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Genetic Analysis of Ectopic Circadian Clock Induction in *Drosophila*

Valerie L. Kilman^{*,†,1} and Ravi Allada^{*,†}

^{*} Department of Neurobiology and Physiology, Northwestern University, Evanston, Illinois

[†] Center for Sleep and Circadian Biology, Northwestern University, Evanston, Illinois

Abstract

Cell-autonomous feedback loops underlie the molecular oscillations that define circadian clocks. In *Drosophila* the transcription factor *Clk* activates multiple clock components of feedback loops many of which feed back and regulate *Clk* expression or activity. Previously the authors evoked similar molecular oscillations in putatively naïve neurons in *Drosophila* by ectopic expression of a single gene, *Clk*, suggesting a master regulator function. Using molecular oscillations of the core clock component PERIOD (PER), the authors observed dramatic and widespread molecular oscillations throughout the brain in flies expressing ectopic *Clk*. Consistent with the master regulator hypothesis, they found that *Clk* is uniquely capable of inducing ectopic clocks as ectopic induction of other clock components fails to induce circadian rhythms. *Clk* also induces oscillations even when expression is adult restricted, suggesting that ectopic clocks can even be induced in differentiated cells. However, if transgene expression is discontinued, PER expression disappears, indicating that *Clk* must be continually active to sustain ectopic clock function. In some cases *Clk*-mediated PER induction was observed without apparent synchronous cycling, perhaps due to desynchronization of rhythms between clocks or truly cell autonomous arrhythmic PER expression, indicating that additional factors may be necessary for coherent rhythms in cells ectopically expressing *Clk*. To determine minimal requirements for circadian clock induction by *Clk*, the authors determined the genetic requirements of ectopic clocks. No ectopic clocks are induced in mutants of *Clk*'s heterodimeric partner *cyc*. In addition, noncycling PER is observed when ectopic *Clk* is induced in a *cry^b* mutant background. While other factors may contribute, these results indicate that persistent *Clock* induction is uniquely capable of broadly inducing ectopic rhythms even in adults, consistent with a special role at the top of a clock gene hierarchy.

Keywords

Drosophila; endogenous; peripheral; ectopic; clock; induction

Circadian rhythms of behavior and physiology derive from cell-based clocks (Hastings et al., 2008). Clock cells show circadian molecular oscillations in core clock gene RNAs, proteins, phosphorylation states, and their cellular location. Cellular oscillations are synchronized to one another and to the environment by cycles of light and other cues, zeitgebers, either directly or through neural/hormonal signaling. In flies and mammals the most familiar circadian behavior, locomotion, is largely controlled by discrete groups of brain pacemaker neurons that continue to oscillate synchronously in constant conditions (Nitabach and Taghert, 2008; Okamura, 2007). Clock genes also oscillate in other neural

¹To whom all correspondence should be addressed: Valerie L. Kilman, Center for Sleep and Circadian Biology, Northwestern University, Evanston, IL 60208; v-kilman@northwestern.edu.

and non-neural tissues (Glossop and Hardin, 2002). These peripheral clocks can entrain to light in flies, but in mammals they respond to SCN signals and other zeitgebers (Plautz et al., 1997; Schibler, 2009).

The molecular-genetic basis of the clockworks is based on well-conserved cell-autonomous transcriptional feedback loops. In *Drosophila* the heterodimeric transcription factor composed of CLOCK and CYCLE drives the transcription of *period* (*per*) and *timeless* (*tim*) (Allada et al., 1998; Rutila et al., 1998). PER and TIM themselves encode transcription factors that heterodimerize and accumulate in the cytoplasm. After timed phosphorylation PER and TIM enter the nucleus, likely separately, where PER homodimers and/or PER/TIM heterodimers bind to CLK-CYC and thereby reduce their own transcription (Darlington et al., 1998; Landskron et al., 2009; Lee et al., 1998; Meyer, 2006). In this way their repression of CLK-CYC is self-limiting and its relief initiates the next daily cycle of CLK-CYC activation. Many, although not all, clock cells are intrinsically light sensitive through the photopigment CRYPTOCHROME (CRY) (Stanewsky et al., 1998). PER/TIM interactions with CRY provide one route of light entrainment for the fly clock (Ceriani et al., 1999; Rosato et al., 2001). *cry* may also have other functions in flies, as a component of the oscillator or in temperature-response pathways (Collins et al., 2006; Kaushik et al., 2007; Krishnan et al., 2001). In mammals it functions as one mediator of negative feedback (Kume et al., 1999). Other zeitgebers can also entrain fly clocks, most notably temperature (Stanewsky et al., 1998; Wheeler et al., 1993).

