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takeout, a Novel *Drosophila* Gene under Circadian Clock Transcriptional Regulation

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We report the identification and characterization of a new *Drosophila* clock-regulated gene, *takeout* (*to*). *to* is a member of a novel gene family and is implicated in circadian control of feeding behavior. Its gene expression is down-regulated in all of the clock mutants tested. In wild-type flies, *to* mRNA exhibits daily cycling expression but with a novel phase, delayed relative to those of the better-characterized clock mRNAs, *period* and *timeless*. The E-box-containing sequence in the *to* promoter shows impressive similarities with those of *period* and *timeless*. However, our results suggest that the E box is not involved in the amplitude and phase of the transcriptional cycling of *to*. The circadian delayed transcriptional phase is therefore most likely the result of indirect regulation through unknown transcription factors.

Circadian (~24-h) behavioral and physiological rhythms are manifest in virtually all organisms. Our understanding of the underlying molecular rhythms comes largely from genetic investigations of five different classes of organisms: plants (28), photosynthetic bacteria (17), *Neurospora* (8), *Drosophila* (32), and mice (44, 47). Recent progress has reinforced the negative feedback regulation of transcription, originally proposed for *Drosophila* (14, 14a, 15a, 50), as a central theme of circadian rhythms in these organisms (9). In particular, *Drosophila* clocks display conservation with mammalian clocks. At the sequence level, many *Drosophila* clock components have one or more mammalian homologs, which are suggested to play similar roles in mammalian rhythms. This further validates *Drosophila* as an animal model system for the study of circadian rhythms.

The first *Drosophila* clock component identified was the *period* (*per*) gene (3, 20, 31). Biochemical and genetic data suggested a transcriptional autoregulatory feedback loop involving PER (14, 14a, 15a, 50). The second essential pacemaker component, *timeless* (*tim*), was subsequently identified, and both *per* and *tim* reciprocally autoregulate at the transcriptional level (29, 39). TIM dimerizes with PER (10, 24, 51), and the interaction is suggested to be important for the posttranscriptional regulation and nuclear entry of both proteins (35, 48). Although their precise biochemical functions are not certain, PER and TIM probably function directly in the negative regulation of transcription (7, 22). In contrast, the biochemical functions of the recently identified clock genes *Clock* (*Clk*) and *cycle* (*cyc*) are apparent from their primary sequences (1, 7, 34). Both CLK and CYC belong to the basic helix-loop-helix (bHLH)–PAS (Per-Arnt-Sim) transcription factor family, members of which are involved in a wide range of other life processes. For example, the mammalian ARNT-AHR heterodimer is involved in xenobiotic resistance (37), and the *Drosophila* SIM-TANGO heterodimer is involved in embryonic development of the central nervous system midline cells (41).

In the *Drosophila* mutants Clk^{jrk} and cyc^{01} (1, 34), the rate of transcription of the two major clock components, *per* and *tim*, is very low. Both mammalian and *Drosophila* CLK and CYC were found to bind the *Drosophila* per 21-bp E-box-containing sequence in yeast one-hybrid assays (7, 11). Binding was also shown by DNA mobility shift assays (23). CLK-CYC was also found to activate transcription from promoters containing four copies of the 18-bp E-box-containing sequence from both the *per* and *tim* promoters in *Drosophila* cell culture, and the activation was dramatically reduced by an E-box central 2-bp mutation (7).

Recent studies have suggested that the CLK-CYC heterodimer may directly regulate circadian output genes as well as central clock genes. The neuropeptide arginine vasopressin is synthesized and released in a circadian manner from suprachiasmatic nucleus neurons. It is involved in peripheral salt and water balance (38) and also has some distinct effects within the central nervous system (16). The vasopressin peptide rhythm was found to be transcriptionally regulated (6). More recently, the vasopressin mRNA rhythm was shown to be abolished in *Clock*/*Clock* mice (18). An E-box-containing sequence in its promoter was found to be necessary for the CLK-BMAL1-mediated transcriptional activation in cell culture, suggesting that CLK-BMAL1 may directly regulate vasopressin transcription (18). Another output gene, *dbp*, which encodes a basic-leucine zipper transcription factor (25), is down-regulated in *Clock*/*Clock* mice, and CLK is found in a protein complex that binds to an E box within the *dbp* first intron (31a). There is therefore strong evidence that the CLK–BMAL1–E-box complex is relevant to output gene as well as clock gene transcription (11, 18). This indicates that CLK-BMAL1 acts directly on this E box.

In this paper, we report the identification, characterization, and transcriptional regulation of a novel clock-regulated output gene, *takeout* (*to*). *to* was identified through a subtractive hybridization that enriched genes differentially expressed in cyc^{01} and wild-type flies. *to* mRNA levels are undetectable in cyc^{01} and Clk^{jrk} mutant flies, and *to* transcription cycles with a delayed phase compared to that of *per* and *tim*. Functional

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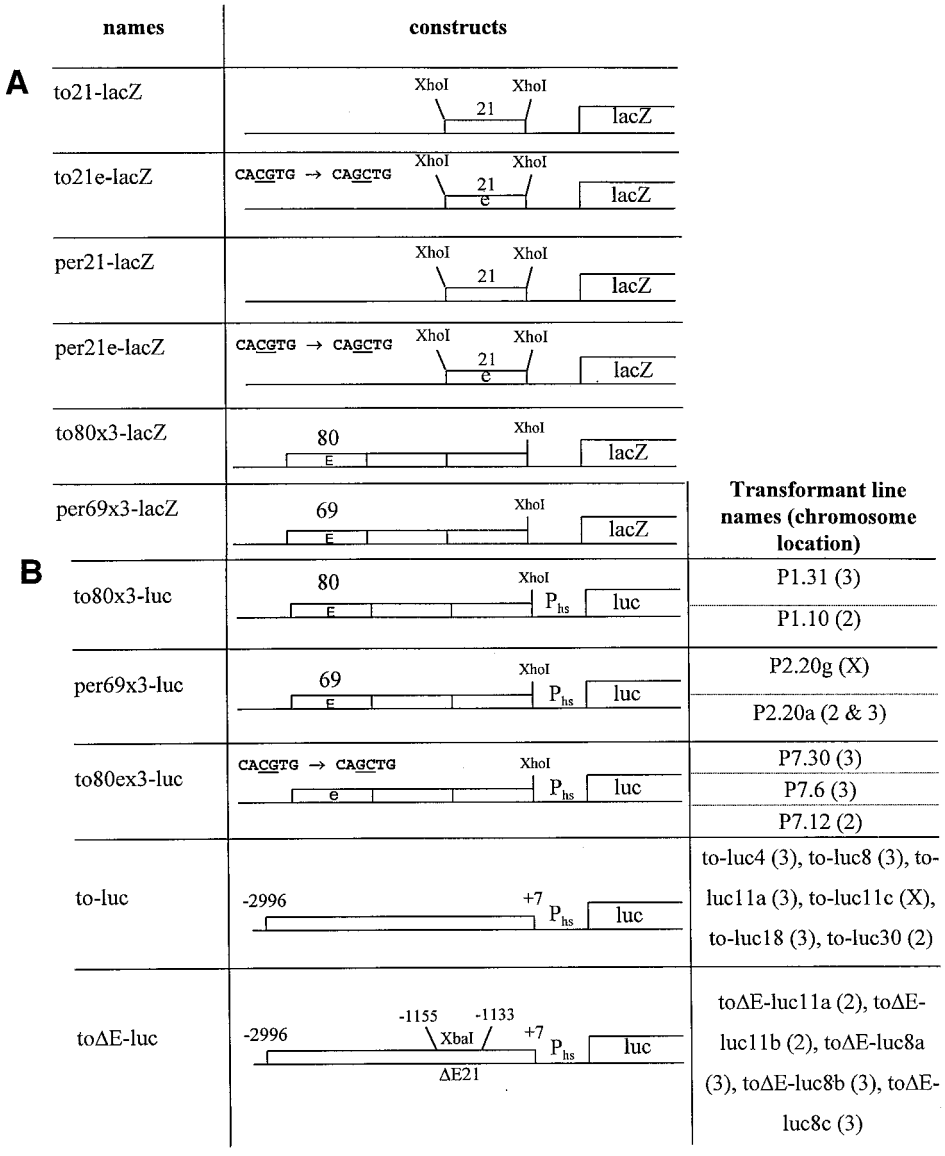

