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Identification of Circadian-Clock-Regulated Enhancers and Genes of *Drosophila melanogaster* by Transposon Mobilization and Luciferase Reporting of Cyclical Gene Expression

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ABSTRACT

A new way was developed to isolate rhythmically expressed genes in Drosophila by modifying the classic enhancer-trap method. We constructed a *P* element containing sequences that encode firefly luciferase as a reporter for oscillating gene expression in live flies. After generation of 1176 autosomal insertion lines, bioluminescence screening revealed rhythmic reporter-gene activity in 6% of these strains. Rhythmically fluctuating reporter levels were shown to be altered by clock mutations in genes that specify various circadian transcription factors or repressors. Intriguingly, rhythmic luminescence in certain lines was affected by only a subset of the pacemaker mutations. By isolating genes near 13 of the transposon insertions and determining their temporal mRNA expression pattern, we found that four of the loci adjacent to the trapped enhancers are rhythmically expressed. Therefore, this approach is suitable for identifying genetic loci regulated by the circadian clock. One transposon insert caused a mutation in the rhythmically expressed gene *numb*. This novel *numb* allele, as well as previously described ones, was shown to affect the fly's rhythm of locomotor activity. In addition to its known role in cell fate determination, this gene and the phosphotyrosine-binding protein it encodes are likely to function in the circadian system.

RHYTHMIC gene expression is a crucial feature of all circadian clocks described so far at the molecular level (reviewed by Young and Kay 2001). In Drosophila the rudiments of clock functioning are as follows (Williams and Sehgal 2001), with emphasis on components that are conceptually and experimentally connected to the current study: Four factors cooperate to generate sustained molecular oscillations with a period of ∼24 hr. Two of these clock genes, *period* (*per*) and *timeless* (*tim*), begin to be transcribed in the late morning; their protein products PERIOD (PER) and TIMELESS (TIM) reach their maximum levels in the late night. During the night both proteins enter the nucleus, where they interact with their transcriptional activators CLOCK (CLK) and CYCLE (CYC). CLK and CYC bind as a dimer to E-box sequences present in the 5′-flanking regions of both *per* and *tim*. The interaction of PER and TIM with the CLK/CYC dimer leads to a repression of its activating function and results in decreasing levels of *per* and *tim* expression. Consequently, PER and TIM levels decline due to degradation of both proteins, ultimately resulting in a release of CLK and CYC inhibition. Such proteolysis, which must be associated with rather short half-lives of the proteins if they are to exhibit abundance fluctuations, is influenced by post-translational modifications affected by the DOUBLE-TIME (DBT) and SHAGGY kinases; among the substrates of these two enzymes are PER and TIM, respectively (Kloss *et al.* 1998; Martinek *et al.* 2001).

Not all clock components exhibit daily fluctuations in their abundance: Products of the *cyc* and *dbt* genes are temporally flat (*e.g.*, Kloss *et al.* 1998; Rutila *et al.* 1998b). But the existence of pacemaking transcriptional regulators that do cycle suggests that they could control—in addition to their own rhythmic expression—oscillations of downstream factors functioning on circadian output pathways. This has already been demonstrated for cyanobacteria, fungi, and mammals (reviewed by Johnson and Golden 1999; Loros and Dunlap 2001; Reppert and Weaver 2001). A few clock-output candidates in Drosophila have been identified in molecular screens for rhythmically expressed genes [*e.g.*, *Drosophila rhythmically expressed gene 5* (*Dreg-5*; Van Gelder and Krasnow 1996) and *Circadianly regulated gene-1* (*Crg-1*; Rouyer *et al.* 1997)]. However, mutant forms of these clock-controlled genes (*ccg*s) are few and far between (although see the *takeout* case of Sarov-Blat *et al.* 2000). Certain output variants that correspond to genes whose mRNAs do not happen to cycle

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are known—via phenotypic screening (Newby and Jackson 1993) or retrospective mutant identification (McNeil *et al.* 1998; Park and Hall 1998; Renn *et al.* 1999; Park *et al.* 2000). There is a flipside of this coin: Would molecular detection of a rhythmically expressed gene necessarily define a *ccg* or, instead, a factor that operates at least in part as a component of the clockworks? The *vrille* (*vri*) gene in Drosophila, whose chronobiological significance was initially pointed to by the fruits of a screen for cycling RNAs, may indeed play the dual roles just indicated (Blau and Young 1999).

Identification of an additional rhythm-related gene in Drosophila, *cryptochrome* (*cry*), showed how novel circadian factors can be discovered by combining mutagenesis with molecularly based phenotyping. Thus, a *cry* mutant was recovered in a screen that involved luminescence rhythms emanating from a *per-luciferase* (*per-luc*) transgene (Stanewsky *et al.* 1998). The short half-life of this reporter enzyme (Plautz *et al.* 1997; Stanewsky *et al.* 1997) facilitated the application of *per-luc* for this and other chronobiological purposes. Analysis of the cryptochrome mutant, detected in a strain that exhibited no *per-luc* cycling, revealed that the gene is involved in daily resetting of the circadian clock mediated by natural cycles of light and darkness (reviewed by Hall 2000). *cry* turned out to be yet another gene for which abundances of the encoded mRNA and protein are subjected to circadian regulation (Emery *et al.* 1998; Egan *et al.* 1999; Ishikawa *et al.* 1999).

The rapid throughput permitted by real-time reporting suggested a further application of this technology, in which new genetic variants would themselves mediate molecular cycling. Thus, we adapted the enhancer-trap technique to identify rhythm-related factors by transposon-induced variants that would elicit daily oscillations of luciferase activity. Enhancer trapping has a long successful history in identifying new genes and their functions in Drosophila (reviewed by Bellen 1999). This method is based on the genetic mobilization of transposable elements that have the tendency to insert in the 5′ regulatory region of genes. The enhancers therein can positively influence reporter-gene activity from the minimal promoter present in most detector constructs, which often reflects at least aspects of the genes' spatial expression pattern (Bellen 1999). Genes in the vicinity of the transposon insertion can easily be cloned by plasmid rescue, inverse PCR, or both (Wilson *et al.* 1989). Even though a small proportion of *P*-element insertions cause overtly defective phenotypes (only 10% lead to visible abnormalities or lethality; Bellen 1999), some of them could induce rhythm-related behavioral defects: Rest-activity cycles (*e.g.*, Renn *et al.* 1999) or periodic eclosion (*e.g.*, Newby and Jackson 1993) could be anomalous. Moreover, in many cases more severely mutated forms of the "trapped" genes can be induced by imprecise excision of the transposon in the presence of a stable source of transposase (Robertson *et al.* 1988).

Against this background, we designed an enhancer-trap vector containing a luciferase-encoding sequence. This reporter was designed to be under the control of a weak constitutive promoter (Figure 1A), but genetic mobilization of the *luc*-containing transposon should allow detection of enhancers that normally control rhythmically expressed genes (Figure 1B). An approach similar to the temporal enhancer-trapping tactic we now report for Drosophila revealed that essentially all genes of the cyanobacterium Synechococcus are expressed rhythmically (Liu *et al.* 1995). Here we show that luminescence-based enhancer trapping is also an efficient method to detect circadianly regulated enhancers in a higher organism. The genes connected to these elements are candidates for factors that encode cyclically expressed input functions (such as CRY), pacemaker molecules (such as PER, TIM, CLK, and VRI), or output functions (such as DREG-5, CRG-1, TAKEOUT, and VRI). Uncovering novel factors in the latter category may be especially important, because little is known about the manner by which core molecular oscillators are linked to overt rhythmicity. In fact, there is a paucity of information about rhythmic *biological* processes in Drosophila. Apart from the aforementioned behavioral and eclosion cycles, the only other circadian rhythm known in Drosophila is one that involves cyclical sensitivity of the olfactory system in the fly's antennae (Krishnan *et al.* 1999, 2001). We propose that a genetically oriented search for fluctuating output functions will simultaneously facilitate two investigatory processes, as the following questions are asked: What rhythmic biological phenomenon is suggested by a given enhancer-trapped strain by virtue of the product encoded at the locus and its spatial expression pattern? Will the periodic phenotype that is putatively predicted be abnormal under the influence of the inserted transposon or variants derived from it?

MATERIALS AND METHODS

Generation of the *luc-sniffer* construct and *P*-element transformation: The firefly *luciferase* (*luc*) cDNA isolated from *Photinus pyralis* was cloned from pJD261 (Luehrsen *et al.* 1992) into the *Sal*I/*Kpn*I sites of pBluescriptII SK (Stratagene, La Jolla, CA) from which parts of the polylinker (between and including the *Hin*dIII and *Xba*I sites) had been removed. The 1.8-kb *luc* fragment was then cloned into the *Not*I and *Kpn*I sites of the *P*-element vector pEG117 carrying the *mini-white*$^+$ gene as visible marker (Giniger *et al.* 1993). In the final *luc-sniffer* construct luciferase expression is under the control of the weak constitutive transposase promoter (Figure 1A). Transformations of *y Df(1)w* embryos (hereafter referred to as *y w*) were performed using standard techniques (*e.g.*, Rubin and Spradling 1986). Newly created transgenic flies were recognized by w^+-mediated eye color. Transposase was supplied by coinjection of the helper plasmid pUChsΔ2-3 (Laski

et al. 1986). Two independent transformant lines were isolated, both located on chromosome *2*.

Mutagenesis: To obtain an *X*-chromosomal jump-start line suitable for generating new autosomal insertion lines, one of the two original insertions on chromosome *2* was mobilized by crossing it to a transposase-producing Δ2-3 strain (Robertson *et al.* 1988). Thirty-five *X*-chromosomal insertion lines were recovered, from 4 of which we determined the frequency of transposition after crossing females of these strains again to Δ2-3. Next, 20–50 individual crosses were set up, in each of which one male carrying both the *luc-sniffer* and Δ2-3-encoded transposase was mated to *y w* females. The transposition frequency was determined by scoring the progeny for orange or red-eyed (*mini-w*$^+$) males, which are produced only if a transposition from the *X* chromosome to an autosome occurred. One line (X-90) with a frequency of 93% (meaning that almost every single cross resulted in a new insertion line) was used as starter line for the mutagenesis.

The mutagenesis scheme is shown in Figure 1 of the supplementary material at http://www.genetics.org/supplemental. Males homozygous for a third chromosome carrying the transposase gene (Δ2-3) and the additional dominant homozygous-viable marker *Kinked* (*Ki*; Lindsley and Zimm 1992) were crossed to homozygous X-90 females. Single F_1 males from the progeny carrying the *luc-sniffer* and one copy of the transposase-encoding chromosome were crossed to virgins heterozygous for a dominantly marked (*Bl*) second chromosome and the *In(2LR)O, Cy* (*CyO*) balancer. In the F_2, crosses were screened for the presence of *mini-w*$^+$ males, indicating that a transposition event from the *X* chromosome to one of the autosomes had occurred. Such individual males were again crossed to *Bl/CyO* virgins to generate stable stocks and to determine the chromosome of insertion. In the F_3, four males of each line (heterozygous for the *luc-sniffer*) were screened for rhythmic expression of luciferase (see below). In case the insertion was not on chromosome *2*, rhythmically expressing lines were balanced by crossing transgenic males to females carrying third-chromosomal marker-bearing and balancer chromosomes [*H* and *In(3LR)TM3, Sb*, respectively].

Isolation of a novel *tim* loss-of-function allele, *tim*^{03}, and generation of a recombinant chromosome carrying this clock mutation and a novel enhancer-trapping transposon: A novel *timeless* loss-of-function allele was isolated after ethyl methanesulfonate mutagenesis of a strain expressing a *period-luciferase* transgene (see Stanewsky *et al.* 1998 for details of the screening procedure). The homozygous mutant strain exhibited arrhythmic luciferase expression and locomotor activity. Complementation analysis with flies carrying the *tim*^{01} mutation (Sehgal *et al.* 1994) indicated that the *tim* gene is mutated in the novel strain. Behavioral arrhythmicity of this mutant could be rescued by introducing a *tim* rescue transgene (*cf.* Rutila *et al.* 1998a) into flies carrying *tim*^{01} over the newly isolated *tim* allele: Of 16 *trans*-heterozygous males without the rescue transgene, all were arrhythmic, whereas 9 out of 10 male flies harboring one copy of the rescue transgene showed robust locomotor rhythms in dark:dark (DD) cycles (average period 23.4 hr ± 0.1). This demonstrates that a novel *tim* allele was isolated. Moreover, Western blot analysis revealed that no TIMELESS protein is produced in this mutant. Sequencing the region mutated in the *tim*^{01} allele (Myers *et al.* 1995) showed that in the novel *tim* allele this region is not affected. Hence, the isolated mutation represents a novel loss-of-function mutation in the *tim* gene and was therefore dubbed *tim*^{03} [*tim*^{01} is an intragenic mutation; the so-called *tim*^{02} allele is a deletion of the entire locus along with neighboring second-chromosomal genes (Myers *et al.* 1995)]. To obtain recombinants between *tim*^{03} (cytological map position 23F3-5) and the *luc-sniffer* insertion in one of our enhancer-trap lines (1-17), we crossed *tim*^{03} males to 1-17/*CyO* virgins that were homozygous *white*. Since the *tim*^{03} chromosome was marked with *black* (*b*; cytological map position 34B) and the transposon of line 1-17 mapped to 57A on chromosome *2*, we selected for recombinants carrying the *mini-w*$^+$ gene and *b*. This was possible only after backcrossing the recombinant chromosomes to the *tim*^{03} *b* strain, since homozygous 1-17 flies are lethal due to the insertion of the *luc-sniffer* into a vital gene (see results). Individuals from recombinant *b* 1-17/*CyO* strains were then crossed to *tim*^{01} and male progeny were tested for the presence of locomotor activity rhythms to assure that they carry the *tim*^{03} mutation (indicated by arrhythmic behavior of the *tim*^{03}/*tim*^{01} mutant flies); note that 1-17/+ flies exhibit normal locomotor rhythms (Table 5).