CLK-CYC also activates expression of a bHLH repressor, *clockwork orange* (*cwo*), that feeds back and represses and/or activates CLK-CYC-mediated activation (Kadener et al., 2007; Lim et al., 2007; Matsumoto et al., 2007; Richier et al., 2008). CLK-CYC activates another feedback loop including the bZIP transcriptional regulators *Par domain protein 1* (*Pdp1*) and *vri* (*vri*) (Blau and Young, 1999; Cyran et al., 2003; Glossop et al., 2003). VRI represses *Clk* transcription perhaps by antagonizing activation by PDP1 to drive cycling transcription of *Clk* (Cyran et al., 2003). Because CLK-CYC activates the components of multiple loops that feed back to regulate CLK, this suggests that CLK may act as a master regulator. Consistent with this hypothesis, we showed that ectopic expression of *Clk* was able to induce circadian *tim* and *cry* oscillations in putatively naïve cells (Zhao et al., 2003). CLK expression was also found to precede that of PER during late embryonic development (Houl et al., 2008). We suggested *Clk* expression might be a critical organizer of clock formation, potentially serving as a master regulator capable of inducing the clock program in any cell (Zhao et al., 2003).

Here we set out to determine minimal requirements for circadian clock induction. We found *Clk* uniquely able among clock genes to induce the core molecular feedback loop. Ectopic clocks share cardinal features with endogenous peripheral clocks including genetic requirements, cycling induction of core loop components, and daily rhythmicity. Adult-restricted *Clk* expression can also induce oscillations in ectopic locations, but such expression must be persistent to sustain clocks. Clocks are induced broadly in the brain; however, in some ectopic locations *Clk* induces noncycling PER, consistent with a requirement for other factors. Despite the likely role of other contributing factors in sustaining and entraining ectopic clocks, these results indicate that persistent *Clock* induction is uniquely capable of broadly inducing ectopic rhythms even in adults, consistent with a special role for *Clk* at the top of a clock gene hierarchy.

MATERIALS AND METHODS

Flies

Flies were raised on standard cornmeal-molasses food at room temperature unless otherwise indicated. Flies were obtained from the following sources: *cry16*, *cry24*, *UClkIII*, *UClkII*, *UAScycHA*, *UAStimII-4*, *UAStimII-5*, *UAStimx*, *tubG80ts*, *pdf01*, *cyc01*, *cryb*, *cry16cryb*, *UClkcryb*, M. Rosbash; *UASV1*, *UASV3*, J. Blau; *UAScwo*, C. Lim/R. Allada; *c316*, *c232*, flytrap.org; *201Y*, L. Restifo; *MJ162*, L. Griffith; *A9AC*; *B3AB* (*UASpdf1*, P. Hardin). Other lines were obtained from the Bloomington Stock Center (Bloomington, IN). Recombination and standard genetic crosses were used to generate: *cry16cyc01* (*UClk*; *cyc01*); (*UClk*; *UcycHA*); (*tubG80ts*; *UClk*, courtesy J. Pitman).

Immunostaining

Flies were entrained at least 2 days in 12-h light:12-h dark conditions at 25 °C unless otherwise indicated. Brain tissue was dissected and fixed in 4% formalin/PBS for 40 min. After PBT rinses (0.3% Triton in PBS), tissue was incubated in primary antibody diluted in 10% normal serum-PBT overnight or up to 48 h on a rotator at 4 °C. Primary rabbit anti-PER (Kaneko et al., 1997) was diluted 1:4000 and mouse anti-PDF (DSHB C7) was diluted 1:600. After PBT washes, secondary antibodies were applied overnight. Donkey anti-mouse AlexaFluor 488 and donkey anti-rabbit AlexaFluor 594 were diluted at 1:600 in PBT-10% normal serum. Alternatively, sometimes PER was visualized with tyramide signal amplification. In this case the secondary antibody was donkey anti-rabbitHRP (Amersham #NA934; Arlington Heights, IL) diluted 1:200, followed by the TSA kit as per directions (Molecular Probes/ICN kit #15; Molecular Probes, Eugene, OR). Following rinses in PBT and PB, tissue was mounted in 80% glycerol/PBS.