FIG. 1. *lacZ* reporter yeast constructs (A) and luciferase reporter constructs and flies (B). P_{hs}, heat shock promoter.

analysis (36) shows that TO is involved in an output pathway which conveys temporal and food status information to feeding-relevant metabolisms and activities. Our data here suggest that a prominent E box in the *to* promoter is not involved in its temporal transcriptional regulation. Therefore, there is most likely an indirect regulation by CLK and CYC which gives rise to the delayed *to* transcriptional phase relative to *per* and *tim*.

MATERIALS AND METHODS

Fly strains. The strain of wild-type flies used was Canton-S. cyc^{01}; ry^{506},per^{01},*yw*;tim^{01},per^{01};tim^{01};ry^{506}, and *yw*;Clk^{jrk} flies were used for analysis. Genomic DNA of the flies was tested with PCR for the presence of *to*.

Constructs and transgenic flies. The *lacZ* constructs were generated by inserting *Sal*I/*Sal*I or *Xho*I/*Xho*I promoter fragments (Fig. 1A) into the *Xho*I-cut p*BglII-lacZ* reporter vector (11). The *to21-lacZ Xho*I/*Xho*I insert sequence is CCGCTCGAGGCAGCTCACGTGATGGAACTCGAGCGG, and the *to21e-lacZ* sequence is CCGCTCGAGGCAGCTCAgcTGATGGAACTCGAGCGG (lowercase letters indicate mutation changes). Likewise, the *per21-lacZ Xho*I/*Xho*I insert sequence is CCGCTCGAGCCGCCGCTCACGTGGCGAACTCT CGAG, and the *per21e-lacZ* sequence is CCGCTCGAGCCGCCGCTCAgcTG GCGAACTCTCGAG. *to80x3-lacZ* was generated by synthesizing the 80 bp around the E box with *Sal*I on one side and *Xho*I on the other. Since *Sal*I and *Xho*I have compatible ends, multimers of the 80 bp were generated by cutting, washing, ligating, and then cutting with both restriction enzymes. Trimers were selected after running on an agarose gel and then ligated to the *Xho*I-cut p*BglII-lacZ* reporter vector. The *per 69x3-lacZ* construct was made likewise.

The luciferase constructs were generated in the CaSpeR4 vector. They contain a basal heat shock promoter (CaSpeR-hs43-lacZ; GenBank accession number X81643) ligated to *Sal*I/*Kpn*I of the luciferase (42). The *Xho*I site at the 5′ end of the basal heat shock promoter allowed insertion of the different *Sal*I/*Xho*I and *Sal*I/*Sal*I promoter pieces (Fig. 1B). Note that *Sal*I and *Xho*I have compatible ends, and ligation of the two overhangs destroys the restriction site. The reporter constructs were then used to generate germ line transformants by injecting *yw*;*Ki* p^P [ry^+ Δ*2-3*]/+.

PCR-based cDNA subtraction and screening. Wild-type and cyc^{01} mutant flies of the same age were entrained at 25°C in a 12-h light–12-h dark (LD) cycle for 2 days before being collected at zeitgeber time 15. (ZT15; zeitgeber time is the time in hours in a 12-h light–2-h dark cycle, where ZT0 is lights on and ZT12 is lights off). Frozen heads were isolated, and total RNA was extracted using TRIzol reagent (GibcoBRL). Poly(A)$^+$ RNA was prepared using the PolyATract mRNA isolation system (Promega). cDNAs were prepared from 2 μg of the poly(A)$^+$ mRNA from each sample and were hybridized according to the Clontech protocol. Conditions of the PCR selection were optimized by

monitoring the subtraction efficiency by measuring the level of controls: *tim*, which is rare and differentially expressed in the two subtraction samples; and *rhodopsin*, which is abundant and equally expressed. The level of the controls in the subtracted and unsubtracted PCR products was measured by slot blot hybridization. Subtracted PCR products were then cloned into *Not*I-digested pBluescript II KS+ and used to transform *Escherichia coli* DH5α (UltraMAX DH5α-FT competent cells; GibcoBRL). Transformation efficiency of 2×10^7 CFU/μg was obtained. Clones were randomly picked, and their plasmid inserts were amplified by PCR. The PCR products were transferred to HyBond-N+ membranes using a dot blot minifold (Schleicher & Schuell, Inc.). The membranes were hybridized to ^{32}P- or fluorescein-labeled subtracted and unsubtracted PCR products. Clones that showed substantial differential expression in the two populations of probes were further examined on Northern blots.

RNA extraction and analyses. For Northern blots, total and poly(A)$^+$ RNAs were prepared as described above. One-microgram samples of poly(A)$^+$ mRNA were loaded on formaldehyde gels and then transferred onto nylon membranes. ^{32}P-labeled probes were prepared by random priming of gel-purified fragments using Prime-It II (Stratagene). Prehybridization (~1 h) and hybridization (~16 h) were performed at 65°C in 10 ml of Church buffer (0.5 M $NaHPO_4$ [pH 7.2], 7% sodium dodecyl sulfate, 1% bovine serum albumin, 1 mM EDTA). The membranes were washed in washing buffer (0.2× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate] 0.1% SDS) twice briefly at room temperature and then twice at 60°C for 20 min before being exposed to film either at room temperature or at −80°C with an intensifying screen, depending on the strength of the signals. RNase protection assays were performed as described by Marrus et al. (27). The RNA probe protects nucleotide (nt) 529 to 839 of the cDNA region.

mRNA in situ hybridization. Flies were entrained to LD cycles. Frozen sections (10 μm) of adult heads and bodies were cut, and in situ hybridization was performed as described by Hasan (14b). Digoxigenin-labeled riboprobes were prepared from the full-length *to* and *per* cDNAs and were hydrolyzed prior to use. All hybridization and washes were performed at 65°C.

Genomic and cDNA library screening. A *Drosophila* genomic library in EMBL3 was generously provided by Ron Blackman. Two *Drosophila* adult head cDNA libraries were screened. Seven clones were sequenced, and a full-length sequence of 1,064 nt was obtained. While this report was under review, the *Drosophila* genome sequence was assembled and annotated. The *to* genomic sequence is confirmed by genomic scaffold accession number AE003751, the CG11853 gene. The λZAPII cDNA library generated with half oligo(dT) and half random primers was generously provided by Thomas Schwarz; the directionally cloned λEXLX(−) cDNA library generated with oligo(dT) primers alone was generously provided by Bruce Hamilton (30). Molecular techniques were performed using standard protocols (2).

Nuclear run-on assay. Nuclear run-on assays were performed as described by So and Rosbash (40). PCR products covering the full-length genomic region (~1.4 kb) were used as probe.

Yeast one-hybrid assay. The yeast one-hybrid assay was performed as described elsewhere (7), using the constructs described above and in Fig. 1A.

Luminescence monitoring and analysis. Luminescence monitoring was performed as described elsewhere (5). The readings were taken every 30 min, and data were analyzed according to the I-and-A software documentation (30a). The food was prepared with 1% Bacto Agar, 5% sucrose, and 15 mM luciferin.