Generation of revertants of enhancer-trap lines 1-17 and 90-3: All flies used for the generation of revertants carried the *y w* markers on their *X* chromosomes (see above). For the homozygous lethal line 1-17, the transposon was mobilized by crossing 1-17/*CyO* males to *CyO*/+; *Ki* Δ2-3/+ virgins. F_1 1-17/*CyO*; *Ki* Δ2-3/+ males with pigmented eyes were individually mated with *Bl/CyO* virgins, and the F_2 was screened for white-eyed 1-17/*Bl* jump-out males. These males were crossed back to *Bl/CyO* females to generate 1-17/*CyO* males and females, which were then crossed together to check for homozygous viability. For line 90-3, homozygous males were mated with *CyO*/+; *Ki* Δ2-3 virgins. The next crosses were analogous to line 1-17. Because homozygosity for the *luc-sniffer* in line 90-3 does not cause a lethal phenotype, jump-out lines had to be screened molecularly for precise excisions. PCR using genomic DNA isolated from homozygous jump-out animals was performed. Primers were chosen from both sides of the transposon such that a 500-bp PCR product resulted in the *y w* control line; no product could be amplified in the original enhancer-trap line 90-3 due to the *luc-sniffer* insertion. Putative revertants were isolated on the basis of the assumption that a perfect revertant should again give a PCR product of 500 bp. Analysis of the PCR products from two putative revertants (90-3^{rev1} and 90-3^{rev2}) showed that their sequences were identical to the relevant autosomal ones in the *y w* strain.

Plasmid rescue: Genomic sequences flanking the sites of the *luc-sniffer* insertions were isolated by plasmid rescue (*cf.* Wilson *et al.* 1989), using the restriction enzymes *Eco*RI or *Sac*II (for 3′ sequences) and *Bam*HI, *Bgl*II, or *Pst*I (for 5′ sequences). After digestion and ligation DNA was transformed into *Escherichia coli* XL1-Blue electrocompetent cells (Stratagene). For each rescue, plasmid-DNA from at least three colonies was isolated and subjected to restriction analyses. On the basis of the restriction pattern, representative clones were chosen for sequence analysis (see below). These data were used to perform BLAST and PSI BLAST searches (Altschul *et al.* 1990, 1997) against the nucleotide and protein sequence databases of the Berkeley *Drosophila* Genome Project and the National Center for Biotechnology Information, respectively (http://www.fruitfly.org/; http://www.ncbi.nlm.nih.gov/). Genomic location of the identified genes associated with the *luc-sniffer* insertions was determined by GadFly (Flybase 1999). To verify the transposon location, *in situ* hybridizations to polytene chromosomes were effected using linearized digoxygenin-labeled *luciferase* DNA as a probe (Blackman 1996). In addition, Southern blots with digested genomic DNA of *luc-sniffer* insertion lines were performed to confirm the restriction pattern of the rescue constructs and to check for multiple insertions. In two cases the results of *in situ* hybridizations and Southern blots indicated the presence of more than one transposon in the genome. These lines were excluded from further analysis.

Nucleotide sequencing: To determine the terminal sequences of plasmid-rescued genomic fragments the following

primers were used: For *Eco*RI, *Sac*II rescues 5′-AGTGGATGTC TCTTGCCGACG-3′ (proximal ends) and 5′-GTGCCACCTGA CGTCTAACGAAACC-3′ (distal ends); for *Bam*HI *Bgl*II, and *Pst*I rescues 5′-CCTCTCAACAAGCAACGTGCACT-3′ (proximal ends) and 5′-TATAGTCCTGCTGGGTTTCGCCACC-3′ (distal ends). Dye primer sequencing (Thermo Sequenase fluorescent-labeled primer cycle sequencing kit, Amersham Biosciences, Freiburg, Germany) was carried out on an ALFexpress DNA sequencer (Pharmacia Biotech).

RNA detection assays: Total RNA was prepared from 50 heads (males and females) or from 10 male bodies per time point as described in Zeng *et al.* (1994). RNase protection assays were performed as described in Emery *et al.* (1998) with the following modifications: A total of 5×10^5 cpm of the *rp49* probe and 1×10^6 cpm of all other probes were used in the hybridization reactions. Templates for the antisense RNA probe synthesis were generated by RT-PCR. The following primers were used to amplify the probes from total RNA extracted from *y w* flies: *CG13432*, 5′-TCCCACAAGGTGC ACTGTTC-3′ and 5′-TCCGATGACGTATGCGTATC-3′; *CG6 145*, 5′-CACTCACATTGGCGCACACATAGG-3′ and 5′-CATG TGCTTCTCCTGCACCAGCCA-3′; *numb*, 5′-GTCACACACG CACGAACCACTCGAGCG-3′ and 5′-CAACGTCTGCTGACG GAAGGATCCGGTC-3′; *twins/CT19500*, 5′-CGGAATTCCAA AGTGCCTGTGCCCAAGA-3′ and 5′-CGGGATCCTTGCTGG CTTCTTGGCTCCAT-3′; *twins/CT36963*, 5′-CGGAATTCGT AAAGTGCGAAATTTGCAAC-3′ and 5′-CGGGATCCACCGA ACAGTTTGCGTCGATT-3′. A different probe was used in Northern blot experiments for detection of both the *CT19500* and the *CT36963* transcripts; this labeled DNA fragment stemmed from amplified cDNA isolated from Canton-S wild-type flies using the primers 5′-GTCAGTGAGCGTGACAAGTC-3′ and 5′-GAATGAGGCGTGATCGTAGT-3′: *CG2207*, 5′-GTGA ATTCTTGGTGATCCAAAAACCCTCAG-3′ and 5′-CATCAAGC TTAGCCTTTTTCTCCGGCG-3′. To proceed with RNase protection assays, the PCR products were digested with *Hae*III (*CG13432*), *Bam*HI (*CG6145*), *Xho*I/*Bam*HI (*numb*), *Eco*RI/*Bam*HI (*twins*, both *CT19500* and *CT36963*), or *Eco*RI/*Hin*dIII (*CG2207*) and subcloned into pBluescriptII SK (Stratagene). The antisense probes were transcribed from these constructs in the presence of [^{32}P]UTP using the T3, T7, or SP6 RNA polymerases. Riboprobes protect the following regions of mRNAs: *CG13432*, 229 bases of transcript CT32789 (the last 68 bases of exon 3 and the first 161 bases of exon 4); *CG6145*; 245 bases of transcript CT19307 (the last 146 bases of exon 1 and the first 99 bases of exon 2) and in addition 99 bases of a second transcript generated from the same gene (corresponding to the first 99 bases of exon 2 of expressed sequence tag LP03268); *numb*, bases 825–1472 (648 nucleotides) of the transcript are referred to as "zygotic" and bases 476–1076 (601 nucleotides) of the transcript are referred to as "maternal" (numbers and denotations according to Uemura *et al.* 1989); *twins/CT19500*, 248 bases (complete exon 2); *twins/CT36963*, 189 bases (exon 2); *CG2207*: 568 bases of transcript *CT7302*. Primer sequences and other information regarding the generation of templates for riboprobe synthesis for the remaining genes listed in Table 4 are available on request. Gels were quantified with a Cyclone Storage Phosphor System phosphoimager (Packard, Meriden, CT) and OptiQuant analysis software.

Luciferase monitoring: Bioluminescence measurements of individual live flies were similar to those described in Stanewsky *et al.* (1997). Every other well of 96-well microtiter plates (Optiplate; Packard, Meriden, CT) was filled with 100 μl food consisting of 1% agar, 5% sucrose, and 15 mm Luciferin (Biosynth, Staad, Switzerland) dissolved in H_2O. After entrainment for at least 3 days to a 12 hr:12 hr (12:12) light:dark (LD) regime, individual flies were placed in the food-containing wells and covered with a small plastic dome to reduce locomotor movements (*cf.* Stanewsky *et al.* 1997). Luminescence emanating from each well was measured once per hour for 5–6 days in LD (same as the entrainment conditions) or for 3 days in LD followed by 2–3 days in constant darkness (DD). The resulting data were analyzed both by visual inspection of the plotted time series and by *f*ast *F*ourier *t*ransform-*non l*inear *l*east-*s*quares analysis (FFT-NLLS) (*cf.* Plautz *et al.* 1997). The outcomes of applying these functions are period and phase values, whereby the latter describes the peak time of luciferase expression during a 12:12 LD cycle. A metric called "relative amplitude error" (rel-amp) for each fly is obtained by dividing the 95% confidence interval of the amplitude estimate by the amplitude estimate (ratio of amplitude error to most probable amplitude). This value ranges from 0 to 1, where 0 indicates a rhythm with infinite precision (zero error) and 1 indicates a rhythm that is not statistically significant (error exceeds amplitude estimate). The rel-amp is used to determine the significance of a given rhythm: rel-amps <0.7 indicate that the bioluminescence rhythm is due to rhythmic gene expression with 95% confidence (see Stanewsky *et al.* 1997). Therefore all flies with rel-amps >0.7 were considered to be arrhythmic for reporter-gene expression.

Behavior: Locomotor activity of adult males was monitored automatically as described in Hamblen *et al.* (1986). The data were processed and analyzed as described in that report and in Hamblen-Coyle *et al.* (1992). Generally, flies were entrained for 1 day in 12:12 LD at 25° and then assayed for locomotor activity for the next 5 days in the same LD regime, followed by 7 days in constant darkness (DD). Activity periods in DD were analytically determined by χ^2 periodogram analysis ($\alpha = 0.05$). The program also indicates the strength of the behavioral rhythm (*cf.* Ewer *et al.* 1992) by computing "power" values (roughly the height of the periodogram peak) and the number of 0.5-hr bins crossing the significance line ("width"). Only flies showing periods in combination with powers ≥20 and width ≥2 were considered significantly rhythmic and had their period values listed in Table 5 (averages for all rhythmic flies from a given genotype).

RESULTS

Enhancer-trap mutagenesis: We generated a modified version of the *P-lacW* enhancer-trap construct (Bier *et al.* 1989) by replacing the transposase-*lacZ* fusion gene with sequences encoding firefly luciferase. In the final *luc-sniffer* construct, designed to sniff out regulatory elements related to temporal control of gene expression, luciferase is expressed under control of the weak constitutive *P*-transposase promoter (Figure 1; bottom of Figure 2). After germline transformation, two autosomal transgenic lines were recovered. One line was used to mobilize the *P* element genetically by crossing it to a fly strain that constitutively expresses transposase (see materials and methods). An *X*-chromosomal *luc-sniffer* insertion line (X-90) was isolated, found to exhibit temporally flat levels of luciferase, and used as a jumpstart line for mutagenesis (Figure 1 of the supplementary material at http://www.genetics.org/supplemental). This was performed by remobilization of the *luc-sniffer* element and subsequent screening for novel autosomal insertion lines (Figure 1 of supplementary material at http://www.genetics.org/supplemental). A

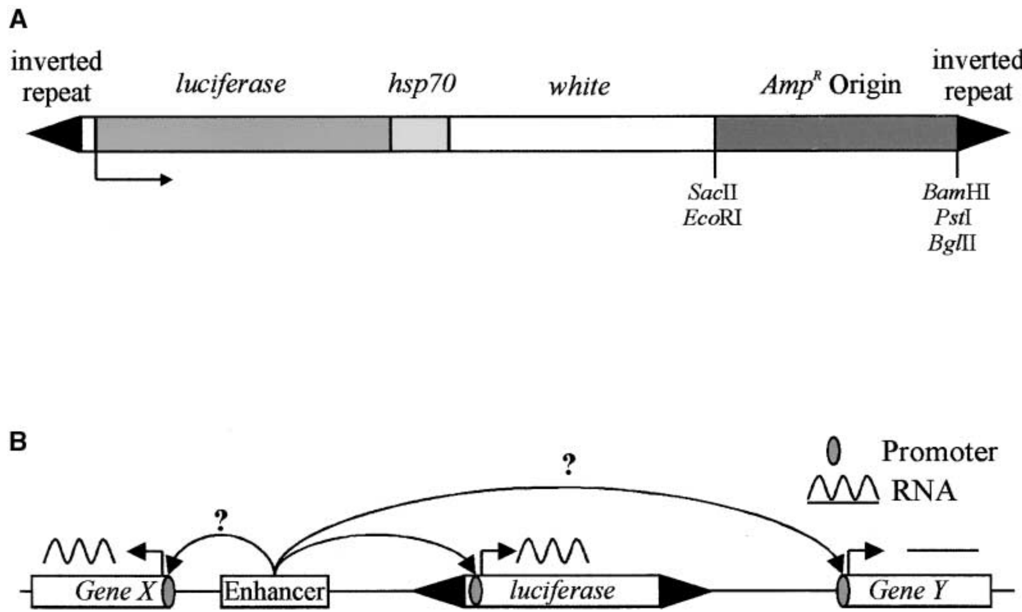


Figure 1.—(A) Schematic map of the *luc-sniffer* transposon. The firefly *luc* cDNA was cloned into the polylinker of a vector called the "universal-sniffer" pEG-117 (Giniger *et al.* 1993). The length of the transposable portion of *luc-sniffer* is 10 kb. *luc* is expressed under the control of the weak constitutive promoter, originating from the (natural) *P* element's transposase-encoding gene (arrow). The reporter gene is upstream of a *hsp70* polyadenylation signal and the intronless *mini-white*$^+$ gene (the latter serving as visible marker for recovery of transformed flies). The bacterial plasmid sequences are surrounded by the unique restriction enzymes indicated, which allow the recovery of genomic fly DNA sequences to both sides of the transposon (see materials and methods). (B) Schematic view of a hypothetical enhancer-trap insertion. The clock-controlled enhancer acts on the promoter in the *luc-sniffer*, resulting in rhythmic luciferase expression. In addition, it regulates rhythmic expression of a gene upstream (but not one downstream) of the insertion site. Given the uncertainty about which of the neighboring loci is controlled by the enhancers, both candidates can be analyzed for temporal RNA expression of the native mRNAs they encode (see text).

total of 1176 lines were generated, among which the *luc-sniffer* was then inserted in chromosome *2* ($n = 550$) or in chromosome *3* ($n = 626$). To determine whether expression of the reporter construct in a given autosomal location is under the control of an enhancer driving rhythmic gene expression, four males of each line were tested for rhythmic bioluminescence during 4–5 days in 12 hr light:12 hr dark (12:12 LD) conditions (Figure 2). The overall reduction of signal levels occurring during the course of such an experiment (Figures 2, 3, and 6) is due to substrate depletion, because the effect can be compensated for by supplying fresh luciferin after flies have been fed the initial substrate-containing food for several days (Plautz *et al.* 1997).

A total of 71 lines (6%) reproducibly showed bioluminescence rhythms with cycle durations in the circadian range, which was determined after performing a numerical analysis of the raw expression data (Table 1). These results indicate that, in many or all of the 71 lines, *luc* has come under the control of an enhancer that would naturally mediate rhythmic expression of an endogenous gene in the vicinity of such a *cis*-acting regulatory element (*cf.* Figure 1B). For 20 lines the exact positions of the transposons were determined (see below), revealing that 2 lines had the *luc-sniffer* inserted at almost identical chromosomal locations. The rhythmically expressing lines were then grouped according to the robustness of the observed oscillations. This was not done subjectively, but by a formal analysis whose outcome is the so-called rel-amp, serving as a measure for rhythm strength (see materials and methods). The 71 lines showing circadian bioluminescence rhythms with associated rel-amp errors <0.7 (*cf.* Stanewsky *et al.* 1997) were considered to reflect rhythmic *luc* gene expression. Flies showing rhythms with rel-amp errors <0.5 were designated class I lines (total of 20, Table 1), representing the lines with the most robust rhythms; those with values between 0.5 and 0.6 were grouped in class II ($n = 30$); and those with rel-amps between 0.6 and 0.7 were placed in class III ($n = 21$), the group with the weakest, although still significant, rhythms (Table 1). Averaged bioluminescence rhythms for one representative line of each class are shown in Figure 2, along with one example of the majority of lines (94%) whose expression did not display a significant rhythm (Figure 2, bottom).