Imaging and Quantification

Experiments were repeated at least twice with 5 to 8 brains/time point in each experiment. Experiments consisted of 6 time points evenly spaced throughout the day. Only peak and trough were collected to examine *tub-GAL80^{ts/+}*; *cry16UClk* flies 4, 8, and 11 days after shift from 29 °C to 18 °C and for flies in *cyc⁰¹* background due to limited viability. Slides were imaged on a Nikon C1 confocal with identical settings within each experiment, with 20× or 60× objective at 1-μm steps. Small ventral lateral neurons (sLN_v) were identified for Figure 1 from PDF colabeling. Cycling was assessed for *cry16* expression in different conditions and genetic backgrounds using individual cells measured from the representative region marked in Figure 1. Cycling was assessed for each GAL4 driver in the regions indicated in figures. Data were quantified from digital images using ImageJ (NIH Image) and Excel. Within an experiment, average intensity for each cell was measured from the largest optical slice through the cell nucleus. An average was then calculated for each time point and scaled so that the typical peak, usually ZT1 for ectopic clocks, was 100 AU (arbitrary units). One exception was in Figure 1C, where 100 AU equals the peak (ZT5) for the control sLN_v, *y w*; *cry16/+*. Two replications were compared with one another to confirm that the results were similar. Experiments were then combined by scaling all individual cells to AU, averaging over both experiments and calculating standard error of the mean (SEM). One-way ANOVA with post hoc Tukey test was used to identify time-dependent cycling ($p < 0.01$). For 2-time-point data we performed *t* tests.

Behavior

Flies were entrained at 25 °C for 5 days in 12:12 LD conditions before release into constant darkness for 7 days. Trikinetics *Drosophila* Activity Monitors (Waltham, MA) were used to

record locomotor activity. Behavior was analyzed with Clocklab software (Actimetrics Inc., Evanston, IL).

RESULTS

Clk Uniquely Induces Ectopic Clocks

We used *crypGAL4-16* (*cry16*) as we had used previously (Zhao et al., 2003) to induce ectopic *Clk*. It expresses broadly both in circadian neurons and in many other nonclock cells including the ellipsoid body, central complex, and many other unidentified cells throughout the brain. We examined PER expression at 6 evenly spaced circadian time points. In light/dark cycling conditions (LD) we found broad intense cycling expression of PER both in the pacemaker sLN_v and in ectopic clocks (Fig. 1). All ectopic locations in the brain cycle roughly similarly (Supplemental Fig. S1). We quantify the region circled in A and B and these data are graphed in D. This region is near olfactory antennal input regions, but without further colabeling we cannot definitively identify these cells. In both control and overexpression, peak intensity was about 2-fold increased over their respective trough levels. (Although the nonzero PER expression in the “ectopic” location of control *cry16* flies, graphed in Fig. 1D, is likely nonspecific labeling, it is possible it reflects endogenous noncycling PER. Further experiments will be necessary to address this.) Oscillations in overexpressing locations also appeared somewhat phase advanced relative to control sLN_v. Thus ectopic clock PER cycling faithfully models endogenous clock molecular cycling in LD.

Next we used *cry16* to express a variety of clock genes including *cyc*, *tim*, *Pdp1*, *vri*, and *cwo* (*per* had been examined in Zhao et al., 2003). None of these genes when similarly expressed was able to induce ectopic PER (Fig. 2). Although these experiments were not done as a group, it was apparent that PER levels were lower in *vri* overexpressing pacemakers than in comparably stained controls. The reduction of PER may be due to VRI repression of endogenous *Clk* transcription (Cyran et al., 2003; Glossop et al., 2003). The *cyc* transgene has been used to rescue molecular cycling in pacemaker neurons, indicating that it is functionally expressed (Peng et al., 2003). The *cwo* transgene can also rescue *cwo* mutant behavior (C. Lim, 2008 personal communication). We verified *Pdp1* overexpression with immunostaining for PDP (Benito et al., 2007) (Supplemental Fig. S2). To get some indication of the effectiveness of other transgenes, we examined behavior in flies overexpressing *tim* and *vri* (Table 1). For expression individually of 3 *tim* (*timII-4*, *timII-5*, *tim-on-x*) and 2 *vri* (*V1*, *V3*) transgenes, rhythmicity is reduced, with less than 10% of flies being rhythmic. These data suggest that despite functional/measurable expression, other clock genes are unable to induce clock molecular oscillations.