RESULTS

***to* is a novel clock-regulated gene.** To identify novel genes involved in circadian rhythms, we performed a PCR-based cDNA subtraction and screening (Clontech; see Materials and Methods) whereby poly(A)$^+$ RNA from heads of *cycle* null mutant (*cyc*^{01}) flies was subtracted from wild-type RNA. We speculated that genes regulated by this transcription factor are inessential and that some of them are related to circadian behavior. Therefore, the aim was to find genes under circadian regulation by identifying genes differentially expressed in wild-type and *cyc*^{01} mutant flies. After screening 108 subtracted clones, we identified three different novel genes that are down-regulated in *cyc*^{01} flies. Here we present the cloning, characterization, and transcriptional regulation of one of them, *to*.

to mRNA expression is down-regulated in *cyc*^{01} flies and in all other circadian mutants tested (Fig. 2A). Its level is undetectable in *cyc*^{01} and *Clk*^{jrk} mutants, as measured by RNase protection and Northern blotting. In contrast, there is detectable *to* mRNA in all other genotypes tested, though it is substantially lower than that in wild-type flies. As there is little or no functional CLK-CYC heterodimer in the *cyc*^{01} and *Clk*^{jrk} backgrounds, the simplest way to explain this observation is

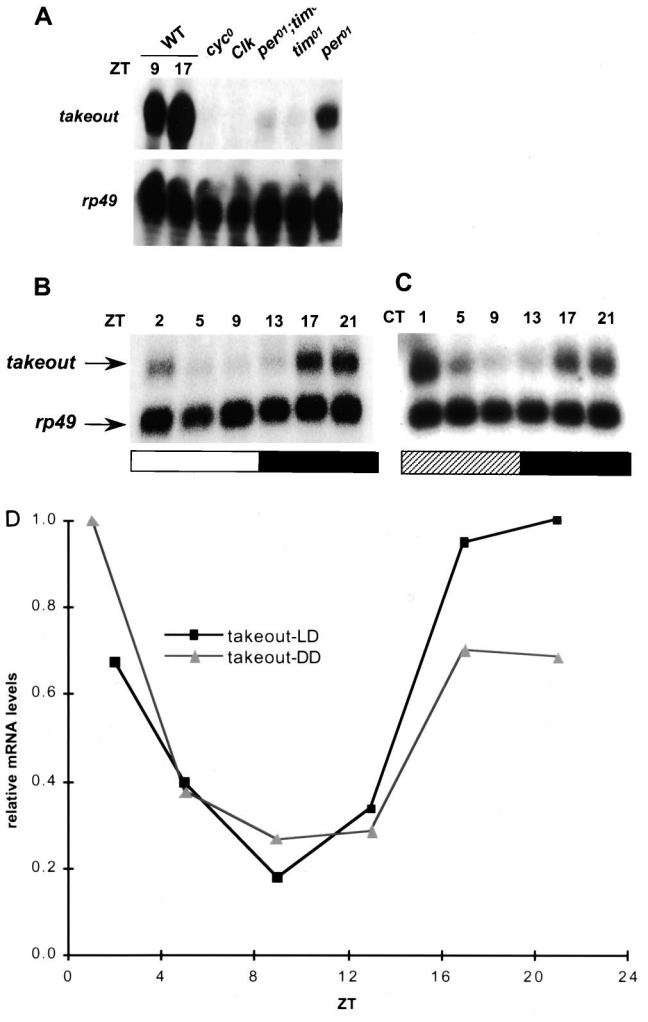

FIG. 2. *to* mRNA expression. (A) RNase protection assay showing that *to* mRNA is down-regulated in clock mutants. WT denotes wild type; *rp49* is an internal control. The gel is overexposed to show the low-level signal in *per*^{01}, *tim*^{01}, and double-mutant flies. (B and C) Northern blots showing the *to* mRNA cycling profiles in LD (B) and constant dark (C) conditions. To obtain comparable signals, the *rp49* probe was about half the length of and 1/40 lower in specific activity than the *to* probe. Open and filled bars represent the time when light was on (ZT0 to ZT12) and off (ZT12 to ZT24), respectively; the hatched bar represents the subjective day (circadian time [CT] 0 to 12). (D) Quantification of *to* mRNA levels in panels B and C. The *to* mRNA signals were normalized to the *rp49* signals. DD, constant dark.

that *to* is directly regulated by CLK and CYC. The higher *to* transcription in *per*^{01}, *tim*^{01}, and *per*^{01} *tim*^{01} double-mutant flies is presumably due to residual functional CLK-CYC heterodimer in these backgrounds (22; L. Sarov-Blat, unpublished data). *per*^{01} flies reproducibly showed a higher level of *to* expression than *tim*^{01}, indicating that PER and TIM may differentially regulate *to* expression. However, the mechanism underlying this difference is still unknown. When mRNA levels at different time points were measured, *to* did not show a significant cycling pattern in the clock mutants tested (data not shown).

***to* mRNA levels cycle with a novel phase in the head.** In wild-type flies, *to* mRNA levels exhibit a daily fluctuation in both cycling LD and constant dark conditions (Fig. 2B to D). The cycling in free-running conditions indicates that this property is a function of the endogenous clock rather than light