Another output from the numerical analysis is the average peak time (here called the *phase*) of the oscillation for each line. Interestingly, the majority of the rhythmic insertion lines (93%) show their expression maxima between midnight and midday (ZT19 to ZT7, Table 1). In those lines peak expression occurs later compared with transgenic *plo* flies, in which *luc* is driven by the promoter region of the clock gene *per*; peak expression of *plo* occurs at *ca.* ZT18 (Stanewsky *et al.* 1997; Table 2; Figure 4A).

Clock control of enhancer-driven bioluminescence rhythms: To ask whether the observed oscillations are mainly driven by light or controlled at least in part by the circadian clock, we analyzed all class I lines in *per*^{01} and *per*^{T} genetic backgrounds. The former is a *per* loss-of-function mutation (*e.g.*, Yu *et al.* 1987) that eliminates

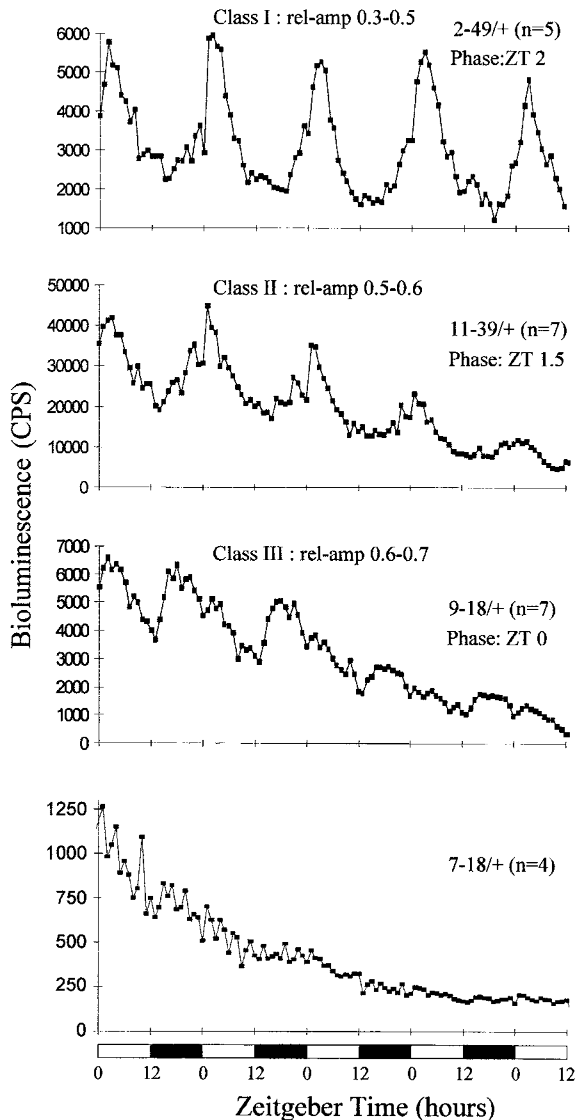


FIGURE 2.—Examples of enhancer-trap lines exhibiting significant bioluminescence rhythms or arrhythmic reporter-gene activity. The 71 lines showing rhythmic *luciferase* expression in a circadian pattern were grouped in classes I, II, or III according to the robustness of the observed rhythms. Rhythm strength (expressed as rel-amp) and peak phase [expressed in hours relative to the Zeitgeber time (ZT)] of expression were determined analytically as described in MATERIALS AND METHODS. ZT0 is conventionally the time of lights on in a 12:12 LD cycle and ZT12 is time of lights off. The bottom graph depicts an arrhythmically expressing line, typical for the majority of lines recovered in the screen. The solid and open bars below the plots indicate when the lights were off and on, respectively, during such cycles. CPS, counts per second.

behavioral rhythms in constant conditions (DD; KONOPKA and BENZER 1971); flies carrying the latter allele exhibit a dramatically shortened free-running period of 16 hr (KONOPKA *et al.* 1994). In addition, both mutations affect molecular rhythmicity in LD: per^{01} results in elimination of rhythmic *per* RNA expression (HARDIN *et al.* 1990), whereas per^{T} leads to an earlier-than-normal rise of PER protein compared with the daily upswing of the wild-type protein (HAMBLEN *et al.* 1998), probably caused by an anomalously early rise time for per^{T} RNA. If enhancer-driven *luc* rhythms are regulated by *per* (and therefore by part of the central clock mechanism), they should be influenced by the two *per* mutations in the same way that *per* expression itself is altered by these alleles.

Figure 3 shows examples of the mutations' effects on different class I lines as well as on *per*-promoter-driven *luc* in the *plo* transgenic type. Figure 4 and Table 2 give a summary of the results of all class I lines analyzed in different clock-mutant genetic backgrounds. In most lines (80%) the trapped enhancer seems to be tightly controlled by the clock, indicated by a clear phase advance of expression in a per^{T} genetic background compared with expression in a per^{+} background and by elimination of rhythmic expression by per^{01} (Figures 3B and 4; Table 2). In such lines the effects of the clock mutations are in fact similar or indistinguishable from those they exert on *per-luc* expression in the *plo* transgenic type (Figures 3A and 4; Table 2). In the remaining 20% of all class I lines expression seemed to be under weak clock control only: For two lines (90-14 and 1-45) rhythmic phases of expression were only mildly advanced by per^{T}, and rhythmic expression was not thoroughly eliminated by per^{01} (Figure 3D; Table 2). In the example shown (line 90-14), 35% of the flies remained rhythmic in per^{01}, compared with 91% rhythmic flies in a per^{+} genetic background (Figure 3D; Table 2). We also analyzed the effects of a different arrhythmia-inducing clock mutation, a loss-of-function allele of the *timeless* gene (tim^{01}; SEHGAL *et al.* 1994). Rhythmic expression was observed in only 8% of the tim^{01}; 90-14 individuals, indicating a potentially independent role of *per* and *tim* gene function in the regulation of this particular enhancer (Figure 3D; Table 2).

Two other lines (1 and 3-50) showed tight clock control based on the effects of per^{T} (*i.e.*, substantial phase advance of peak expression; Figure 3C; Table 2), whereas the high number of rhythmically expressing flies in per^{01} (37 *vs.* 65% in per^{+} for line 1; 47 *vs.* 89% for line 3-50) points to weaker influence of the clock (Figure 3C; Table 2). We have no explanation for this substantive discrepancy, but it is possible that the enhancers we classified as "weakly clock controlled" are additionally influenced by light. In a clockless per^{01} genetic background, a light-inducible enhancer would still be rhythmically active in LD cycles even if it is simultaneously regulated by the clock.

An independent way to demonstrate clock control of gene expression is to measure molecular rhythms in constant conditions (all dark, no temperature fluctuations), which rules out effects on gene expression caused by environmental fluctuations. A problem with this kind of analysis is that even the molecular rhythms of the *per* and *tim* clock gene products dampen rapidly

TABLE 1

Classification of enhancer-trap lines according to the strength of their bioluminescence rhythms

Lines (no.)	Chromosome location		Phase (ZT)				
	2	*3*	<19	19–21	22–24	1–3	>3
Class I (20)	6	14	0	6	6	8	0
Class II (30)	18	12	3 (ZT16, ZT18)	7	9	9	2 (ZT4, ZT7)
Class III (21)	10	11	2 (ZT16, ZT18)	3	6	9	1 (ZT4)
Total (71)	34	37	5	16	21	26	3

All enhancer-trap lines exhibiting significant bioluminescence oscillations were grouped in three different classes according to the strength of their rhythm. Such strengths were inferred from the average rel-amp (see MATERIALS AND METHODS) for each line after testing at least eight individuals from each strain for 5–6 days in LD conditions. Class I lines exhibit (by definition) the most robust rhythms (rel-amp ≤0.5), class II lines strong rhythms (rel-amp between 0.5 and 0.6), and class III lines weak but still significant rhythms (rel-amp between 0.6 and 0.7). Chromosomal locations of the novel transposon inserts were determined genetically after crossing the *luc-sniffer* insertion lines to flies carrying the relevant marker-bearing and balancer chromosomes (MATERIALS AND METHODS). ZT values refer to the calculated peak time of bioluminescence expression in a 12:12 LD cycle (ZT0 = lights on, ZT12 = lights off). ZT values given in parentheses in the "<19" and ">3" columns of class II and class III lines indicate the specific ZTs for these lines. "Phase" indicates the average peak level of expression for a given line, as determined by FFT-NLLS (see MATERIALS AND METHODS).

in DD (*e.g.*, STANEWSKY *et al.* 1997). This is probably caused by internal desynchronization of clock gene cyclings within a given individual animal. Nevertheless, DD analysis of *per*-promoter-driven *luciferase* expression in the *plo* transgenic line showed that 48% of the individuals display significant circadian bioluminescence rhythms (Figures 3A and 4B; Table 2; *cf.* STANEWSKY *et al.* 1997). Thus, we also analyzed expression of the class I insertion lines in DD. In most lines ($n = 10$) expression was rhythmic in 27–67% of individuals (Figure 4B; Table 2), comparable to what was observed for *plo* flies. Surprisingly, seven lines showed a higher percentage of rhythmic expression compared with *plo*. Between 80 and 97% of the individuals from those strains showed rhythmic *luc* expression in DD. Interestingly, line 90-14—which showed residual rhythmicity in a per^{01} genetic background (see above)—was most robustly rhythmic in DD (97% of all individuals tested; Figures 3D and 4B; Table 2), indicating that it is regulated both circadianly and by light (see above). The remaining three lines (3-70, 6-4, and III129) showed rhythmic expression in only 11–13% of all individuals tested. Since these three lines showed tight clock control of expression in the per^{01} and per^{T} genetic backgrounds, we assume that in these cases the rhythmic enhancers are active in tissues that are more sensitive to internal desynchronization (see above). Note also that in lines 3-70 and III129 the *luc-sniffer* construct is inserted in the same gene, only 51 bp apart from each other (Table 3; see below).

A subset of the enhancer traps was also tested in the per^{T} genetic background in DD. As already observed in LD, this mutation has a negative effect on rhythmic luciferase expression of all lines (except 7-32A and 90-14; Table 2). The free-running period associated with the luminescence oscillations of the few significantly rhythmic per^{T} individuals was between 16 and 19 hr (see legend to Table 2).

Identification of genes adjacent to the clock-regulated enhancers: Given that the great majority of the trapped class I enhancers is regulated by the circadian clock, we set out to isolate the genes in the vicinity of *luc-sniffer* insertions by plasmid rescue (WILSON *et al.* 1989). First, by *in situ* hybridization to polytene chromosomes we determined the location of each transposon in all class I lines (Table 3). This cytological mapping revealed that in each of the 20 lines a single insertion event had occurred. The *luc-sniffer* transposon was designed to allow recovery of genomic DNA sequences in the 3′ direction of the insertion, using the *Sac*II and *Eco*RI restriction enzymes; 5′-flanking material could be recovered after digestion of genomic DNA with *Bam*HI, *Pst*I, *or Bgl*II (Figure 1A). The rescued DNA fragments were partially sequenced using *P*-element-specific primers (see Figure 1 and MATERIALS AND METHODS), and these data were aligned to the whole Drosophila genome sequence (ADAMS *et al.* 2000). This information was then used to confirm the band location determined by *in situ* hybridization and to identify the nearest genes neighboring the transposon (Table 3). Except for one line (2-49) we were able to isolate genomic DNA sequences next to the *luc-sniffer*. In total, our insertions occurred near or in (i) 20 genes with a known function, *i.e.*, those connected

TABLE 2

Quantitative analysis of bioluminescence oscillations in class I enhancer-trap lines

A. Transgenic line	LD per^+ genetic background: No. of rhy/tested (% rhythmic)	Period ± SEM	Rel-amp error ± SEM	Phase ZT ± SEM	DD per^+ genetic background: No. of rhy/tested (% rhythmic)	Period ± SEM	Rel-amp error ± SEM
plo/plo	134/176 (76)	24.3 ± 0.1	0.48 ± 0.01	18.2 ± 0.2	32/67 (48)	23.6 ± 0.2	0.57 ± 0.02
1	37/57 (65)	24.0 ± 0.1	0.47 ± 0.02	19.4 ± 0.5	14/33 (42)	24.5 ± 0.3	0.56 ± 0.03
1-17/+	55/56 (98)	24.0 ± 0.1	0.33 ± 0.01	0.5 ± 0.3	41/48 (85)	24.8 ± 0.3	0.48 ± 0.02
1-45/1-45	25/29 (86)	24.3 ± 0.1	0.47 ± 0.02	1.1 ± 0.5	8/16 (50)	25.8 ± 0.6	0.50 ± 0.04
2-49	44/51 (86)	24.0 ± 0.1	0.41 ± 0.02	3.3 ± 0.4	13/36 (36)	24.9 ± 0.6	0.58 ± 0.02
3-50/+	17/19 (89)	24.5 ± 0.3	0.48 ± 0.03	20.9 ± 0.7	5/16 (31)	23.5 ± 0.6	0.56 ± 0.04
3-70/+	17/35 (49)	24.4 ± 0.2	0.49 ± 0.03	20.7 ± 0.9	3/27 (11)	23.2 ± 0.6	0.65 ± 0.03
3-80/+	40/45 (89)	24.3 ± 0.1	0.37 ± 0.02	0.5 ± 0.3	13/15 (87)	25.9 ± 0.3	0.50 ± 0.02
3-89/+	22/23 (96)	24.6 ± 0.2	0.41 ± 0.02	22.8 ± 0.5	4/8 (67)	24.7 ± 0.3	0.50 ± 0.06
4-16/+	11/24 (46)	24.3 ± 0.2	0.46 ± 0.05	21.6 ± 0.9	4/15 (27)	23.2 ± 1.1	0.50 ± 0.06
4-29	43/53 (81)	23.9 ± 0.1	0.43 ± 0.02	1.4 ± 0.4	15/37 (41)	26.6 ± 0.2	0.50 ± 0.03
5-1/+	14/27 (52)	24.1 ± 0.2	0.50 ± 0.03	21.8 ± 0.9	2/7 (29)	25.6 ± 2.6	0.52 ± 0.06
6-4	28/32 (88)	24.3 ± 0.1	0.44 ± 0.02	0.5 ± 0.6	1/8 (13)	25.3	0.34
6-48	15/19 (79)	24.0 ± 0.2	0.46 ± 0.03	2.3 ± 0.5	14/17 (82)	26.9 ± 0.4	0.44 ± 0.03
6-63/+	15/18 (83)	24.1 ± 0.2	0.41 ± 0.03	20.4 ± 0.8	6/12 (50)	22.4 ± 0.6	0.55 ± 0.04
7-32A/+	20/20 (100)	24.2 ± 0.1	0.37 ± 0.02	23.7 ± 0.4	12/14 (86)	24.5 ± 0.3	0.47 ± 0.03
8-35/+	36/39 (92)	23.8 ± 0.1	0.45 ± 0.02	22.0 ± 0.5	28/32 (88)	26.1 ± 0.2	0.48 ± 0.02
9-24/+	10/15 (67)	24.8 ± 0.3	0.51 ± 0.04	19.6 ± 0.7	5/12 (42)	25.1 ± 0.6	0.62 ± 0.03
90-3	41/47 (87)	24.3 ± 0.1	0.39 ± 0.02	23.6 ± 0.3	41/51 (80)	24.6 ± 0.2	0.47 ± 0.02
90-14	50/55 (91)	23.9 ± 0.1	0.30 ± 0.01	2.2 ± 0.2	35/36 (97)	23.5 ± 0.1	0.45 ± 0.02
III129/+	19/22 (86)	24.4 ± 0.2	0.40 ± 0.03	20.8 ± 0.6	2/15 (13)	25.3 ± 0.1	0.68 ± 0.01