It is possible that other genes may induce clocks in different cell types. For instance other cells may need only *cyc*, the heterodimeric partner of *Clk*, to become clocks. To test this, we expressed *cyc* pan-neuronally using *elavGAL4* and ubiquitously with *actinGAL4*. These flies showed no ectopic PER staining (data not shown). Also we were not able to detect additional PER staining relative to *y w*; *cry16/UClk* in flies that also coexpressed either *cyc* or *Pdp1* (data not shown) with *Clk*. These data make it likely that *Clk* is the only clock gene capable of organizing the molecular feedback loop central to the circadian clock.

Clk Can Induce Clocks in Differentiated Adult Cells

Clk's ability to induce clock cell oscillations could require the presence of development stage-specific cofactors. In this case, *Clk* may have a critical period during development to induce clocks and may not be able to induce clocks in differentiated adult cells.

To test this possibility, we sought to inducibly express *Clk* in adulthood and assay effects on ectopic clock cycling. *GAL80* is a yeast repressor of *GAL4*. Ubiquitous expression of a temperature sensitive allele (*tubulinGAL80^{ts}*) can be used to repress *GAL4* activation at 18 °C. Raising the temperature to 29 °C inactivates the *tubGAL80^{ts}* and relieves the repression of *GAL4*, permitting inducible tissue-specific expression (McGuire et al., 2004). We had previously identified 2 phenotypes of interest in *cry16/UClk* flies: ectopic PER staining and circadian behavioral changes (loss of the LD evening peak, short period in DD, reduced rhythmicity) (Zhao et al., 2003). To test the effectiveness of *GAL80* repression, we evaluated these phenotypes in *tubGAL80^{ts}/+; cry16/UClk* flies. By these criteria, *Clk* expression was effectively repressed in *tubGAL80^{ts}/+; cry16/UClk* progeny raised and tested at 18 °C, resulting in wild-type behavior and PER staining (Fig. 3A, Table 1).

The effect of *Clk* expression restricted to adulthood was then assessed by raising crosses at 18 °C and shifting adult progeny (3–10 days old) to 29 °C. Flies shifted for 24 h showed no detectable ectopic PER expression at ZT1. However after being held at 29 °C for 4 days, ectopic PER staining was broad, intense, and robustly cycling (Fig. 3B), similar to flies raised at 29 °C. Thus robust ectopic clock induction was possible even in adult differentiated cells and is not restricted to a developmental critical period.

Ectopic Clocks Require Persistent *Clock* Expression

We then asked whether *Clk* acts as a developmental switch capable of directing cells irrevocably into a particular cell fate, that of a circadian clock cell. In this model, ectopically supplied *Clk* would activate the endogenous *Clk* gene initiating the clock program. This positive feedback loop would sustain the clock program and remove the requirement for sustained transgenic *Clk* expression. This would be evident as sustained cycling after transient *Clk* activation.

We asked if ectopic clocks were sustained after transgene expression was terminated. As in the previous experiments we raised *tubGAL80^{ts}/+; cry16/UClk* crosses at 18 °C and shifted adult progeny to 29 °C for 4 days to fully induce ectopic clock cycling. We then shifted flies back to 18 °C (permissive for *tub-GAL80^{ts}*) and assessed PER over successive days at peak and trough time points ZT1 and ZT9. Four days later, we found ectopic PER was still expressed and cycling in many locations (Fig. 3C). Only 8 to 11 days after the shift back to 18 °C did ectopic PER levels drop below detectability. Thus ectopic clocks were long lived within the time frame of clock oscillations. They did, however, eventually fade without transgenic drive. These data indicate that molecular cycling only gradually ceases once external activation of *Clk* is removed. These results indicate that *Clk* is not sufficient to initiate a self-sustaining program, suggesting a requirement for other cofactors. This could be implemented through developmentally restricted cofactors required for clock cell induction, resulting in long-lasting chromatin modifications. This is roughly the model accepted for other developmental programs, for instance eye and muscle development (Baylies and Michelson, 2001; Kozmik, 2005). Alternatively, the ability to express clock cell oscillations may be a general property of cells and not restricted to developmentally preprogrammed classes.