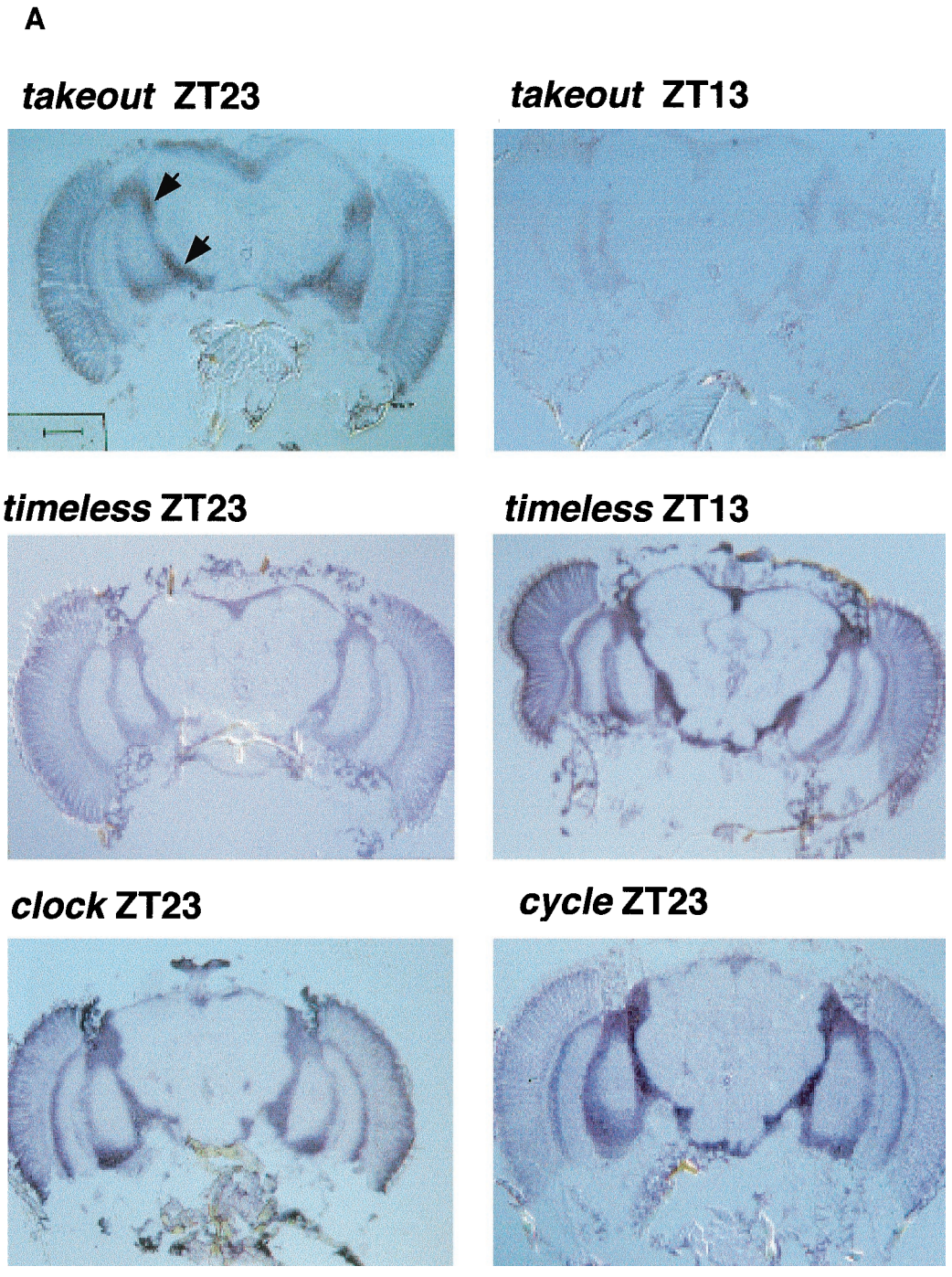

FIG. 3. (A) *to* mRNA is colocalized with *tim* and exhibits cycling expression in adult head sections. Shown are in situ hybridizations of antisense digoxigenin RNA probes for *to*, *tim*, *Clk*, and *cyc*, frontal sections of adult head at ZT13 and ZT23. Arrows point to where the lateral neurons are located. The mRNA expression of *to* and *tim* cycles with a different phase. Expression of both *to* and *tim* was detected in the photoreceptors and brain cortex, especially in the optic lobe regions. *tim* has broader expression, e.g., in the glial cells and in the central body of the central complex. *Clk* and *cyc* have the same expression pattern as *tim*. In situ hybridizations using sense RNA probes did not show obvious signals. (B) Alignment of the *to* family members. Boxes indicate the signature motifs that define the *to* family. Out of the 10 family members identified so far, 7 that have full-length or almost full-length conceptual translation sequences are shown. Five of the members, including *to* and *0.9kb* (GenBank accession number AL024485), AA696925, and AA142273, are from *Drosophila melanogaster*. Two, including AI142207, are from *Manduca sexta*. Three, including AU002769 and AU004740, are from *Bombyx mori*. The original EST sequences were obtained from the BDGP/HHMI *Drosophila* EST project. The EST sequences shown here are refinements from the different clone sequences, and accession numbers of the representative ones are used. Black background with white letters indicates identity; grey background with black letters indicates similarity.

driven. The amplitude (peak-to-trough ratio) of cycling is about 5, significantly smaller than those of *per* (~10) and *tim* (>10) mRNA expression. Interestingly, from several Northern blot analyses and RNase protection assays, the mRNA levels peak at about ZT17 to ZT20, a 2- to 5-h phase delay with respect to the *per* and *tim* cycling profiles.

Since *to* expression is down-regulated in the clock mutants, it was of interest to learn if the regulation is directly via CLK

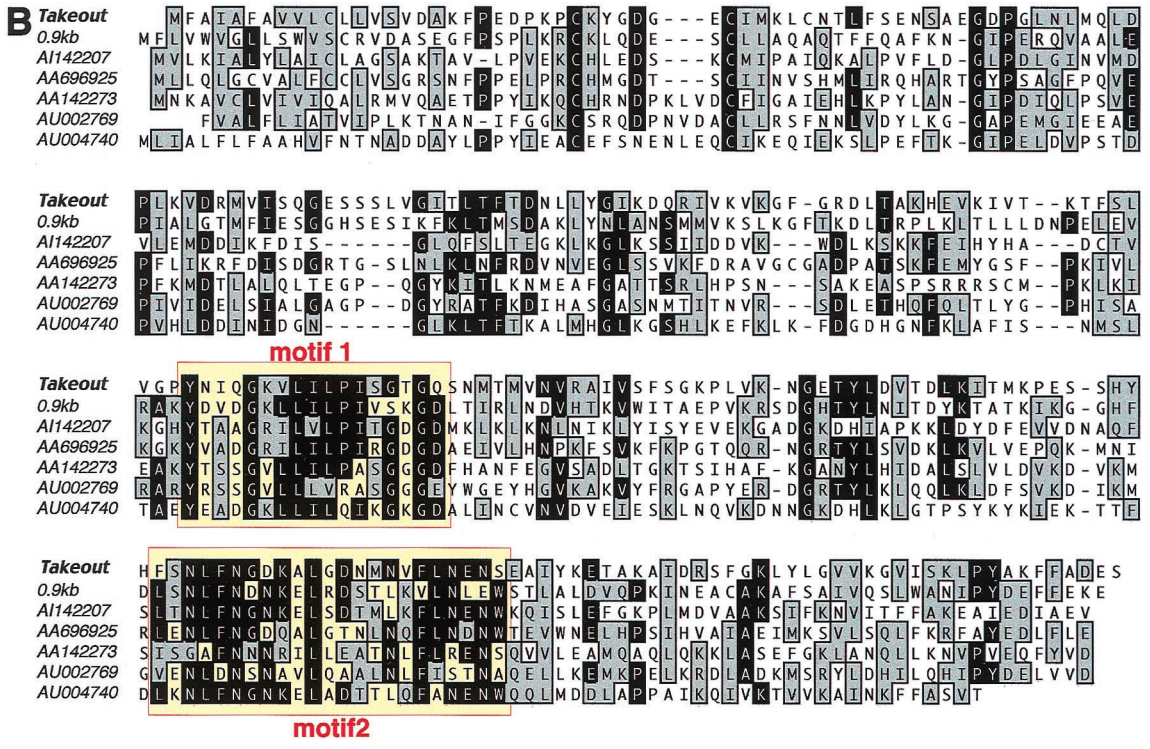

FIG. 3—*Continued.*

and CYC. To determine the localization of *to* expression, mRNA in situ hybridization to fly head sections was performed. For comparison, clock-expressing cells in the brain were also visualized by assaying *tim* expression as well as *Clk* and *cyc* expression. The in situ results showed very similar expression patterns at each gene's high time points (Fig. 3A). All four genes are expressed throughout the brain cortex, especially in the region between the optic lobe and the central complex, where the lateral neurons are located. They are also expressed in photoreceptor cells, although the *to* photoreceptor signal is generally lower than that in the brain cortex. However, *to* was not observed in other *tim*-expressing cells such as the glial cells in the optic lobe, the central body in the central complex, and the proboscis (data not shown). In general, the *tim* expression pattern is very similar to that of *Clk* and *cyc*. In bodies, the *to* expression pattern is a subset of the *tim* expression pattern (36). The data here suggest that *to* is expressed in a significant subset of clock-expressing cells in the head (see Discussion). However, due to the low resolution of mRNA in situ hybridization, we cannot rule out the possibility that *to* is expressed in cells adjacent to clock cells.

***to* is a member of a novel family.** The full-length sequence of the 1,064-nt *to* gene was obtained through screening of two *Drosophila* adult head cDNA libraries (see Materials and Methods). The sequence was later confirmed with the expressed sequence tag (EST) clones derived from an adult *Drosophila* head cDNA library (BDGP/HHMI *Drosophila* EST project; accession numbers AI403166 for the 5′ sequence and AI107005 for the 3′ sequence). A BLAST search against the nonredundant database using the open reading frame indicates that *to* is a novel gene. It has sequence similarity with a *Drosophila* gene called *0.9kb* (26) (see Discussion) and a group of EST clones from insects (Fig. 3B). They form a novel protein family with approximately 250 amino acid residues. Sequence similarity extends throughout the entire protein, with two stretches of highly conserved regions defined as motifs 1 and 2. These regions were used during database searching as criteria for defining family members. Family members have been found only in insects, and sequence analysis suggests a ligand-binding function (see Discussion).