B. Transgenic line	LD per^{01} genetic background: No. of rhy/tested (% rhythmic)	Period ± SEM	Rel-amp error ± SEM	Phase ZT ± SEM	LD per^T genetic background: No. of rhy/tested (% rhythmic)	Period ± SEM	Rel-amp error ± SEM	Phase ZT ± SEM
plo/plo	6/111 (5)	24.8 ± 0.1	0.58 ± 0.03	3.3 ± 0.5	24/50 (48)	24.4 ± 0.1	0.53 ± 0.02	11.5 ± 1.0
1	11/30 (37)	24.1 ± 0.2	0.53 ± 0.03	19.3 ± 0.8	13/39 (33)	24.0 ± 0.2	0.57 ± 0.03	11.1 ± 0.7
1-17/+	1/21 (5)	25.5	0.57	20.7	20/22 (91)	24.0 ± 0.1	0.35 ± 0.02	19.5 ± 0.5
1-45	4/10 (40)	24.5 ± 0.3	0.57 ± 0.06	2.7 ± 1.4	9/14 (64)	23.5 ± 0.2	0.51 ± 0.05	22.3 ± 1.2
2-49	4/47 (8)	24.2 ± 0.6	0.57 ± 0.03	11.1 ± 2.9	32/49 (65)	24.2 ± 0.1	0.37 ± 0.02	20.7 ± 0.4
3-50/+	7/15 (47)	23.7 ± 0.1	0.51 ± 0.03	20.4 ± 1.8	9/15 (60)	24.6 ± 0.2	0.51 ± 0.03	16.1 ± 0.6
3-70/+	0/17 (0)				3/24 (13)	24.3 ± 0.7	0.62 ± 0.08	17.5 ± 2.3
3-80/+	1/22 (5)	23.8	0.38	4.9	14/32 (44)	24.3 ± 0.2	0.45 ± 0.03	20.5 ± 0.7
3-89/+	0/16 (0)				10/21 (48)	24.3 ± 0.2	0.43 ± 0.03	18.7 ± 1.1
4-16/+	2/29 (7)	23.8 ± 0.2	0.68 ± 0.01	2.1 ± 3.5	4/24 (17)	23.1 ± 0.3	0.59 ± 0.03	18.4 ± 0.3
4-29	3/40 (8)	24.4 ± 0.2	0.55 ± 0.08	19.8 ± 1.5	28/36 (78)	24.2 ± 0.1	0.43 ± 0.02	19.5 ± 0.5
5-1/+	1/13 (8)	25.0	0.50	2.1	3/22 (14)	24.3 ± 0.4	0.41 ± 0.05	12.3 ± 1.2
6-4/+	2/22 (9)	23.6 ± 1.0	0.58 ± 0.00	2.2 ± 3.3	14/43 (33)	23.6 ± 0.2	0.56 ± 0.02	20.0 ± 1.0
6-48/+	1/16 (6)	23.7	0.68	6.2	17/27 (63)	23.7 ± 0.1	0.46 ± 0.03	19.1 ± 0.5
6-63/+	1/11 (9)	25.4	0.65	13.6	13/21 (62)	24.1 ± 0.2	0.42 ± 0.04	17.1 ± 0.7
7-32A/+	1/10 (10)	23.6	0.54	3.2	25/25 (100)	24.2 ± 0.1	0.33 ± 0.02	21.5 ± 0.2
8-35/+	0/18 (0)				24/30 (80)	23.8 ± 0.1	0.46 ± 0.02	16.8 ± 0.5
9-24/+	0/13 (0)				1/16 (6)	23.2	0.70	12.9
90-3	2/18 (11)	23.9 ± 0.4	0.51 ± 0.01	5.3 ± 0.2	7/13 (54)	24.8 ± 0.2	0.40 ± 0.03	15.1 ± 0.7
90-14/+	9/26 (35)	24.4 ± 0.3	0.52 ± 0.03	2.8 ± 0.9	23/24 (96)	23.9 ± 0.1	0.40 ± 0.02	2.22 ± 0.6
III129/+	1/14 (7)	24.6	0.38	15.4	14/27 (52)	24.2 ± 0.2	0.45 ± 0.04	18.8 ± 0.8

(*continued*)

with a given protein as a whole; (ii) 16 genes that encode proteins with previously apprehended domains or that share homologous sequences from genes with known functions isolated in various organisms; and (iii) 12 genes with unknown functions, not even with appreciable motifs within the overall deduced amino acid sequence (Adams *et al.* 2000; Table 3).

In no case had an insertion occurred in the close

TABLE 2

(Continued)

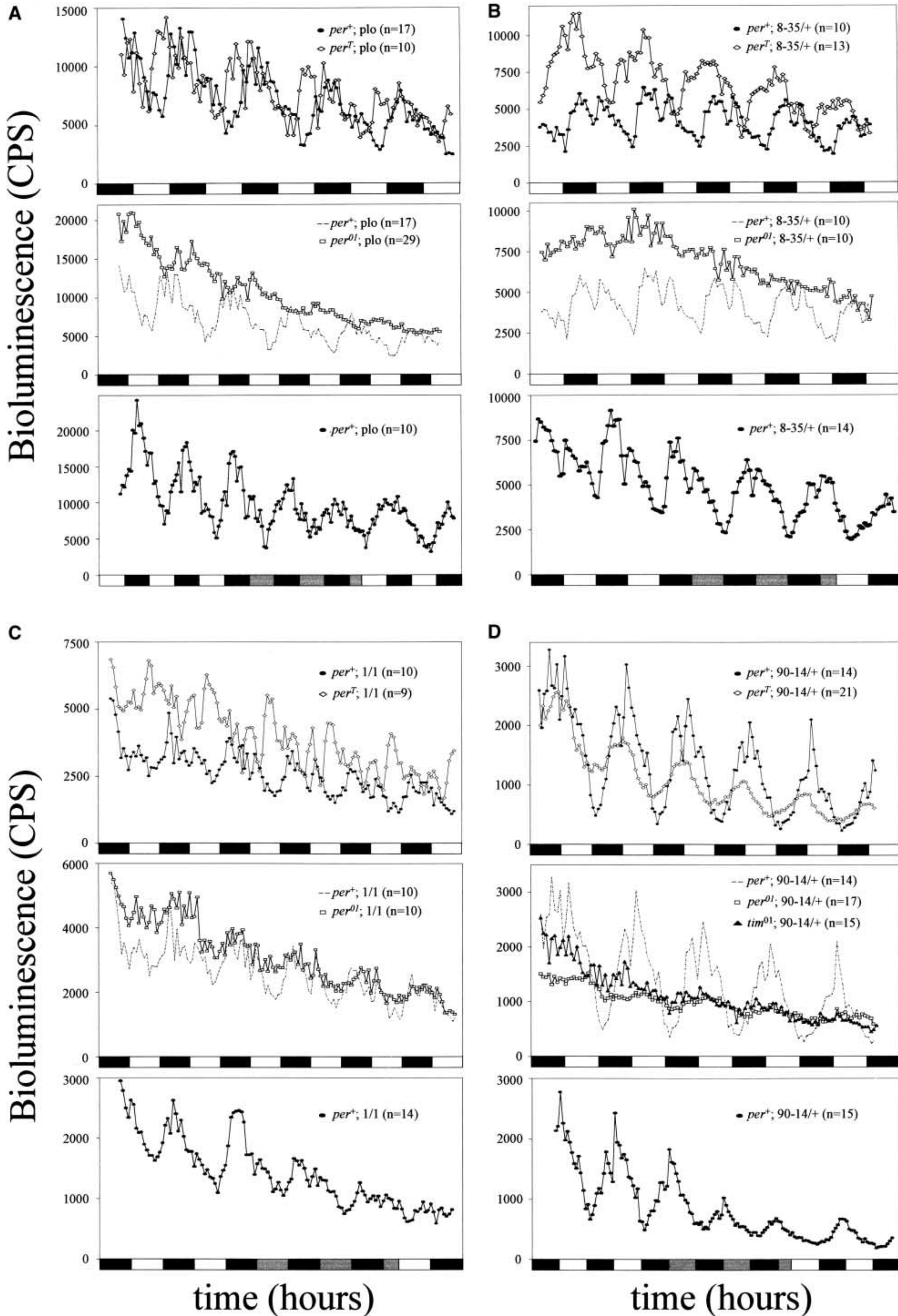
C. Transgenic line and genetic background	LD No. of rhy/tested (% rhythmic)	Period ± SEM	Rel-amp error ± SEM	Phase ZT ± SEM
tim^{01}; 90-14/+	3/37 (8)	23.0 ± 0.3	0.60 ± 0.03	5.2 ± 0.8
1-17/+; cyc^{01}	0/19 (0)			
1-17/+; Clk^{Jrk}	3/20 (15)	22.8 ± 0.2	0.70 ± 0.00	9.3 ± 0.8
tim^{03} 1-17/tim^{01}	1/29 (3)	23.2	0.55	23.2

Bioluminescence of each fly was measured for 5–6 days in the photic conditions indicated for the appropriate columns or table subsection (LD, 12:12 LD cycles; DD, constant darkness). These [counts per second (cps)] data were subjected to quantitative analysis to determine rhythmicity, period (cycle duration in hours), and phase (peak cps per day, with reference to ZT). Adult males heterozygous or homozygous for a given transposon had their luciferase activity so analyzed. "Tested" flies are all those analyzed for a given genotype that survived until the end of a given experiment. "Rhythmic" indicates the subset of flies that gave rel-amps (reflecting robustness of daily oscillations) ≤0.7 (see MATERIALS AND METHODS) and period values in the ranges of 24 ± 2.5 hr (LD) or 24 ± 5 hr (DD). Data listed in the "Period," "Rel-amp," and "Phase" columns are means (±SEM) resulting from all significantly rhythmic individuals from a given line. (A) LD and DD results from strongly rhythmic class I enhancer-trap lines and from the *plo* (*per-luc* fusion) transgenic in clock-normal genetic backgrounds (see examples in Figure 5). (B) LD results from class I lines and from *plo* in per^{01} and per^{T} genetic backgrounds (males hemizygous for either such *X*-chromosomal clock mutation). Several per^{T} flies were also tested in DD: 1-17 (5 flies out of 16 were rhythmic, average period length [τ] = 16.8 ± 0.9 hr); 2-49 (1/11, τ = 18.1); 3-70 (0/12); 3-80 (5/16, τ = 19.3 ± 0.3 hr); 4-29 (0/3); 8-35 (1/4, τ = 19.2 hr); 90-3 (1/19, τ = 16.5 hr); 90-14 (2/4, τ = 18.9 ± 1.2 hr). (C) LD results from lines 90-14 and 1-17, in a *tim*-null genetic background, and from line 1-17 in backgrounds that included homozygosity for the third-chromosomal cyc^{01} or Clk^{Jrk} clock mutations (*cf.* Figures 3, 4, and 6).

vicinity of a gene known to function in the circadian system of *Drosophila melanogaster* (Table 3). Also, we did not identify genes that encode a PAS domain, a protein-protein interaction motif found in several clock proteins (*e.g.*, those encoded by the *per*, *Clk*, and *cyc* genes in this species, as reviewed by Young and Kay 2001). Two insertions (3-89, 7-32A) occurred in the vicinity of novel genes containing sequences homologous to those encoding a *helix-loop-helix* domain. This protein dimerization domain is found within members of transcription factor families, including those crucial for clock function (*i.e.*, the CLK and CYC proteins). Other genes identified by our approach with a potential function in the circadian system include those encoding kinases, phosphatases, and proteases (Table 3). These are intriguing, because several clock proteins undergo daily changes in their phosphorylation pattern (*i.e.*, PER, TIM, and CLK), and such catalytic events are associated with the timed disappearance of these proteins (Young and Kay 2001).

Kinases involved in PER and TIM phosphorylation have been described: the casein-kinase Iε, encoded by the *dbt* gene (Kloss *et al.* 1998; Price *et al.* 1998), and a glycogen synthase kinase-3 (GSK-3), encoded by *shaggy* (Martinek *et al.* 2001). Additional findings related to DBT and its PER substrate suggest that the former is not the only enzyme that mediates phosphorylation of the latter protein (Suri *et al.* 2000); thus additional rhythm-related kinases await identification, as may have now occurred (Table 3).