Clk Can Induce Clock Gene Expression in a Broad Range of Cells

We were interested to know whether *Clk* could induce clocks in any cell, or whether only certain cells, perhaps preprogrammed in some way, were capable of expressing clock molecular oscillations. One approach to this question would be to express *Clk* using a wide variety of *GAL4* drivers to assess the susceptibility of different cell types to clock induction. One difficulty with this approach is that *Clk* expression is lethal in combination with many (though not all) drivers, often at pupal stage. When *tubGAL80^{ts}* is coexpressed, flies can be

raised at 18 °C to repress *Clk* and circumvent developmental lethality. Even so, shifting to 29 °C to relieve repression in adulthood often then sickens flies and increases mortality, making evaluation of cycling difficult. In addition, flies that survive best are often those with relatively limited expression patterns or low-level expression. This suggests that misexpression of *Clk*, a powerful transcriptional activator, can have detrimental effects in both development and adulthood in some cells. These toxic effects may reflect *Clk* functions in development (Park et al., 2000) or they may be an artifact of excessive and ectopic activation of *Clk* target genes normally insensitive to wild-type *Clk* levels. Lethality is also partially a result of the higher temperature, as all flies show somewhat increased mortality when shifted to 29 °C as well. Nevertheless, we tested a number of different GAL4 drivers with adult-specific expression in this manner. One broad line that remained viable, *elavGAL4*, expresses exclusively in neurons and appeared to drive cycling PER expression throughout the brain (Supplemental Fig. S3A). However, the expression was so broad that tracking individual cell groups was difficult. We turned instead to drivers with more restricted, usually neural expression patterns. Some, such as *MJ162* (used in Zhao et al., 2003; non-core α/β , γ lobes (Joiner and Griffith, 1999; G. Liu, 2009 personal communication) expressed in the mushroom body and other brain regions (Supplemental Fig. S3B), and *c232* (Fig. 4A) expressed almost exclusively in the ellipsoid body, exhibit ectopic cycling with phase similar to that seen with *cry16* (Fig. 1). However, some drivers showed abundant PER expression but did not reliably cycle. For instance, *repoGAL4*-driven CLK activates expression of ectopic PER in glia (the only primarily non-neural driver tested), but this PER does not cycle coordinately (Fig. 4B and Supplemental Fig. S3E). We did not observe any consistent ectopic rhythms. The variance at individual time points was higher in this genotype than in coordinately cycling lines (Supplemental Fig. S3E"). This variability could reflect either lack of entrainment or mixed populations of cycling and noncycling cells sampled inconsistently. PER is normally rhythmic in a (small) subset of glia (Zerr et al., 1990). However, we were unable to quantify PER rhythms specifically in normally PER-expressing glia because ectopic PER expression in most glia prevented us from identifying this rhythmic subset (Zerr et al., 1990). *c316* (Waddell et al., 2000) expressed in the dorsal paired median neurons (DPM) and elsewhere (Supplemental Fig. S3C) induced PER expression that cycled. The mushroom body driver *201Y* (core α/β , γ lobes, Yang 95) also expressed noncycling PER (Supplemental Fig. S3D) despite the cycling evident in *MJ162* (noncore α/β , γ lobes), which also shows mushroom body expression (Supplemental Fig. S3B). Again, this may be due to expression in different subtypes of cells, either within (core vs. noncore α/β) or outside the mushroom body (for example, neural vs. glial). This reflects an underlying heterogeneity in the ability of clocks to express molecular cycling, perhaps reflecting a cofactor requirement. Alternatively, *Clk* overexpression may not as effectively induce entrainment mechanisms as well as it does core cycling components. Further work will be necessary to address this distinction. Nonetheless, *Clk* appears necessary and sufficient to induce ectopic expression broadly in the brain.

Genetic Requirements for Ectopic Clocks

If similar to endogenous clocks, ectopic clocks might serve as a model to study the induction of the central molecular feedback loop in natural clocks. We asked what the genetic requirements for ectopic clocks were. Using *cry16*, we expressed ectopic clocks in various clock mutant backgrounds.

