in females *Df(1)cho2* (3E;4A) and *Df(1)cho23* (3E-F;4F). The *Df(1)cho* lines were provided by M.Steinmann-Zwicky, all the others by the Bloomington Stock Center.

Accession numbers

The cDNA sequences have been submitted to the EBI database with the accession numbers: Y12584 (Crg-1A), Y12585 (Crg-1B) and Y12586 (Crg-1C).

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