A phosphatase with potential relevance for the circadian system is PP2A, whose regulatory subunit B is encoded by the *twins* (*tws*) gene (Uemura *et al.* 1993). *tws* functions in pattern formation during metamorphosis and is required for normal mitosis in neuroblasts of the larval central nervous system (*e.g.*, Mayer-Jaekel *et al.* 1993; Shiomi *et al.* 1994). In line 90-14 (see above) the rhythmically expressed *luc-sniffer* element is inserted within the *tws* locus (Table 3). A chronobiological role played by this gene in adults could be related to a known PP2A function in mammals, for which this enzyme has been shown to inactivate the transcription factor cAMP response element binding protein (CREB; Hunter 1995; Hafen 1998). CREB is usually activated by phosphorylation via Ca^{2+}- and cAMP-dependent signal transduction pathways (Hunter 1995). In a chronobiologically important mammalian brain structure, the hypothalamic suprachiasmatic nucleus, CREB activation is regulated by the circadian clock (Ginty *et al.* 1993; Ding *et al.* 1997). In Drosophila, a homolog of mammalian CREB, *dCREB2* is also involved in circadian rhythm function, in that it is required for normal temporal expression of *per* and *tim* (Belvin *et al.* 1999). Moreover, in the chick pineal gland, the RNA encoding the PP2A subunit that is homologous to the one encoded by *tws* is expressed rhythmically (J. Olcese, personal communication). There were no obvious additional candidates among the sequenced genes (pointed to by our enhancer trapping) known or suspected to play a role in the circadian system (Table 3).



A
B
C
D
Bioluminescence (CPS)
time (hours)
per+; plo (n=17)
perT; plo (n=10)
--per+; plo (n=17)
per01; plo (n=29)
per+; plo (n=10)
per+; 8-35/+ (n=10)
perT; 8-35/+ (n=13)
--per+; 8-35/+ (n=10)
per01; 8-35/+ (n=10)
per+; 8-35/+ (n=14)
per+; 1/1 (n=10)
perT; 1/1 (n=9)
--per+; 1/1 (n=10)
per01; 1/1 (n=10)
per+; 1/1 (n=14)
per+; 90-14/+ (n=14)
perT; 90-14/+ (n=21)
--per+; 90-14/+ (n=14)
per01; 90-14/+ (n=17)
tim01; 90-14/+ (n=15)
per+; 90-14/+ (n=15)

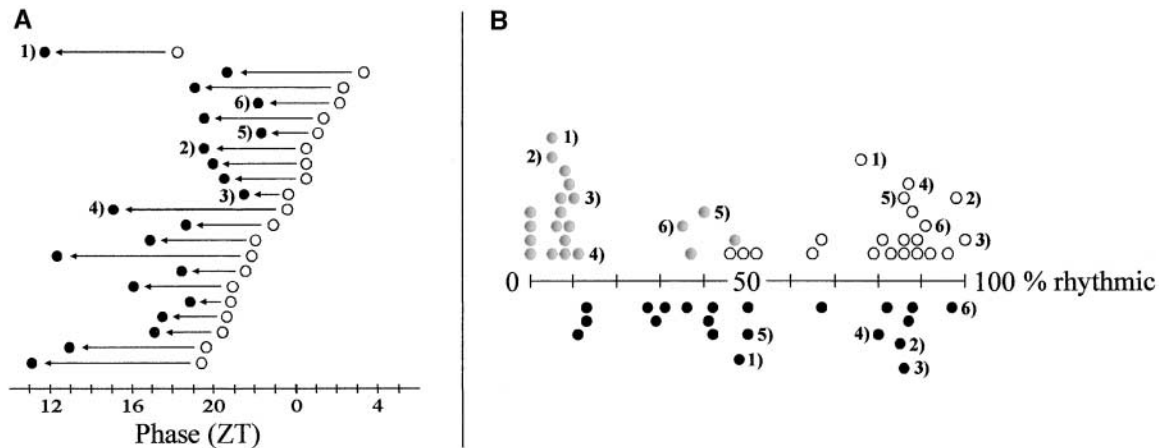


FIGURE 4.—Overview of clock regulation in class I enhancer trap lines. Rhythmic bioluminescence of all such lines (see Table 2) was analyzed in two different clock-mutant backgrounds and in DD to determine the extent to which these rhythms are clock controlled. Luminescence cycling mediated by the *per-luc-only* (*plo*) transgenic type (in which *per* promoter sequences are fused directly to *luc*) is plotted (1) to exemplify circadian-pacemaker regulation of the *period* gene's 5′-flanking DNA. In addition, enhancer-trap lines in which a rhythmically expressed gene was identified in the vicinity (*cf.* Table 4) are indicated: (2) 1-17, (3) 7-32A, (4) 90-3, (5) 1-45, and (6) 90-14 (although cycling of the gene trapped in the last of these lines turned out to be irreproducible; see text). (A) Change of peak-phase *luc* expression in LD cycles in clock-normal per^+ (open circles) *vs.* per^T (solid circles) genetic backgrounds. Except for the *plo* transgenic type (1), all lines were sorted according to their peak phase in a clock-normal genetic background (*cf.* Table 2). ZT, Zeitgeber time in hours. (B, top) Average percentage of rhythmic individuals in each line in an LD cycle in clock-normal per^+ (open circles) and per^{01} (shaded circles) genetic backgrounds. (B, bottom) Percentage of individuals showing circadian expression of *luc* in DD per^+ (solid circles). Note that some class I lines exhibit stronger rhythmicity in DD compared with the *plo* transgenic type.

Identification of novel rhythmically expressed genes: To determine whether the circadianly regulated enhancers controlling *luc* rhythms also influence circadian expression of endogenous genes (*cf.* Figure 1B), we analyzed temporal RNA patterns of a subset of those we identified. To this end we analyzed 12 genes by performing RNase protection assays (RPAs) with total RNA isolated from wild-type flies at at least six different times during a 12:12 LD cycle (Table 4). As protecting probes we generated radiolabeled antisense RNA fragments of the respective genes and one designed to detect the constitutively expressed *rp49* gene as a control for equal loading of RNA (see MATERIALS AND METHODS). We assumed that a given gene might be expressed rhythmically in only a subset of the tissues in which its products are made. Therefore we analyzed RNA expression in heads and bodies separately, and given that bioluminescence rhythms were measured in male flies only, we restricted body-RNA analysis to this sex. Among the 12 genes analyzed, 4 showed reproducible RNA oscillations with amplitudes ranging from two- to fourfold (Table 4; Figure 5). This demonstrates that the bioluminescence-based enhancer-trap approach is a suitable way to isolate rhythmically expressed genes in Drosophila. We now describe the most heavily analyzed subset of the sniffed-out genetic loci, from the perspective of their informational contents and temporally varying expression of the gene products.

***anon1A4 (CG2207)*:** The *CG2207* DNA sequence is located 3 kb upstream of the *luc-sniffer* insertion in line 1-45. This gene (sharing no homologies with others) is also known as *anon1A4* (an arbitrarily designated "anonymous" factor) and was identified in a screen for fast-evolving genes in Drosophila (SCHMID and TAUTZ

FIGURE 3.—Clock control of *per*-regulated and enhancer-driven bioluminescence rhythms. To determine whether rhythmic expression in a given enhancer-trapped line is affected by circadian-pacemaker mutations, the different class I enhancer-trap lines were crossed into period-altering (per^T, top graphs in A–D) or rhythm-eliminating (per^{01}, center in A–D) mutant genetic backgrounds. In addition, expression was analyzed in clock-normal backgrounds in LD conditions (top and center in A–D) and after transfer from LD to constant darkness (DD, bottom in A–D). Solid and open bars are as in Figure 2; shaded bars indicate when the lights would have been on in an LD cycle. (A) Bioluminescence rhythms of the *plo* transgenic type, in which luciferase is expressed under control of the *per* promoter (*cf.* BRANDES *et al.* 1996). Rhythms are phase advanced in per^T, eliminated in per^{01}, and continue (although dampened) in DD, indicative of strong clock control of *per* gene expression. (B) Similar results as in A were obtained for *luc-sniffer* line 8-35. (C) Enhancer-trap line 1 shows advanced peak expression in a per^T genetic background, but weak rhythmicity is retained in per^{01}. (D) Line 90-14 shows only mild phase advances of expression in per^T and residual rhythmicity in per^{01} (*cf.* Table 2B). 90-14 expression was caused to be aperiodic by tim^{01} (*cf.* Table 2C) and continued to be rhythmic in DD in a clock-normal genetic background (*cf.* Table 2A).

TABLE 3

Genes identified in the vicinity of class I enhancer-trap lines

Line	Map location	Distal gene	Gene trapped	Proximal gene
1	85E	*CG9495* (1.6 kb) *sex combs on midleg* (*Scm*)	*CG8327* spermidine synthase	*CG9429* (0.3 kb) *Calreticulin* (*Crc*)
1-17	57A	*CG13434* (4.1 kb)	*CG13432* Zona pellucida (ZP) domain protein	*CG13431* (1.2 kb) α-1,3-mannosyl-glycoprotein beta-1,2-N-acetylglucosaminyltransferase (*MGAT1*)
1-45	39E	*CG3549* (21 kb)	*CG2201* choline kinase	*CG2207* (3 kb) *anon1A4*
2-49	86A	ND	ND	ND
3-50	82A	*CG9780* (12 kb) ABC transporter motif	*complexin*, GenBank accession no. AF260578	*CG9766* (4 kb) ankyrin repeat protein
3-70	100E	*CG2245* (0.5 kb)		*CG2210* (19 bp) *abnormal wing discs* (*awd*)
3-80	97E/F	*CG5889* (7 bp) *malate dehydrogenase* (*Mdh*)		*CG6051* (23 kb)
3-89	95C	*CG12268* (0.6 kb) helix-loop-helix domain		*CG5320* (34 bp) *Glutamate dehydrogenase* (*Gdh*)
4-16	94E	*CG13826* (4kb)		*CG4467* (3 kb) peptidase
4-29	56D	*CG7563* (1.6 kb) *Calpain-A* (*CalpA*)	*CG9325 hu li tai shao* (*hts*)	*CG10460* (27 kb) peptidase
5-1	100F	*CG2053* (20 kb)		*CG2003* (25 kb) transporter
6-4	47A	*CG2368* (15 kb) *pipsqueak* (*psq*)	*CG12052 longitudinals lacking* (*lola*)	*CG18378* (2.5 kb)
6-48	67C	*CG6721* (1.2 kb) *GTPase-activating protein 1* (*Gap1*)		*CG10809* (1 kb) cytoskeletal structural protein
6-63	62A	*CG13927* (2 kb) *gamma-glutamyl carboxylase* (*GP*)	*CG13928*	*CG17248* (3 kb) *n-synaptobrevin* (*n-syb*)
7-32A	30B	*CG3769* (25 kb) helix-loop-helix domain		*CG3779* (17 bp) *numb*
8-35	93C/D	*CG5862* (2.6 kb)	*CG17299 SNF4Agamma*	*CG7000* (−12 kb) scavenger receptor
9-24	90C	*CG7660* (1 kb) *peroxinectin-related* (*pxt*)	*CG7467 osa*	*CG7477* (1 kb) DNA binding
90-3	50B	*CG6152* (1.3 kb) NAD-kinase	*CG6145* NAD-kinase	*CG6139* (4.4 kb)
90-14	85F	*CG6241* (1 kb) transcription factor	*CG6235 twins* (*tws*)	*CG6217* (1.6 kb)
III129	100E	*CG2245* (0.5 kb)	*CG2210 abnormal wing discs* (*awd*)	*CG1896* (−12 bp)

After isolation of genomic DNA surrounding the *luc-sniffer* insertions by plasmid rescue, genomic nucleotide sequences adjacent to the insertion site were determined. Those sequences were aligned with the whole *D. melanogaster* sequence to confirm the map position of a given insertion and to identify the neighboring or trapped genes (Flybase 1999). The map locations listed are according to Flybase (1999) and in each case matched with the one determined by *in situ* hybridization. The genes on either side of the transposon insertion site are listed as CGs [for computed gene, although annotation of CGs is not limited to computational methods (Flybase 1999)]. The approximate distance relative to the insertion site is indicated in kilobases (kb) or base pairs (bp). In cases for which the insertion occurred within a transcription unit, the respective gene is listed as "gene trapped." In those cases the distance of the adjacent genes is given relative to nearest end of the trapped gene. Negative values (in base pairs or kilobases) indicate the degree of overlap between the trapped gene and a neighboring one. If known, gene names in addition to "CG" are noted by their formal designators and abbreviations in italic type. Inferred gene functions based on a known domain or homology to genes from other species are indicated in roman type. Note that lines 3-70 and III129 have their *luc-sniffer* insertions immediately upstream of or within the *awd* gene, respectively. All other lines represent unique insertion events. ND, not determined.

1997). Interestingly, *per* was found in that same screen by virtue of showing a similarly high rate of amino acid substitutions between *D. yakuba* and *D. melanogaster* as *anon1A4* (this rate was used by Schmid and Tautz 1997 as a measure to determine the speed of evolution for genes analyzed in this study). *anon1A4* encodes the nuclear phosphoprotein Df31, which is thought to be involved in chromatin folding (Crevel *et al.* 2000). *CG2201* mRNA isolated from adult heads showed reproducible oscillations, whereas body RNA did not oscillate (Table 4 and data not shown).