***to* mRNA cycling is transcriptionally regulated.** To determine the level of regulation of *to* mRNA cycling, the in vivo transcription rate was measured by a nuclear run-on assay. The results show that the *to* transcription rate exhibits a daily fluctuation (Fig. 4A and B). The peak of transcription is about 3 to 4 h in advance of the mRNA peak (Fig. 2B to D), as expected for a gene regulated at the level of transcription (40). Consistent with the mRNA comparisons, the transcription profile of *to* is delayed by about 3 to 4 h with respect to the well-characterized *per* and *tim* profiles (40). This is most apparent by comparing the rising phase of transcription: *per* and *tim* transcription starts to rise at about ZT5 (40), whereas *to* transcription starts at about ZT9.

The phase delay was reproduced with a *to* 3.0-kb promoter driving a luciferase reporter gene in transgenic flies (*to-luc* [Fig. 4C]). A clear cycling of luminescence was exhibited by all six lines. The luminescence intensity is about two- to threefold higher than that of *plo* (luciferase driven by *per* promoter only [5, 42]), consistent with the estimated mRNA expression level difference between *to* and *per* observed in Northern blots and RNase protection assays (data not shown). The cycling peaks at about ZT23 and is about 5-h phase delayed compared to *plo*, which peaks at about ZT18 (5, 42). This strongly suggests that the regulatory information for the *to* phase of transcriptional cycling is contained within this 3.0-kb promoter fragment.

***to* promoter sequence contains an E box similar to those of the *per* and *tim* promoters.** The *to* promoter sequence revealed a remarkable sequence identity with the E-box region of the

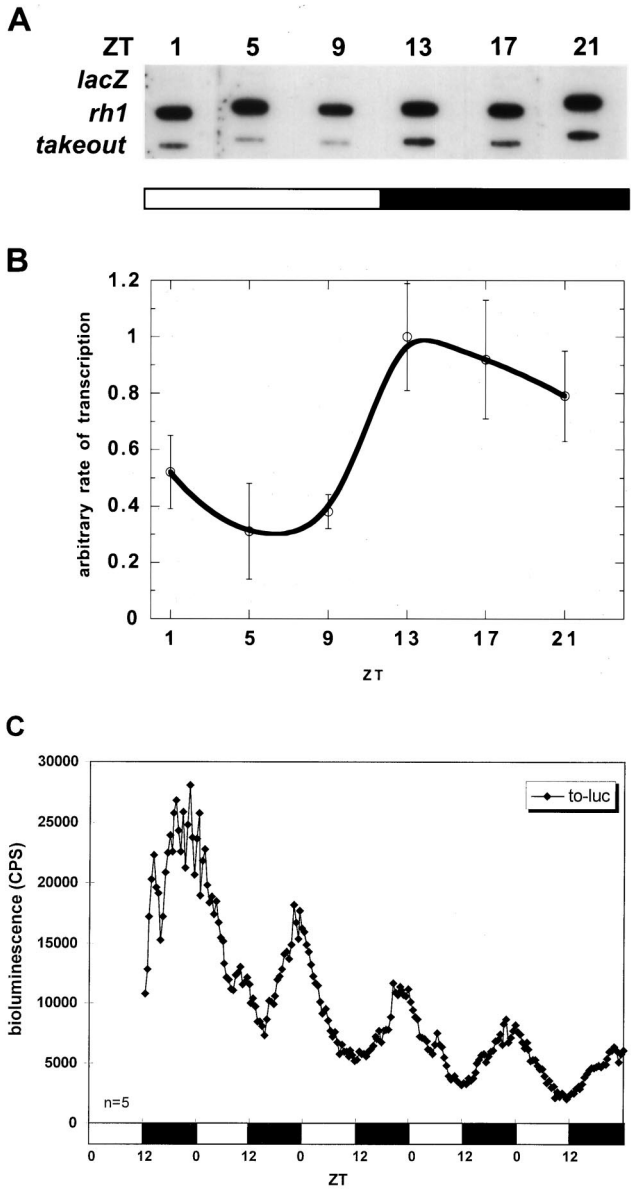

FIG. 4. Rate of transcription in wild-type flies. (A) Representative blot from a nuclear run-on assay. Each column is an individual hybridization blot from flies entrained and collected at the time indicated above the blot. Each row shows hybridization signals from the genes indicated on the left. The *lacZ* gene is a negative control, and the *rhodopsin* gene (*rh1*) is an internal control that has constant transcription rates throughout the day. (B) Quantification of rate of *to* transcription. The run-on signals were normalized to *rh1* signals. Averaged data are shown. Error bars indicate the standard deviation ($n = 3$ to 4). (C) Temporal luminescence from *to-luc* flies in LD. Average bioluminescence of representative lines is shown. Flies are heterozygous for the transgene. The number of flies used for the analysis is shown at the bottom left; open bars indicate lights on, and filled bars indicate lights off.

per and *tim* promoters (Fig. 5A). In particular, there is a 9-bp sequence identity around this E-box sequence. The other E-box sequences known in circadian genes usually share the 6-bp core sequence or the core sequence with an additional A (CACGTGA), which has shown to be strongly preferred by the mammalian BMAL1-MOP4 bHLH-PAS transcription factor heterodimer (15). In fact, the *to* and *per* promoters share 13 out of the 18 bp shown to be sufficient to drive transcriptional activation in S2 cells (7). This is also consistent with the fact that *to* mRNA is undetectable in cyc^{01} and Clk^{jrk} mutants (Fig. 2A), suggesting that CLK-CYC regulates *to* transcription directly. This would be similar to *per* and *tim* transcriptional regulation, despite the phase difference.

Binding of CLK and CYC to a 21-bp *per* E-box-containing fragment has been shown by a yeast one-hybrid assay (7). To determine if CLK and CYC also bind the *to* 21-bp E-box-containing sequence, similar yeast one-hybrid assays were performed (see Fig. 1A for constructs tested). Binding to the sequence is signaled by activation of the *lacZ* gene, resulting in blue color. As shown in Fig. 5B and similar to the *per* 21-bp control, both CLK and CYC are required to bind the wild-type *to* 21-bp sequence, but they do not bind to the identical sequence containing a mutated E box (CACGTG to CAGCTG, the same central 2-bp transversion mutation as in reference 7). The slight difference in the intensity of the blue coloration may be due to the different 2 μm plasmids used for the expression and reporter constructs.

Previous studies have shown that a 69-bp E-box-containing *per* upstream sequence fragment is sufficient to drive robust high-amplitude circadian cycling of reporter gene expression in flies (12). Since the *to* sequence and the yeast results suggest a similar *to* E box, we tested a comparable *to* 80-bp upstream sequence for its effect on in vivo transcription. The sequence was chosen so that the E box sits in the middle of the 80 bp (Fig. 5A). Because the nuclear run-on data indicate that the *to* transcription rate is lower than that of *per* and *tim* (reference 40 and Fig. 4A), a trimer of the 80-bp fragment (*to80x3-luc*) was assayed. At the same time, a mutated E-box reporter construct was also assayed (*to80ex3-luc*; the same 2-bp transversion mutations used in the yeast one-hybrid assays). As a control, a *per* 69-bp trimer (*per69x3-luc*) was examined in parallel.