We asked whether ectopic clocks require its heterodimeric bHLH-PAS partner CYC. *cyc⁰¹* mutant flies have undetectable levels of PER in endogenous clocks. Ectopic CYC expression is not sufficient to induce ectopic clocks (Fig. 2). To test whether *Clk* requires *cyc* to activate cycling, we ectopically expressed *Clk* in a *cyc⁰¹* mutant background. We found that *Clk*

expression in *cyc⁰¹* mutants induces neither endogenous nor ectopic PER (Fig. 5A). This indicates *Clk*'s ability to induce the central feedback loop is entirely dependent upon *cyc*, consistent with the hypothesis that CLK does not dimerize with other bHLH partners. These data also suggest that either *Clk* induces *cyc* broadly or that *cyc* is broadly present in cells susceptible to ectopic *Clk* action.

CRY provides the cell-autonomous light input to the central oscillator (Emery et al., 1998; Stanewsky et al., 1998). The *cry^b* allele contains a point mutation that prevents light sensing. In these mutants the pacemakers entrain to light due to input from the eye, but some peripheral oscillators may not (Ivanchenko et al., 2001; Krishnan et al., 2001; Stanewsky et al., 1998). We found in *cry16-cry^b/UClk-cry^b* flies ectopic PER was broadly expressed, but it did not detectably cycle (Fig. 5B, B'). Given that the sLNv cycle robustly in a *cry^b* background, this suggests that sLNv are not able to synchronize ectopic clocks in these flies. Instead they appear most like peripheral clocks, entraining directly to light. Thus, sufficient induction or preprogrammed expression of *cry* may be necessary for coordinate cycling in light/dark cycles.

DISCUSSION

We showed previously that *Clk* expression was able to induce cycling *tim* and *cry* in naïve cells or ectopic clocks (Zhao et al., 2003). Here we further explore the role of the *Clk* gene in induction of molecular clocks. We find that *Clk* is unique among all other core clock genes in its ability to induce clocks. It is able to induce cycling broadly throughout the brain and even in differentiated adult cells, indicating that there is no critical period for molecular clock induction. However, we also reveal evidence that this “master regulator” function of *Clk* may be limited. Ectopic *Clk* can induce PER but without coordinate cycling, perhaps reflecting a lack of CRY. Ectopic cycling also fades 8 to 10 days after deinduction of the *Clk* transgene, suggesting additional factors are required to sustain rhythmicity. Notably *Pdp1* alone, which may form a positive feedback loop with *Clk* transcription, is not sufficient to sustain ectopic clocks. These data suggest CLK expression is a primary determinant of clock behavior in cells, serving as a molecular switch to initiate the core cycling program.

There is interest in the development of circadian clocks. The *Clk* gene seems a logical candidate to control the induction of clocks because it activates transcription of many core clock genes, including at least *per*, *tim*, *vri*, *Pdp1*, *cwo*, and indirectly *cry* (Allada et al., 1998; Blau and Young, 1999; Cyran et al., 2003; Lim et al., 2007; Matsumoto et al., 2007; Zhao et al., 2003). Dominant negative and hypomorphic/partial loss of function CLOCK alleles block expression of clock genes in flies (Allada et al., 2003; Allada et al., 1998; Houl et al., 2008), and the developmental onset of PER cycling in pacemakers has been correlated with the initiation of *Clk* expression (Houl et al., 2008). Our work has shown that *Clk* expression is sufficient to induce oscillating expression of enough components of the core molecular oscillator (*tim*, *cry*, PER) and in appropriate ratios to one another such that approximately normal clock cycling results. Taken together, these data show *Clk* is a master regulator at the top of the circadian hierarchy.

We have shown that ectopic clocks provide a good model of cycling in endogenous clocks. Although we have previously shown that ectopic rhythms persist until day 2 of constant darkness (Zhao et al., 2003), we cannot exclude the possibility that ectopic PER rhythms observed here may be light driven. The phase of cycling in ectopic clocks is similar to that of *Clk* overexpressing sLNv. Relative to wild-type, clock gene levels are very high but cycle 2-fold just as endogenous clocks do. Phase is slightly advanced but not greatly offset from wild type. The recessive *Clk^{ar}* allele also reduces clock gene levels without large phase changes (Allada et al., 2003) suggesting transcriptional mechanisms regulate rhythm

amplitude rather than phase. It is interesting that such a large increase in gene products does not alter cycling more destructively. *Clk* may activate expression of all core clock genes in ratios relative to one another such that robust cycling amplitudes are maintained. Consistent with this hypothesis, cultured mammalian fibroblasts continue to cycle robustly even when overall transcription rates are experimentally reduced (Dibner et al., 2009).