***CG13432*:** In line 1-17 the transposon is inserted in the first intron of *CG13432*, a gene showing homology to *nompA* (Kernan *et al.* 1994). This gene encodes a

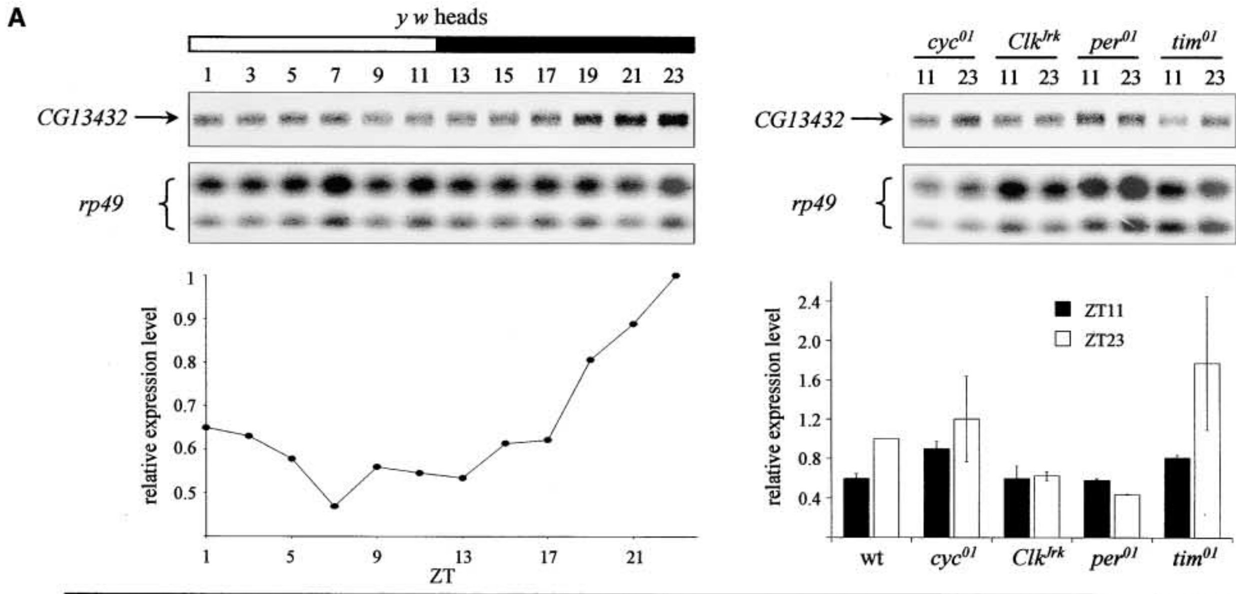
TABLE 4

Genes analyzed for rhythmic RNA expression by RNase protection assays

Line	Neighboring gene	RPA (no.)	Expression
1-45	*CG2207*	H (3)	Rhythmic (twofold amplitude)
		B (1)	AR
1-17	*CG13432*	H (4)	Rhythmic (twofold amplitude)
	ZP-domain protein	B (1)	Weakly rhythmic
3-70	*CG2210* (*awd*)	H (2)	AR
III129	Nucleotide diphosphate kinase	B (4)	AR
3-89	*CG5320* (*Gdh*)	H (1)	AR
	Glutamate dehydrogenase	B (1)	AR
4-29	*CG7563* (*CalpA*) protease	H (1)	AR
6-4	*CG12052* (*lola*)	H (1)	AR
		B (1)	AR
7-32A	*CG3779* (*numb*)	H (1)	Weakly rhythmic
		B (3)	Rhythmic (fourfold amplitude)
8-35	*CG17299* (*SNF4Agamma*)	H (1)	AR
	Protein kinase	B (1)	AR
	CG7000	H (1)	AR
	Scavenger receptor	B (1)	AR
9-24	*CG7467* (*osa*)	H (1)	AR
90-3	*CG6145* NAD kinase	H (3)	Rhythmic (twofold amplitude)
90-14	*CG6235* (*tws*)	H (5)	AR
	Protein phosphatase	B (1)	AR

Summary of all genes analyzed by RPA for rhythmic expression of transcript abundances (AR, arrhythmic). RNA expression of neighboring or trapped genes of a given *luc-sniffer* insertion in a class I enhancer-trap line was analyzed for at least six different time points during a 12:12 LD cycle. The tissues from which total RNA was isolated (H, heads; B, male bodies), along with the number of experiments performed, are indicated. RNA was isolated from Canton-S wild-type or *y w* flies. Gene-specific radiolabeled antisense riboprobes were generated as described in MATERIALS AND METHODS. Note that initially the *tws* gene was found to be expressed rhythmically by Northern blot analysis; this result proved irreproducible after performing additional Northern blot and RPA experiments ($n = 3$ for the former and 5 for the latter).

zona pellucida domain protein, which is required to connect mechanosensory dendrites to sensory structures (Chung *et al.* 2001). Analysis of the mRNA encoded by this gene revealed that it is rhythmically expressed in heads (Figure 5A; Table 4), whereas only weak fluctuations were observed in bodies (Table 4 and data not shown). Peak RNA levels occurred late at night (ZT19-ZT23), substantially later than the times of peak expression levels for either *per* or *tim* RNA (*ca.* ZT15). To compare the actual mRNA peaks with those of the *luc*-reported bioluminescence oscillations, one has to consider that the *plo*-reported expression peak occurs ~3 hr later relative to the *per* mRNA peak (Stanewsky *et al.* 1997). Similarly, the peak of bioluminescence in line 1-17 (ZT0.5) occurs several hours after that of the *CG13432* RNA, indicating that the luciferase activity in this enhancer-trapped line reflects expression of the endogenous gene (Table 2). To determine whether *CG13432* is regulated by the circadian pacemaker, we analyzed RNA levels in different clock-mutant genetic backgrounds. In particular we analyzed expression in per^{01}, tim^{01}, Clk^{Jrk}, and cyc^{01}, each of which causes behavioral arrhythmicity and elimination of molecular rhythms of clock molecules (Young and Kay 2001). Similarly, these mutations abolished RNA rhythms of *CG13432*, with the intriguing exception of tim^{01} (Figure 5A). In three independent tests RNA levels were found to be higher at ZT23 compared with ZT11. To confirm this result we analyzed bioluminescence rhythms of 1-17 flies in the same mutant backgrounds (Figure 6A). Since both *tim* and *CG13432* are located on chromosome *2*, a doubly variant chromosome was created by meiotic recombination between a *tim* mutation and 1-17. For this, we applied a novel loss-of-function *tim* allele, dubbed tim^{03} (see MATERIALS AND METHODS for the origin of this mutation and the manner by which this double variant was recovered). Bioluminescence was measured from flies carrying the recombinant chromosome in heterozygous condition with tim^{01}, which is noncomplementing with tim^{03} (Figure 6A). In each of the four clock-mutant genetic backgrounds (including the *tim*-null one) bioluminescence oscillations were abolished (Figure 6A; Table 2C). Assuming that *luc* cycling in line 1-17 reflects that of endogenous *CG13432* expression, this result in conjunction with the actual RNA data (Figure 5A) indicates that *CG13432* is clock controlled. We have no explanation for the discrepancy between observed head RNA fluctuations and constitutive biolumi-


A
y w heads
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CG13432
rp49
relative expression level
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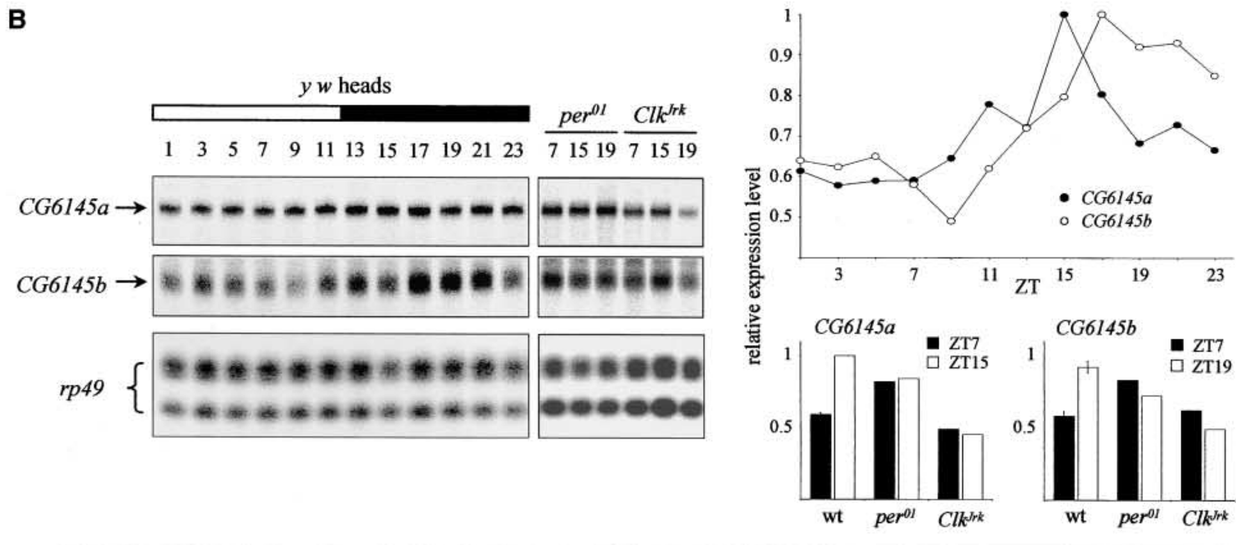
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ZT15
ZT19
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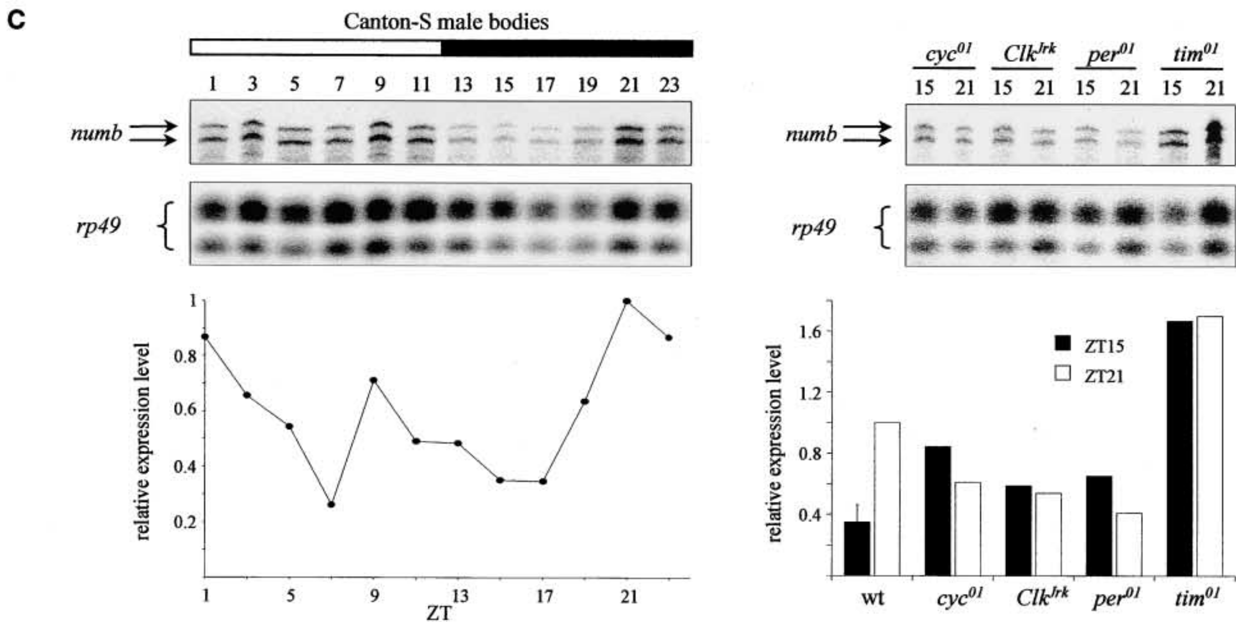
C
Canton-S male bodies
1 3 5 7 9 11 13 15 17 19 21 23
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ZT21
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nescence expression in a *tim*-null background (Figures 5A and 6A; Table 2C). It could be that different tissues give rise to the bioluminescence signal compared with the rhythmic head signals stemming from tissue homogenates. This would suggest that the *timeless* clock gene plays only a minor role in regulation of *CG13432* rhythms in the head. To examine this matter further, a more dense *CG13432* RNA time course in a *tim*-null background needs to be conducted. Another 1-17-related issue is that levels of bioluminescence emanating from this *luc-sniffer* insertion were low in genetic backgrounds that included homozygosity for the Clk^{Jrk} or cyc^{01} mutations (luciferase-mediated counts per second were equivalent to trough levels observed in a Clk^{+} cyc^{+} background; Figure 6A). These findings are consistent with the roles played by these two genes as transcriptional activators (Williams and Sehgal 2001; Young and Kay 2001). Levels of 1-17-mediated luminescence in per^{01} or tim^{01}/tim^{03} mutant backgrounds were low to medium, suggesting that these genes have only minor effects on the expression level of *CG13432* (Figure 6A). These differential effects of *Clk* and *cyc* mutations, on the one hand, and *per* and *tim* mutants, on the other hand, were not observed at the actual RNA level. Here *CG13432* RNA abundances under the influence of either Clk^{Jrk} or per^{01} were found to be near the trough levels observed with clock-normal fly extracts (left-most pair of histogram bars in Figure 5A), whereas the cyc^{01} and tim^{01} mutations caused *CG13432* RNA to stay at the normal peak level (Figure 5A). A reason for this discrepancy could be the apparently different levels of *rp49* expression in the various clock-mutant backgrounds (Figure 5A, right), which here complicated an intergenotype comparison of *rp49* normalized expression values. Given the low amplitude of normal *CG13432* RNA cycling (Figure 5A, left), even small errors associated with such normalizations can obscure the actual RNA levels in the different genetic backgrounds. In any event, cycling of the *CG13432* transcript was abolished by most arrhythmia-inducing clock mutations and in terms of reporter-gene cycling by all four clock-mutant alleles applied here, demonstrating that expression of this gene comes under the sway of the circadian pacemaker.

***CG6145*:** In line 90-3 the *luc-sniffer* got inserted at the *CG6145* locus, whose conceptual protein has homology to NAD kinases (Table 3). Detailed analysis of this transcription unit revealed that two alternatively spliced transcripts are generated from this locus, each encoding a polypeptide with a different N terminus (data not shown, but see materials and methods). The insertion site is situated at an intragenic site corresponding simultaneously to the first intron of the larger transcript (*CG6145a*) and to the upstream region of the first exon of the smaller transcript (*CG6145b*). The riboprobe used is able to detect both transcripts, and RPA analysis revealed that both RNAs are rhythmically expressed in fly heads with slightly different temporal profiles (Figure 5B; Table 4). The *CG6145a* transcript exhibited a similar temporal expression pattern as that described for *per* and *tim* RNAs (see above), with maximum levels observed at about ZT15. The shorter *CG6145b* transcript peaks 2–6 hr later, reaching its highest levels between ZT17 and ZT21 (a time course similar to the profile described in the previous subsection for the *CG13432* transcript). The maximum bioluminescence for line 90-3 occurred 5–6 hr later compared with the *plo*-mediated (*per*-reporting) peak (Table 2), indicating that the *luc* rhythm in this enhancer-trapped type probably reflects expression of the shorter transcript. RNA fluctuations of both transcripts were abolished in genetic backgrounds that included Clk^{Jrk} or per^{01} mutations, demonstrating that *CG6145* is a clock-controlled gene (Figure 5B). In agreement with this conclusion, 90-3-mediated *luc* rhythms were eliminated in a per^{01} background and phase advanced in per^{T} flies (Figures 4 and 6B; Table 2), indicating that the *luc*-reported expression closely reflects that of *CG6145* RNA.