Flies transformed with *to80x3-luc* showed surprisingly weak cycling of luminescence (Fig. 6A). Although the cycling was observed in every line, it was not observed in all experiments (data not shown). Compared to the *per69x3-luc* control (Fig. 6B), not only was the cycling amplitude from the *to80x3-luc* flies much lower, but luciferase expression was much weaker (note the difference in scale between Fig. 6A and B). Therefore, the *to* E-box-containing 80-bp fragment does not drive robust transcriptional cycling in vivo. Moreover, the mutated version, *to80ex3-luc*, showed an identical weak cycling pattern, suggesting that this E box is not relevant to the transcriptional pattern. Consistent with this view, overexpression of CLK driven by a heat shock promoter had no detectable effect on *to80x3-luc* expression(Fig. 6C), whereas it clearly induced *per69x3-luc* expression (Fig. 6D). Note that all of these trimer promoter constructs, wild type and E-box mutated, resulted in higher luminescence than the monomer constructs, *to-luc* (Fig. 4C) and *per-luc* (*plo* in reference 5, 42). This is most likely due to cooperative activity of transcription factors that binds to these regulatory elements.

The failure to observe robust expression and transcriptional oscillation with the *to* 80-bp fragment suggested that the positive yeast two-hybrid result with the *to* 21-bp fragment was misleading. We therefore tested a larger *to* fragment in the yeast system. Consistent with the transgenic fly data (Fig. 6), binding of CLK and CYC to the *to* E box in yeast became undetectable when the E-box-containing sequence was extended from 21 bp (Fig. 5B) to 80 bp (Fig. 7). A similar negative result was observed with 1.5 and 3.0 kb of *to* upstream sequence (data not shown). On the other hand, binding of CLK and CYC to the *per* E box was unaffected by the increase in sequence length from 21 to 69 bp. This suggests that there is

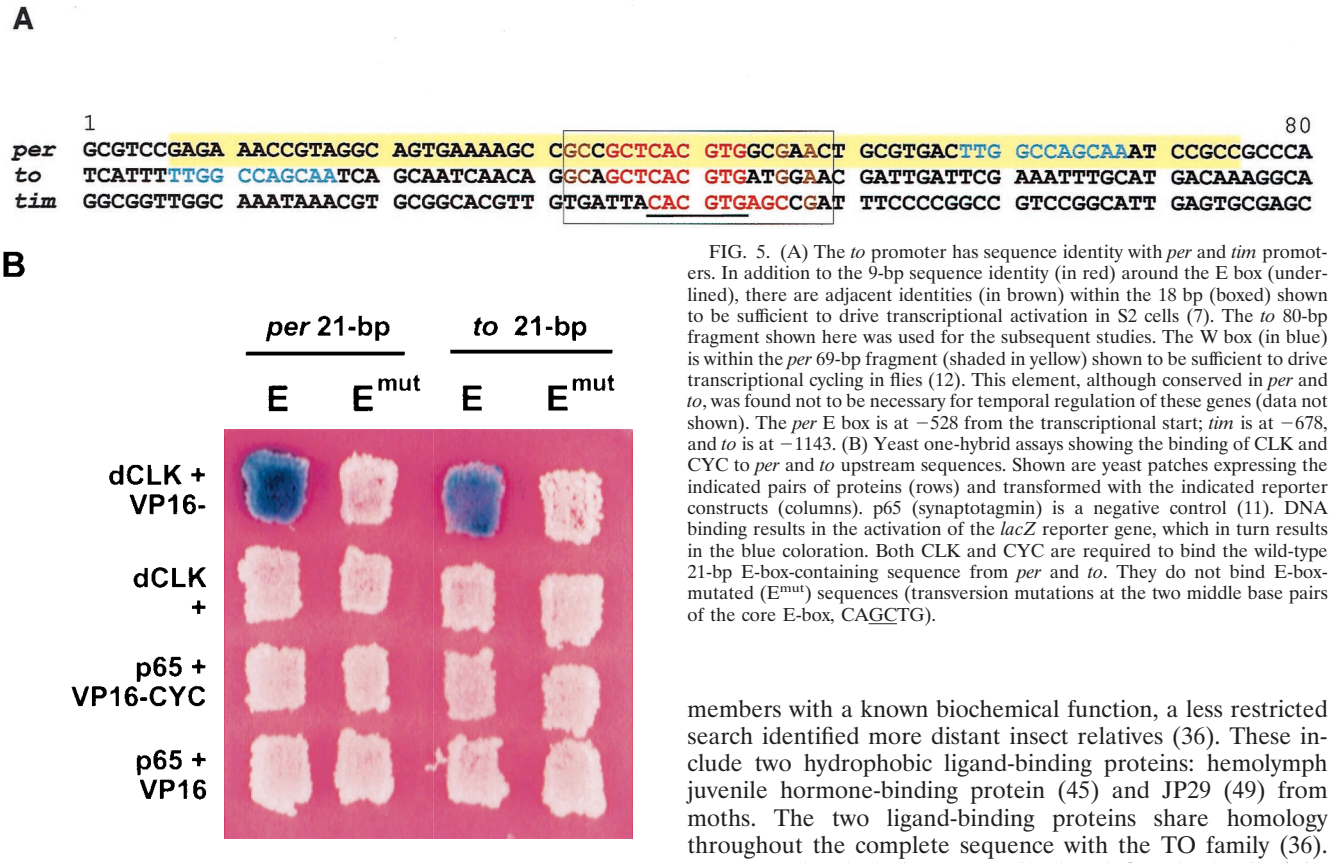

FIG. 5. (A) The *to* promoter has sequence identity with *per* and *tim* promoters. In addition to the 9-bp sequence identity (in red) around the E box (underlined), there are adjacent identities (in brown) within the 18 bp (boxed) shown to be sufficient to drive transcriptional activation in S2 cells (7). The *to* 80-bp fragment shown here was used for the subsequent studies. The W box (in blue) is within the *per* 69-bp fragment (shaded in yellow) shown to be sufficient to drive transcriptional cycling in flies (12). This element, although conserved in *per* and *to*, was found not to be necessary for temporal regulation of these genes (data not shown). The *per* E box is at −528 from the transcriptional start; *tim* is at −678, and *to* is at −1143. (B) Yeast one-hybrid assays showing the binding of CLK and CYC to *per* and *to* upstream sequences. Shown are yeast patches expressing the indicated pairs of proteins (rows) and transformed with the indicated reporter constructs (columns). p65 (synaptotagmin) is a negative control (11). DNA binding results in the activation of the *lacZ* reporter gene, which in turn results in the blue coloration. Both CLK and CYC are required to bind the wild-type 21-bp E-box-containing sequence from *per* and *to*. They do not bind E-box-mutated (E^{mut}) sequences (transversion mutations at the two middle base pairs of the core E-box, CAGCTG).

a major difference between the *per* and *to* E-box regions, which explains the different biological activities in flies.

The E box is not necessary for *to* transcription in S2 cells. To provide yet another test of the *to* E box, we transformed S2 cells with a luciferase reporter gene driven by the 3.0-kb *to* promoter (*to-luc*). Luminescence was measured in the presence or absence of cotransfected CLK. There was a 3-fold induction of expression of *to-luc* by the *Clk* construct, much less than the 60- and 94-fold induction from the *per* and *tim* promoter fragments, respectively (Fig. 8A). Furthermore, an E-box deletion from the 3.0-kb *to* promoter did not diminish the level of transcriptional activation, unlike the E-box deletions from the *per* and *tim* promoters (data not shown). The results indicate that the *to* E box may not contribute to clock-regulated *to* transcription, suggesting that *to* transcription requires factors other than CLK and CYC.

The E box is not necessary for *to* transcription in vivo. To test the in vivo role of the E box in *to* transcriptional cycling, transgenic flies carrying *to-luc* were compared with a 21-bp deletion version that removes the E box (*toΔE-luc*) [Fig. 1B]. Consistent with the S2 cell data (Fig. 8A), *toΔE-luc* flies also showed cycling of luminescence with amplitude and phase comparable to that of *to-luc* (Fig. 8B). This indicates that the E box is not required and that additional elements outside this 21-bp region are sufficient for cycling expression.