Ectopic clocks also behave like endogenous clocks in the context of clock mutant backgrounds. *cyc* is absolutely required for *Clk*-dependent expression of clock genes, implying CLK/CYC transcriptional activation mediates clock induction. Given how widespread ectopic clocks can be, this suggests either *cyc* is very widely expressed in the fly brain independent of *Clk*, or else that *Clk* can also drive *cyc* expression. We favor the former explanation given the molar excess of *cyc* over *Clk* observed in whole-head experiments (Bae et al., 2000).

Our results also suggest an essential role for the photoreceptor *cry* in mediating coordinate cycling of ectopic clocks. Ectopic clocks fail to cycle coordinately in *cry^b* flies, just as endogenous peripheral clocks do. As temperature entrainment is able to entrain most peripheral clocks in *cry^b* flies (Glaser and Stanewsky, 2005; Stanewsky et al., 1998) with the exception of antennae (Krishnan et al., 2001), this lack of cycling is likely due to a lack of entrainment. Alternatively, *Drosophila cry* also plays a core clock repressor role specifically in peripheral clocks (Collins et al., 2006; Krishnan et al., 2001) as it does in its mammalian counterpart (Kume et al., 1999).

Our work indicates that a broad range of cells can be induced to show clock molecular oscillations. We were able to induce ectopic clocks with a number of drivers with no known circadian function. With *elav-GAL4* the brain as a whole appeared to cycle, indicating many neurons can cycle. This suggests the ability to be a clock is not restricted to a small number of preprogrammed classes of cells. The GAL4-UAS system employed here almost certainly results in tonic transcriptional activation of *Clk* given the stability of GAL4. Yet in the majority of cells we examined, clock gene expression was cycling rather than tonic, consistent with our previous work (Zhao et al., 2003) indicating CLK can transduce tonic transcriptional input into a cycling program. Transgenic *Clk* may indirectly induce rhythmically expressed endogenous *Clk*. However, CLK protein rhythms are substantially intact even with reverse phase *Clk* transcript oscillations (Kim et al. 2002). Moreover, CLK protein does not show a substantial rhythm in pacemaker neurons (Houl et al., 2006). Thus, transcriptional regulation of *Clk* is not necessary for molecular or behavioral rhythms, indicating the importance of posttranscriptional and posttranslational modifications in rhythm generation.

Despite the broad range of *Clk* induction, evidence suggests that other factors are necessary to induce fully functioning clocks because there were groups of cells that expressed PER but did not cycle. These could be truly arrhythmic, or these cells could be asynchronous within their group, reflecting poor entrainment.

Clk does not appear to alter cell fate in its induction of molecular cycling because the ability to initiate oscillations is not developmentally restricted. It can induce normal clock oscillations in differentiated adult cells. These clocks are dependent on transgene expression but fade only gradually after deinduction. Long-term clock maintenance does require continuous *Clk* expression, suggesting there is an activator(s) of *Clk*, as yet undiscovered, that is necessary for clock cell fate determination. We have demonstrated that *Clk* is a unique master regulator of circadian molecular oscillations. *Clk* induces relatively normal entrained PER oscillations broadly in the brain, even in differentiated cells, indicating it is

sufficient in most cells to organize the coordinated cycling of core clock elements. The factors upstream of *Clk* remain to be determined and will be of interest in future work.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Ectopic clock molecular PERIOD (PER) oscillations mimic those of endogenous clocks. (A, A') PER in the endogenous pacemaker small ventral lateral neurons (sLNv) is near peak at ZT1 and trough at ZT13. (B, B') sLNv and ectopic PER in *cry16^{G4}/UClk* flies also oscillates similarly. (C) sLNv PER quantified at 6 time points throughout LD. Both controls and *Clk* overexpressing flies show robust PER oscillations (ANOVA, $p < 0.01$); however, PER peak is nearly double in the latter and is phase-advanced relative to control. sLNv (arrows) were identified by PDF colabeling (data not shown). (D) PER quantified from the region circled in A (ANOVA shows no significant time-dependent differences) and B (ANOVA, $p < 0.01$). Ectopic oscillations in *cry16/UClk* are similar to those in the *Clk* overexpressing sLNv. Nonzero PER intensity in the control *cry16/+* is likely nonspecific

labeling (see text). AU = arbitrary units, $\approx$ % Peak; $\pm$ SEM. See Methods for calculation. See also Supplemental Figure S1.¹

¹Additional data (Supplemental Figures S1, S2, and S3) are available online at <http://jbr.sagepub.com/supplemental>.