***numb (CG3779)*:** This gene is located immediately up-

Figure 5.—Temporal RNA-expression profiles of genes in the vicinity of clock-regulated enhancers. Total head or body RNA (as indicated) from *y w*, Canton-S wild-type, or different clock-mutant backgrounds was isolated at the different Zeitgeber times (ZT), indicated above each gel image. The RNA amount at each ZT was determined by RPAs using gene-specific riboprobes (see materials and methods). In addition, a probe detecting the constitutively expressed ribosomal *rp49* gene was included to control for RNA loading. Band intensities were quantified (after standardization to the *rp49* signal) using a phosphoimager. Solid and open bars are as in Figure 2. (A, top) Head RNA expression of *CG13432* (identified by line 1-17) in a clock-normal background with 2-hr time resolution during a 12:12 LD cycle. To the right, expression in four different arrhythmic clock mutants is shown. (Bottom) Quantification of two—or three for ZT11 and ZT23—independent experiments with 2-hr time resolution. Right, quantification of signals obtained from three independent experiments involving the various clock-mutant backgrounds compared to wild type. Maximum expression in a clock-normal genetic background was set to 1.0. (B) Head RNA expression of *CG6145a* and *CG6145b* transcripts (identified by line 90-3), analyzed by RPA with 2-hr time resolution in a 12:12 LD cycle. In addition, in one experiment abundance levels at three different time points in per^{01} and Clk^{Jrk} mutant backgrounds were determined. (Top, right), quantification of three experiments for *CG6145a* (solid circles) and two experiments for *CG6145b* (open circles) in a clock-normal genetic background are shown. (Bottom) Quantification of *CG6145* transcript levels in clock-mutant backgrounds. Maximum expression for each transcript in a clock-normal genetic background was set to 1.0. (C, top) *numb* RNA isolated from male bodies at 2-hr resolution in a 12:12 LD cycle. Arrows point to protected fragments of the two alternatively spliced transcripts described (Uemura *et al.* 1989). Right, *numb* expression in four different clock-mutant backgrounds at two different ZTs. (Bottom) Quantification of two independent experiments performed with 2-hr resolution. Right, quantification of one experiment in which *numb* body RNA levels were determined in various clock-mutant backgrounds. Maximum expression in a clock-normal genetic background was set to 1.0.

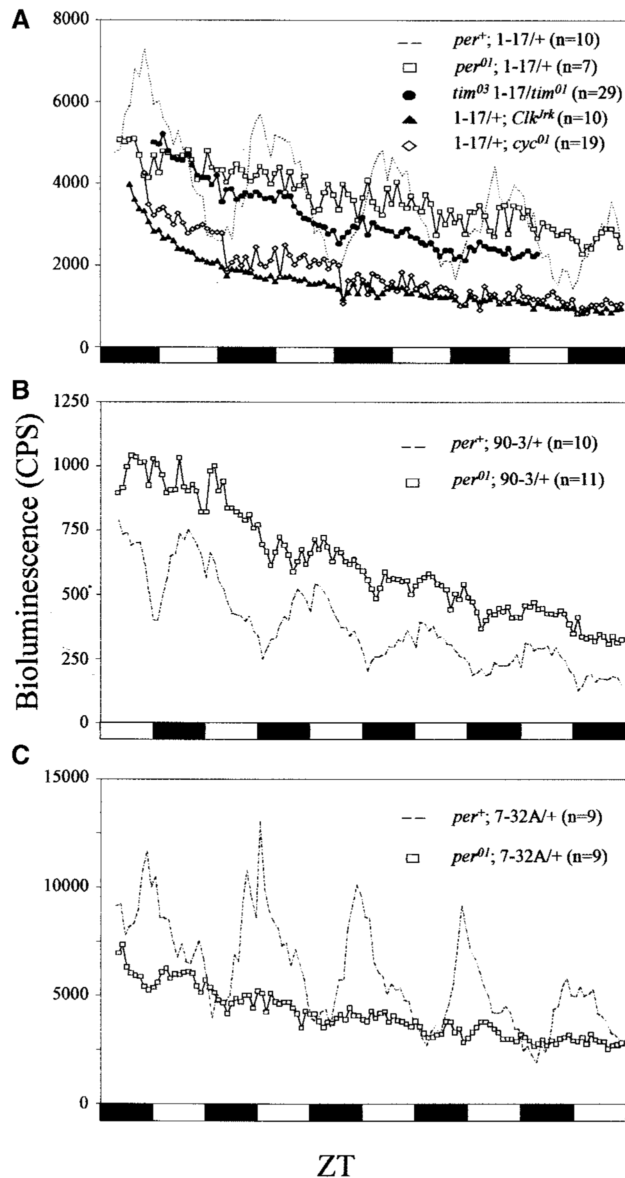

FIGURE 6.—Effects of clock mutations on enhancers regulating circadian gene expression. To verify the results obtained by RPA, bioluminescence expression of class I lines that led to the identification of rhythmically expressed genes in various clock-mutant genetic backgrounds is shown. Expression in a clock-normal background is indicated with a stippled line in each case. Solid and open bars are as in Figure 2. (A) Bioluminescence rhythms normally observed in line 1-17 (identifying *CG13432*) are abolished by all four clock mutations applied (see also Table 2). Note also that expression in a *tim* loss-of-function background became arrhythmic (*cf.* Figure 5A and Table 2C). (B and C) Bioluminescence rhythms occurring in the 90-3 (*CG6145*-identifying) and 7-32A (*numb*-identifying) lines were also abolished by the per^{01} mutation, confirming the RPA results.

stream of the transposon insertion in line 7-32A. *numb* is involved in cell fate decisions during embryogenesis and adult sensory bristle formation (reviewed by JAN and JAN 2000). A general role for mammalian *numb* in endocytic processes was suggested (SANTOLINI *et al.* 2000). The riboprobe we generated to detect *numb* RNA recognizes the two alternatively spliced transcripts, encoding proteins with different N termini (UEMURA *et al.* 1989; also see MATERIALS AND METHODS). We found that both *numb* transcripts are present in male bodies and that expression is robustly rhythmic with an approximately fourfold amplitude (Figure 5C). Peak expression occurred between the late nighttime and early morning (ZT21 to ZT1), and the lowest levels of *numb* transcripts were observed between ZT11 and ZT17. *numb* RNA peaks are therefore delayed by 6–10 hr compared with the *per* and *tim* RNA peaks, similar to what has been described for the *Clk* and *cryptochrome* RNA time courses (BAE *et al.* 1998; EMERY *et al.* 1998). The later-than-*per* and -*tim* peaks were nicely reflected by the 6-hr delay of the bioluminescence peak in line 7-32A compared with that of *plo* (Table 2). When we examined *numb* RNA levels in the genetic background of different arrhythmic clock mutants at peak and trough time points of expression (ZT15 and ZT21), in no instance were fluctuations comparable to those in a clock-normal background observed (Figure 5C). These (raw molecular) results are similar to the elimination of bioluminescence rhythms seen for line 7-32A as affected by per^{01} (Table 2; Figure 6C). Moreover, the actual RNA levels in this arrhythmia-inducing genetic background stayed at levels comparable to the normal trough abundance; the same was observed for the bioluminescence levels determined for per^{01}; 7-32A males (Figures 5C and 6C). We also observed *numb* RNA cycling in temporally collected RNAs isolated from heads, but here the peak-to-trough amplitude was less pronounced compared to that of male-body RNA oscillations (data not shown). Taken together, the results stemming from recovery of the 7-32A line show that *numb* is a clock-controlled, rhythmically expressed gene and that *numb* RNA levels are reflected by the bioluminescence expression observed in line 7-32A (Figures 4, 5C, and 6C).

The remaining eight genes we analyzed did not show reproducible RNA rhythms (Table 4). In a Northern blot time course of the transcripts from the *tws* gene, head-mRNA oscillations were observed (data not shown). This result supported the potential function of this gene in the circadian system (see above). Interestingly, *tws* functions in the same cell fate decision pathway as the rhythmically expressed *numb* gene (SHIOMI *et al.* 1994), suggesting that both factors could also be involved in the same process in the circadian system; this might in turn be related to the circadian regulation and chronobiological significance of *dCREB2* (see above). But when we repeated the temporal RNA analysis of *tws* expression by performing both Northern blotting and RPA experiments, the transcript rhythms originally observed were irreproducible (Table 4; data not shown). This makes a role for *tws* in the circadian system ambiguous for the time being (but see DISCUSSION).

Locomotor activity rhythms in flies mutant or potentially mutant for the rhythmically expressed genes: A useful feature of the enhancer trapping is that the (modified) *P* element frequently induces a mutation in the targeted gene, which can lead to inferences about the nature of the function encoded at that locus. Therefore we analyzed the locomotor behavior of flies homozygous for a given *luc-sniffer* insert in cases for which the transposon had inserted near a gene revealed to be rhythmically expressed (*i.e.*, by more than reporter-enzyme oscillations). If the rhythmically expressed gene functions somewhere along the clock-output pathway regulating circadian locomotor activity or upstream of it in the central clock works, one would expect alterations of behavioral rhythmicity in cases for which an insertion interferes with gene function.

In Table 5 free-running periods of the relevant strains are listed along with the percentages of flies exhibiting rhythmic behavior. In no case was drastic alteration of free-running periodicity or overall rhythmicity observed. Enhancer-trap line 1-45 is inserted 3 kb downstream of the rhythmically expressed gene *CG2207* and therefore probably does not interfere with the function of that gene. In line 1-17 the element is located in the first intron of *CG13432* (see above), and homozygosity for this insertion causes lethality. That developmental death is indeed caused by the *luc-sniffer* insertion was revealed after recovery of homozygous viable revertants of the element associated with this line (data not shown, but see MATERIALS AND METHODS). We could test only 1-17 heterozygous males for locomotor activity, and those flies turned out to be normal for this behavior (Table 5). In line 90-3 the insertion also occurred in intron 1 of the corresponding rhythmically expressed transcript (*CG6145a*) or upstream of its first exon (*CG6145b*; see above). Homozygous 90-3 flies are viable, which means that *CG6145* is not a vital gene or that the transposon does not drastically interfere with gene function. Northern blot and RPA analyses suggest that the latter is the case, since both *CG6145* transcripts appear to be generated in normal amounts and size in homozygous 90-3 flies (data not shown). The free-running periods of homozygous and heterozygous 90-3 flies were ~0.5 hr longer compared with control flies (Table 5). Since revertants created by mobilization of the transposon show the same degree of period lengthening (Table 5), this effect is not caused by the insertion in *CG6145*, but rather represents a genetic background phenomenon.

In line 7-32 the *P* insertion occurred immediately upstream of the *numb* gene. As was the case for line 1-17 this insertion causes lethality in the homozygous condition. Testing flies in which the 7-32 element was placed (separately) over the lethal $numb^1$, $numb^2$, and $numb^3$ mutations (UEMURA *et al.* 1989) revealed that the insert in this enhancer-trap line disrupts *numb* function: 7-32, when heterozygous with any of the *numb* mutations just named, did not lead to viable flies; hence we dubbed this allele $numb^{nuts}$. Interestingly, three of the four lethal alleles showed an ~0.5-hr period lengthening in heterozygous condition compared with the controls (Table 5). In addition the hypomorphic, viable allele $numb^{SW}$ (WANG *et al.* 1997) caused even longer locomotor periods in homozygous conditions (almost 25 hr) and ~0.5-hr lengthenings when heterozygous with $numb^+$ (Table 5). That this gene indeed might play a role in determining the period of locomotor activity is indicated by the significant intermediate period lengthenings (*i.e.*, between 0.5 and 1 hr) observed in flies carrying $numb^{SW}$ over one of the period-lengthening lethal alleles (Table 5). This potentially pertinent player in the fly's rhythm system would be another example of a developmentally vital gene that carries out a separate kind of function

TABLE 5

Free-running behavioral rhythms of enhancer-trap lines and other genetic variants

Genotype	Period (hr)	*n*	% rhythmic
y w	24.0 ± 0.1	44	95
1-45/1-45	24.1 ± 0.2	6	86
1-17/+	24.2 ± 0.1	19	95
90-3/90-3	24.7 ± 0.1	44	80
90-3/+	24.6 ± 0.1	26	62
$90\text{-}3^{rev1}/90\text{-}3^{rev1}$	24.7 ± 0.3	7	71
$90\text{-}3^{rev2}/90\text{-}3^{rev2}$	24.6 ± 0.2	7	57
$numb^{nuts}/+$	24.4 ± 0.1	43	91
$numb^{SW}/numb^{SW}$	24.9 ± 0.2	8	88
$numb^{SW}/+$	24.4 ± 0.0	28	100
$numb^{nuts}/numb^{SW}$	24.7 ± 0.1	11	92
$numb^1/+$	24.4 ± 0.1	12	92
$numb^2/+$	24.2 ± 0.1	16	100
$numb^3/+$	24.4 ± 0.1	9	69
$numb^1/numb^{SW}$	24.6 ± 0.1	14	93
$numb^2/numb^{SW}$	24.3 ± 0.1	15	94
$numb^3/numb^{SW}$	24.6 ± 0.1	15	79

Summary of behavioral analyses performed on enhancer-trap lines with insertions close to a rhythmically expressed gene. Locomotor activity rhythms were recorded in LD and DD and analyzed as described in MATERIALS AND METHODS. In no case was LD behavior altered compared with control flies (data not shown). Where possible, enhancer-trap lines were tested in homozygous condition (1-45 and 90-3). In cases for which homozygosity of the *luc-sniffer* insertion causes developmental lethality (1-17 and 7-32A), only flies heterozygous for the transposon insertion were tested. In addition, for line 90-3, two independently isolated transposon-less revertants were analyzed ($90\text{-}3^{rev1}$ and $90\text{-}3^{rev2}$). The insert associated with line 7-32A disrupts the vital function of the *numb* gene (see text). Therefore, other lethal *numb* alleles ($numb^{1\text{-}3}$, UEMURA *et al.* 1989) and the viable $numb^{SW}$ allele (WANG *et al.* 1997) were also included in the analysis. Differences in free-running periods between the various *numb* mutations (and combinations thereof) and *y w* were statistically significant (ANOVA with Dunnett test, $P < 0.05$; data were normally distributed as determined by Kolmogorov-Smirnow test), except for $numb^2/+$ ($P = 0.8$), $numb^2/numb^{SW}$ ($P = 0.11$), and $numb^3/+$ ($P = 0.07$).

later in the life cycle (*cf.* Newby and Jackson 1993; Price *et al.* 1998; Martinek *et al.* 2001).

DISCUSSION

Identification of rhythmically regulated enhancers: We adapted the enhancer-trap method to identify gene regulatory factors whose *cis*-acting functions are influenced by the circadian clock. From the 20 lines studied in more detail, only 2 had their insertion close to the same gene. This suggests that most of the 71 rhythmically active insertions (out of 1176 total lines analyzed) occurred at different positions, suggesting in turn that ~6% of the Drosophila genes are rhythmically expressed. A similar estimate, based on temporally differential gene expression determined by microarray technology, has been made in one study of *Arabidopsis thaliana* (Harmer *et al.* 2000; although see Schaffer *et al.* 2001).