DISCUSSION

The amino acid sequence of the TO protein indicates that *to* is a member of a novel gene family, found only in insects. Although the search shown here failed to reveal any family members with a known biochemical function, a less restricted search identified more distant insect relatives (36). These include two hydrophobic ligand-binding proteins: hemolymph juvenile hormone-binding protein (45) and JP29 (49) from moths. The two ligand-binding proteins share homology throughout the complete sequence with the TO family (36). However, they lack the two motifs that define the family (Fig. 3B), implying that they form a superfamily with TO. The shared biochemical function of the superfamily is presumably to bind a hydrophobic ligand. However, the different family members may bind different ligands.

The *to* ligand is unknown, but our recent data suggest that it may contribute to feeding-related functions. This is based on the *to* expression pattern in a few relevant body tissues. A *to* mutant strain also shows an unusual behavioral response and dies rapidly when subjected to starvation conditions. Finally, *to* mRNA levels increase in response to starvation, indicating that *to* expression is regulated by food availability as well as by the circadian pacemaker (36).

The only other family member with some relationship to circadian rhythms is the *0.9kb* gene, initially identified as a gene adjacent to the *per* locus (3, 31). *0.9kb* mRNA levels rise shortly before eclosion and decrease within a few hours after eclosion (26). As flies eclose under circadian clock control, the transcript is indirectly under circadian regulation but with only a single burst of expression at the beginning of the adult stage (26). This prior relationship with circadian rhythms therefore may be fortuitous. Preliminary assays on two other *Drosophila* EST clones in the *to* family also showed no obvious daily cycling expression by Northern blot analysis (data not shown). Therefore, the *to* family members may be regulated differently as well as bind different ligands and contribute to different physiological processes.

to cycling is due in large part to transcriptional regulation, as previously described for *per* and *tim*. However, the cycling apparently does not require a prominent *cis*-acting E box, unlike the transcription of *per* and *tim*. It is surprising that the *to* 80-bp and *per* 69-bp E-box-containing sequences, which have such striking nucleotide identities, are recognized so differ-

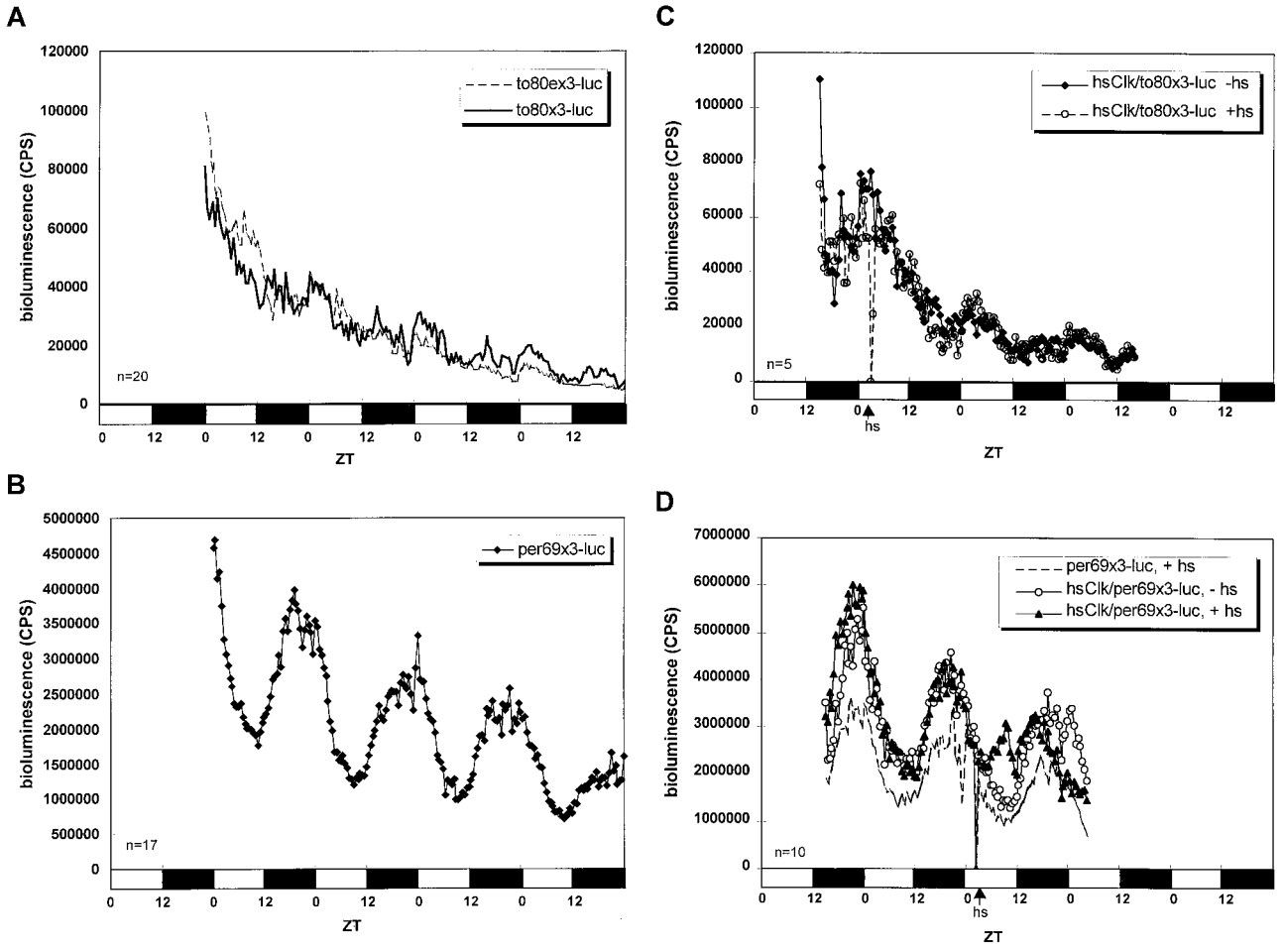

FIG. 6. The 80-bp E-box-containing sequence in the *to* promoter is not sufficient for robust transcriptional cycling. (A) Comparison of the wild-type *to80x3-luc* flies with the mutated *to80ex3-luc* flies. Both showed a very weak cycling of luminescence. Representative lines and experiment are shown. Flies are heterozygous for the transgene. (B) The *per69x3-luc* flies showed an impressive rhythmicity of bioluminescence with strong intensity and robust cycling amplitude. (C) Overexpression of CLK in *hsClk*/*to80x3-luc* flies did not show an observable change in bioluminescence. hs, heat shock. (D) Overexpression of CLK in *hsClk*/*per69x3-luc* flies phase advanced the luciferase reporter gene expression. Heat shock of the *per69x3-luc* flies alone without the *hsClk* transgene is also shown as a control. Arrows indicate the time of a 30-min 37°C heat shock. Average bioluminescence of representative lines and experiments is shown. Numbers of flies used for the analyses are shown at the bottom left; open bars indicate lights on, and filled bars indicate lights off.

ently: the *per* 69-bp sequence is sufficient to drive robust transcriptional cycling, whereas the *to* counterpart is not (Fig. 5A). The *to* 80-bp fragment may be missing a separate sequence element required for CLK-CYC binding and activity, it may contain adjacent sequences that are inhibitory to strong binding and activity, or it may be missing an elusive E-box feature. The last possibility is consistent with indications that the core E-box sequence is necessary but not sufficient for potent binding of the mammalian CLK-containing heterodimer (15). The absence of a separate sequence element is consistent with experiments indicating that a single *per* 18-bp E-box sequence is not sufficient for cycling reporter gene expression in flies (P. Hardin, personal communication). We cannot exclude the possibility that the *to* 80-bp region contributes more directly to spatial or developmental regulation, as the *per* 69-bp region alone has shown to be sufficient to mediate proper developmental and spatial expression (13). But the weak CLK-CYC binding and activity suggests that the *to* E box is not a bona fide, relevant sequence important for *to* regulation. Moreover, *to*Δ*E-luc* cycling is identical to *to* cycling.