Figure 2.
CLOCK uniquely induces ectopic PERIOD (PER) cycling. Other clock genes similarly expressed by *cry16* do not show ectopic PER expression. Shown, peak time point (ZT0–1).

Figure 3.

Ectopic clocks can be induced in adults and require sustained transgene expression. (A, A') *tubGAL80^{ts}/+; cry16GAL4/UClk* flies raised and tested at 18 °C show normal PERIOD (PER) staining and oscillations (ZT1, 9) but no ectopic PER. (B, B') Four days after shifting to 29 °C these flies show robust ectopic clocks indistinguishable from those induced by developmental expression of *Clk* (ZT1, 9). When returned to 18 °C to allow *tubGAL80^{ts}* repression of GAL4, ectopic PER gradually fades. (C, C') Four days after shift back to 18 °C, ectopic PER is present and cycling but beginning to fade (ZT1, 9).

Figure 4.

Different cell type responses to *Clk* expression. *tubGAL80^{ts}* was used to allow adult-specific expression of *Clk* with a variety of GAL4 drivers. Although some cell groups show typical circadian cycling of PER (e.g., *c232GAL4*; ANOVA, $p < 0.01$), other cell types did not show coordinate cycling (e.g., *repoGAL4*; ANOVA, $p < 0.01$, but data highly variable and replicate experiments differed). See also Supplemental Figure S3.

Figure 5.

Genetic requirements are similar for ectopic and endogenous clocks. (A) In a *cyc*⁰¹ background, *Clk* overexpression does not induce ectopic PERIOD (PER); endogenous PER is also lost. (B, B') In *cry*^b mutant flies, ectopic PER is expressed but does not cycle coordinately (ZT1, 14 shown).

Table 1

Circadian Behavioral Analysis

Genotype	Period $\pm$ SEM	Power $\pm$ SEM	% Rhythmic	<i>n</i>
<i>cry16/+</i>	25.8 $\pm$ 0.1	61.5 $\pm$ 5.7	100	29
<i>UClk/+</i>	23.4 $\pm$ 0.0	67.5 $\pm$ 5.6	96.4	28
<i>UClk/cry16</i>	22.3 $\pm$ 0.5	15.6 $\pm$ 3.9	66.7	15
<i>Uvri-1/+</i>	23.6 $\pm$ 0.1	70.8 $\pm$ 8.4	91.7	24
<i>Uvri-1/cry16</i>	26.0 $\pm$ 0.0	3.0 $\pm$ 1.9	3.2	31
<i>Uvri-3/+</i>	23.5 $\pm$ 0.2	53.2 $\pm$ 9.6	74.1	27
<i>Uvri-3/cry16</i>	22.8 $\pm$ 3.3	2.7 $\pm$ 1.4	7.1	28
<i>Utim11-4/+</i>	23.6 $\pm$ 0.1	85.2 $\pm$ 8.4	92.3	26
<i>Utim11-4/cry16</i>	25.3 $\pm$ 0.4	7.9 $\pm$ 4.7	9.7	31
<i>Utim11-5/+</i>	23.8 $\pm$ 0.1	43.0 $\pm$ 7.5	72.4	29
<i>Utim11-5/cry16</i>	N/A	0.3 $\pm$ 0.2	0.0	31
<i>Utimx/Y</i>	23.1 $\pm$ 0.1	114.9 $\pm$ 6.6	100	15
<i>Utimx/Y; cry16/+</i>	N/A	0.2 $\pm$ 0.2	0.0	16
<i>tubG80ts/+; UClk/cry16</i> raised 18°C tested 18°C	23.8 $\pm$ 0.2	20.2 $\pm$ 3.5	72	25

NOTE: Genotype is followed by period $\pm$ standard error of the mean (SEM), power of the rhythm (power significance in Clocklab, Actimetrics) $\pm$ SEM, % rhythmic flies ($P - S \geq 10$), *n* = total number tested.