Most enhancer-trap lines (93%) showed their maximum reporter-gene activity between midnight and midday, which is several hours later compared to the *per*-promoter-driven luminescence peak of *plo* flies (Table 1). Moreover, all class I lines exhibited delayed peak phases of luminescence compared to *plo* (Figure 4A). The same delay was observed at the actual RNA level for three of the newly identified *ccg*s (Figure 5), suggesting that transcriptional activity of these genes is biased toward the night and early morning. A similar distribution was observed in a study where 20 *ccg*s were analyzed (Van Gelder *et al.* 1995). Here, 17 genes showed early-evening expression peaks, although the time resolution applied in this study did not allow us to determine if expression occurred with a delay compared to *per* expression. Moreover, both *Crg-1* (Rouyer *et al.* 1997) and *to* (takeout; So *et al.* 2000) exhibit their RNA peaks in the early or late evening, respectively. The high percentage of evening-specific genes and enhancers suggests that they might be regulated by the binding of CLOCK and CYC to E-box sequences. So far all genes known to be regulated by this mechanism show their highest RNA expression values during the early night (*per*, *tim*, and *vri*; Williams and Sehgal 2001; Young and Kay 2001).

Therefore we searched the vicinity of the *luc-sniffer* insertions of all class I enhancer-trap lines for the presence of E-box sequences. The results are presented in the supplementary material at http://www.genetics.org/supplemental and indicate that only a subset of the enhancers might be regulated by the known feedback interactions (see above). The presence of morning-specific enhancers (Table 1; Figure 4A) and genes (*numb*, Figure 5C) indicates that in these cases regulation might occur via the *Clk* feedback loop similar to what has been discussed for the *Clk* and *cry* genes (Glossop *et al.* 1999). In other cases rhythmic regulation might involve cAMP response elements, since it has been shown that the protein binding to these sequences is under circadian control (Belvin *et al.* 1999; supplementary material at http://www.genetics.org/supplemental). Also, given certain results discussed below, it is clear that other regulatory mechanisms exist, which depend only on the function of subsets of the known clock genes.

Identification of rhythmically regulated genes: We succeeded in isolating novel rhythmically expressed genes in the vicinity of certain enhancers. However, not all the genes subsequently analyzed by RNase protection showed rhythmic mRNA accumulation. Reasons for such cases of noncongruence are discussed as follows. In cases where we analyzed expression of only one gene located to the left or to the right of the *luc-sniffer* insertion (lines 4-29 and 6-4), it is possible that the other neighbor is the rhythmically expressed one (Tables 3 and 4; Figure 1B). In those instances where even a gene trapped by the *luc-sniffer* turned out to be expressed constitutively in a wild-type fly (as was the case for the genes identified by recovery of lines III129, 6-4, 8-35, 9-24, and 90-14), it is still possible that the enhancer controls rhythmic expression of a gene farther away from the insertion site. That this is unlikely is suggested by the property of one line (8-35) for which both the trapped gene and its proximal neighbor were analyzed; each exhibited no RNA rhythms in fly heads and bodies (Table 4). Further findings pertinent to this issue came from analysis of such tissue extracts from flies of line 90-14: Actual reporter RNA levels were found to fluctuate with a fivefold amplitude during the course of a day (data not shown), even though the mRNA levels of the gene trapped by this line (*tws*) are constant over time (Table 4). Because the reporter gene is inserted within the second intron of the *tws* gene in line 90-14, it is likely that *tws* is transcribed rhythmically, too, but that *tws* mRNA is more stable compared with *luc* RNA, obscuring the rhythmic activity of this gene. This set of experiments points to a more likely explanation for (interline) discrepancies between certain of the reporter and mRNA time courses. Thus, reporter-gene expression reflects rhythmic *transcriptional* activity of a gene, as driven by the trapped circadian enhancer. But rhythmic transcriptional activity would lead to oscillating levels in mRNA abundance (which is what one measures by Northern blotting or RPA) only if the half-life of this RNA is relatively short (Wuarin *et al.* 1992). Hence, relatively long-lived mRNAs emanating from a gene that had been trapped in our screen would result in noncycling mRNA levels, even if transcription is rhythmic.

These suppositions lead to the question as to why a gene should be transcribed in a rhythmic fashion although its mRNA levels do not oscillate. One possibility is that circadian enhancers are more or less randomly distributed across the fly genome, leading to rhythmic transcription of many genes—~6% according to our

results [a higher proportion than inferred from microarray analyses of similar phenomena in this species (Claridge-Chang *et al.* 2001; McDonald and Rosbash 2001)]. But only those genes that really depend on cyclically varying mRNA levels to fulfill their rhythm-related function would have a sufficiently short RNA half-life to exhibit oscillating RNA levels. Consider in this regard that basically the whole genome of cyanobacteria is transcribed rhythmically (Liu *et al.* 1995), but a far lower proportion of the genes so identified generate mRNAs that oscillate accordingly (Johnson and Golden 1999). Therefore, it is conceivable that in Drosophila and other higher eukaryotes some genes may still be regulated rhythmically at the transcriptional level in a manner that does not connect with oscillatory functions of gene products; such molecular-genetic cases would reflect evolutionary remnants of more global rhythmic regulation. In this scenario, the lack of function for steady-state RNA abundance rhythms could have resulted in the loss of selective pressure on mechanisms that would have maintained short RNA half-lives, ultimately resulting in rhythmically transcribed but constitutively expressed mRNAs.

Alternatively, mRNA stability of a systematically fluctuating transcript could be regulated differentially in different tissues. For example, if the spatial expression of a rhythm-related factor overlaps with that of other clock genes, mRNA turnover could depend on factors expressed in circadian pacemaker cells, *e.g.*, those that are responsible for post-transcriptional RNA regulation, which has been described for *per* (So and Rosbash 1997; *cf.* Stanewsky *et al.* 1997, 2002). That certain chronobiologically important gene products are regulated differentially depending on the tissue in which they are expressed is not new: (i) In Drosophila ovaries *per* RNA is constitutively expressed as opposed to the daily oscillations of abundance of this transcript in all other tissues examined (Hardin 1994; Hall 1995); (ii) the LARK protein (which functions in an output pathway leading to rhythmic eclosion, as noted in the Introduction) oscillates only in specific neurons among many other cells in which it is expressed (Zhang *et al.* 2000); and (iii) in mammals rhythmic expression of the clock-regulated output gene *vasopressin* is restricted to a small portion of the hypothalamus, whereas in other brain regions (including separate hypothalamic ones) RNA levels are constitutive (Reppert *et al.* 1987; Jin *et al.* 1999). These precedences are among the several considerations that warrant determination of the tissue expression patterns of the genes we identified by temporally based enhancer trapping (for example, by *in situ* application of antibodies against luciferase). Such histological assessments should be accompanied by temporal expression analysis of the native gene products within the identified tissues.

Lethal effects of mutations at loci corresponding to certain rhythmically expressed genes: A crucial advantage of the enhancer-trap approach, compared with purely molecular methods that are usually applied to isolate rhythmically expressed genes, is the potential to isolate a mutant allele of the identified gene (*cf.* Bellen 1999). Two of the lines (1-17 and 7-32A) that resulted in the isolation of circadianly regulated genes (*CG13432* and *numb*, Table 4) were overt mutants, because homozygosity for either insert is lethal (as are most previously identified *numb* mutations). Thus, both of these vital genes are essential for the fly's development in addition to their prospective function within the circadian system. Lethal mutations naturally complicate analysis of circadian phenotypes, since only heterozygous animals can be analyzed (*cf.* Newby and Jackson 1993; Price *et al.* 1998; although see Martinek *et al.* 2001). However, it has been shown for certain clock genes and a certain clock-output gene that altering the gene dose can cause changes of the free-running period. For example, reducing the normal dosage of the *vrille* locus (like *lark*, *dbt*, and *sgg*, a rhythm-related vital gene) causes shorter-than-normal free-running periodicity (Blau and Young 1999), and decreased or increased dosage of $lark^+$ leads, respectively, to earlier- or later-than-normal peak times of eclosion (Newby and Jackson 1996). Against this background, we observed period-lengthening effects for three out of the four lethal *numb* alleles as well as in the hypomorphic and homozygous viable $numb^{SW}$ allele (Table 5). These behavioral findings for *numb* harken back to the canonical case of *per*, for which null-mutant heterozygotes lead to period lengthenings and extra doses of per^+ cause shortenings (*e.g.*, Smith and Konopka 1982). Analogous elements of *numb*'s behavioral effects indicate its potential function in the circadian system.

The NUMB protein contains a phosphotyrosine-binding domain, which is involved in the formation of multiple protein complexes and can bind a diverse array of peptide sequences (Zwahlen *et al.* 2000). It is therefore conceivable that this protein interacts with one or more of the known clock factors or alternatively with those involved in clock-output processes (*e.g.*, Renn *et al.* 1999; Sarov-Blat *et al.* 2000). However, one problematical feature of *numb*'s behavioral genetics is that $numb^2$ led to no discernible effect on locomotor rhythmicity (Table 5). But given that the molecular nature of the existing *numb* mutations is not known, it is possible that $numb^2$ does not interfere with the potential clock-related function associated with this gene. Such speculation about the nature of the $numb^2$ mutation is supported by the fact that this was the only lethal allele tested that, when heterozygous with $numb^+$, resulted in 100% rhythmicity for the locomotor-monitored flies (Table 5).

A second lethal mutation, caused by the *luc-sniffer* insertion in line 1-17 at the *CG13432* locus, had no effect on free-running locomotor period when tested in heterozygous condition with the normal allele (Table 5). This result is similar to the outcome of dosage-manipulating the *timeless* clock gene (Rothenfluh *et al.* 2000),

in which heterozygosity for tim^{01} or a deletion of the locus led to no period changes. Thus further interpretation of the 1-17/$CG13432^{+}$ genotype awaits analysis of other circadian phenotypes, such as, for example, eclosion and sensitivity of the olfactory system (*cf.* Krishnan *et al.* 1999, 2001), to demonstrate a potential function of this factor in the circadian system. Alternatively, the gene might be involved in the circadian regulation of so far unknown rhythmic biological processes. In this case, determination of the spatial reporter-gene expression in line 1-17 (*e.g.*, Plautz *et al.* 1997; Stanewsky *et al.* 1997), of endogenous *CG13432* expression, or of both might help to allude to the function of this gene. Indeed, the chronobiological significance of broad spatial expression patterns of clock genes (*e.g.*, Hall 1995; Kaneko and Hall 2000; So *et al.* 2000; Kloss *et al.* 2001) is unknown. But such paucity of knowledge is one reason for studying novel pacemaker output factors. Elucidating the function of a gene such as *CG13432*, or bearing in mind what was previously discovered for *numb*, can provide clues to what the encoded proteins are doing in a given tissue and thus what kind of biological rhythm might naturally emanate from it.

Viable mutants associated with certain rhythmically expressed genes: With respect to two lines identified in our screen (1-45 and 90-3) homozygosity for the transposon did not result in lethality nor did it have obvious effects on locomotor activity (Table 5). These inserts led to the identification of the rhythmically expressed genes *anon1A4* and *CG6145*. In the 1-45 line, the transposon is inserted 3 kb downstream of *anon1A4*, so the function of this gene might not be affected by the insertion. If true, deletions may have to be created by imprecise excisions of the transposon to allow for a more meaningful analysis of circadian biological phenotypes. In line 90-3 the *luc-sniffer* is inserted within the first intron of the gene *CG6145*, but its putative chronobiological function seems not to be affected (see results). Here, too, deletions of or within *CG6145* are likely to be required to establish a potential function in the circadian system (see above).

Conclusions: In summary, we have established a novel method to identify circadianly regulated enhancers and rhythmically expressed genes in eukaryotes. Compared with contemporary microarray methods, ours is likely to be less efficient for identifying *ccg*s. That is because the latter approach directly reveals that a given RNA fluctuates (*e.g.*, Harmer *et al.* 2000; McDonald and Rosbash 2001; Schaffer *et al.* 2001), whereas enhancer trapping usually resulted in several candidate *ccg*s in the vicinity of the *luc-sniffer* (Table 3). Nevertheless, real-time enhancer trapping has crucial advantages compared to standard molecular approaches: First, among the rhythmically expressed genes we isolated were some whose mRNA oscillations occurred with rather low amplitudes (Table 4). These loci were initially identified by the repeated-measures feature of real-time recording (Figures 2 and 3). In these cases, it is questionable whether the requisite clones could have been isolated with molecular techniques, owing to the relative difficulty of generating RNA samples from multiple time points for a given 24-hr period, let alone for several consecutive days (which would be necessary to home in molecularly on a gene that mediates mild but nevertheless convincing day-after-day cycling of its products).

In addition, the enhancer-trap feature of our approach permits identification of candidates for rhythmically expressed genes whose mRNAs oscillate only in a subset of cells in which the molecules are expressed (discussed above in a separate context). In contrast, extracting mRNAs from fly heads at different time points could lead to swamping of transcript-abundance oscillations that occur in a small subset of the brain. Enhancer trapping not only proved suitable for detecting rhythmically expressed genes, but in addition allowed certain studies of the manner by which they are regulated. This was accomplished by combining a given enhancer-trapped *luc-sniffer* with a series of clock mutations (Figures 3, 4, and 6; Table 2). In most cases the influences of such mutations on cyclical expression of a *ccg* paralleled the effects of these pacemaker variants on reporter-gene expression of the *luc-sniffer* line that led to the identification of the particular *ccg* (compare Figures 5 and 6). Interestingly, in a few cases the arrhythmic clock mutants per^{01} and tim^{01} had different consequences for *luc* expression (Figure 3D; Table 2) or for abundance of an endogenous *ccg* transcript (Figure 5A). That different clock genes do not necessarily regulate separate clock-output factors in the same way has been shown in previous studies (*e.g.*, Park *et al.* 2000; So *et al.* 2000), and our results (and the data presented in supplementary materials at http://www.genetics.org/supplemental) indicate that this specificity of certain clock genes in regulating a particular output gene might be more common than previously assumed, on the basis of the assumption that *ccg*s are regulated by the same molecular circadian feedback loops operating in the central clock (see, for example, Loros and Dunlap 2001).

Enhancer trapping has the further advantage of providing an immediate entry point for mutational, and therefore functional, analysis of novel circadianly regulated genes (Table 5). In this regard, a potential improvement of our strategy, which might also result in a higher efficiency of identifying rhythmically expressed candidates, would be to switch to the "gene-trap" technology that has been recently exploited in Drosophila by Lukacsovich *et al.* (2001). Using a modified gene-trap vector containing *luc* instead of *gal4* would allow for a screen, analogous to the present one, to be performed with the advantage that a given cycling gene-trap line would unequivocally specify the rhythmic candidate gene *and* destroy gene function at the same time (*cf.* Lukacsovich *et al.* 2001).

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Note added in proof: After this article was accepted, rhythmic expression of *CG6145* was also reported by Claridge-Chang *et al.* 2001).

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