There is the complication that a very modest cycling amplitude from the *to* 80-bp fragment is observed. This might reflect a cryptic, cycling (non-E-box) element within this 80 bp. The poor activity from the *per* 18-bp fragment may also reflect the lack of this element. Although there are some additional se-

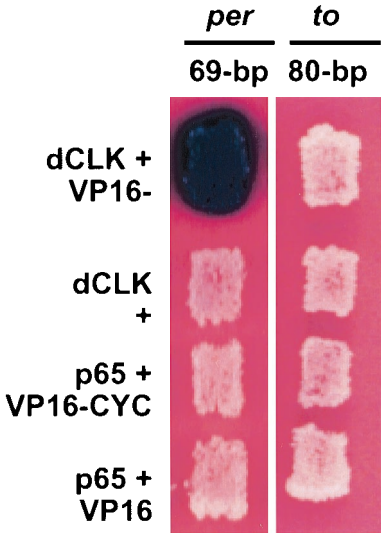

FIG. 7. Yeast one-hybrid assays showing that CLK and CYC bind a longer upstream sequence from the *per* promoter (69 bp) but not from the *to* promoter. Shown are yeast patches expressing the indicated pairs of proteins (rows) and transformed with the indicated reporter constructs (columns). DNA binding results in the activation of the *lacZ* reporter gene, which in turn results in the blue coloration.

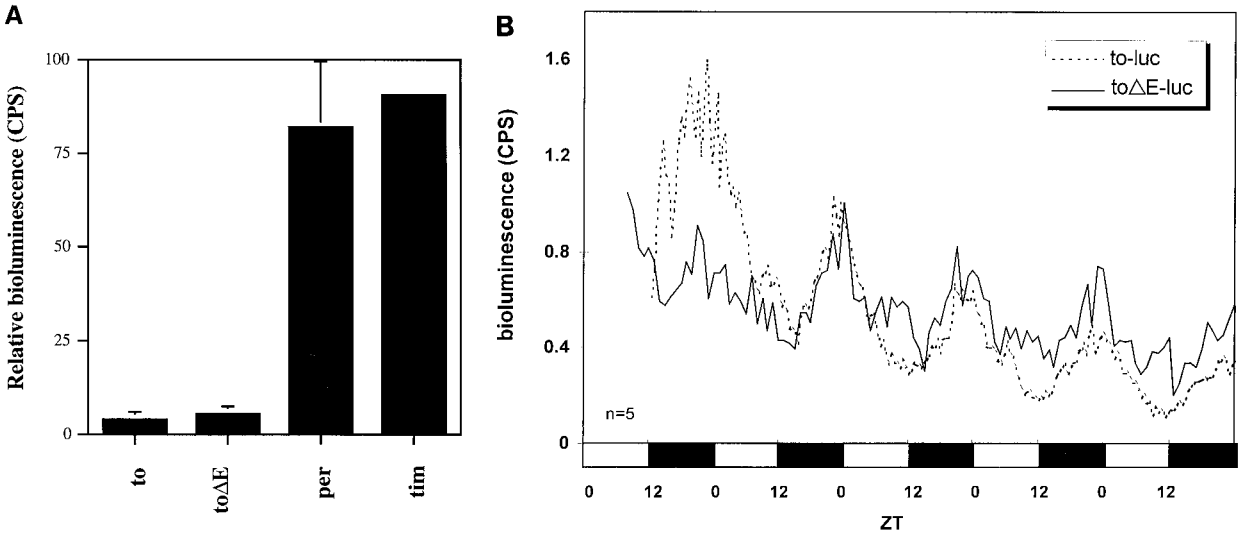

FIG. 8. (A) Bioluminescence assays were performed on several promoter-luciferase fusion constructs in S2 cells. Cells were cotransformed with the 3-kb *to* promoter containing a wild-type or mutant E box and CLOCK-V5 to investigate CLK-CYC-dependent activation of the promoter. Full-length *per* and *tim* promoter-luciferase fusions are included as positive controls to demonstrate CLK-CYC-dependent activation on promoters of known circadian genes. The second peaks of the two sets of bioluminescent intensities are set to 1 for easier comparison, because the absolute intensities were slightly different. (B) Comparison of the temporal luminescence from the *to-luc* and *to*Δ*E-luc* flies in LD. The 21-bp deletion that removes the E box (*to*Δ*E-luc*) does not have an obvious effect on the cycling of bioluminescence. Average bioluminescence of representative lines is shown. The second peaks of the two sets of bioluminescence intensities are set to 1 for easier comparison, because the absolute intensities were slightly different. Flies are heterozygous for the transgene. The number of flies used for the analysis is shown at the bottom left; open bars indicate lights on, and filled bars indicate lights off.

quence similarities between the *to* 80-bp and *per* 69-bp fragments, we have been unable to define any contribution from non-E-box elements to the circadian transcriptional oscillations of *to* (data not shown). This points to a simple conclusion: the weak cycling amplitude from the *to* 80-bp element is irrelevant, making little or no contribution to circadian transcription. This hypothesis implies that the key *cis*-acting circadian elements lie elsewhere within the 3.0-kb promoter fragment, a conclusion consistent with our experiments (Fig. 8B). These elements may even include another, more relevant E box. However, a sequence comparison of the entire 3.0-kb *to* promoter (outside the 80 bp) with the *per* 69 bp also does not show any striking identities, including the lack of any additional E-box elements within the entire *to* genomic region (data not shown). These considerations suggest that *to* transcription is regulated by sequence elements different from those that govern *per* and *tim* transcription and only indirectly by CLK and CYC.

In the *Drosophila* system, the most dramatic phase differences are between the RNA profiles of *Clk* mRNA and *cryptochrome* mRNA on the one hand and those of *per* and *tim* on the other (2a, 43). Although it is not certain how this antiphase regulation takes place, it has been suggested that there is significant similarity with the canonical clock gene model but that PER and TIM might be positive regulators of *Clk* mRNA cycling (23). As they are negative regulators of their own transcription, this would explain the antiphase relationship. The much more modest phase delay of *to* transcription has not been previously reported for any clock gene or clock output gene. Our negative E-box results suggest a different explanation for the few-hour phase difference between *per* transcription and *to* transcription than for the antiphase genes, namely, that CLK-CYC regulation is entirely indirect and that there is another transcription factor or perhaps even more complicated regulatory features interposed between CLK and CYC on the one hand and the *to* promoter on the other.

Most clock-related RNA cycling previously reported exhibits a phase similar to that of the canonical *per* and *tim* patterns (33, 46). This includes output genes that appear directly hardwired to the clock machinery, i.e., that are directly regulated by the CLK-CYC heterodimer or its mammalian equivalent, indistinguishably from the two canonical clock genes. In mammals, these include vasopressin and the transcription factor gene *dbp*, which then regulates a secondary set of output genes (21, 25, 31b). In flies, the transcription factor gene *Vrille* is regulated by CLK-CYC and probably regulates important downstream output genes (4). The *Vrille*-encoded protein or another putative transcription factor (e.g., see reference 33) could regulate *to* transcription. The time needed to accumulate active transcription factor would account for the lag between clock gene transcription and *to* transcription. The identification of the key *cis*-acting regions responsible for *to* transcription should help support this model and identify this putative clock-regulated transcription factor.

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