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VRILLE Feeds Back to Control Circadian Transcription of *Clock* in the *Drosophila* Circadian Oscillator

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Summary

The *Drosophila* circadian oscillator consists of interlocked *period* (*per*)/*timeless* (*tim*) and *Clock* (*Clk*) transcriptional/translational feedback loops. Within these feedback loops, CLK and CYCLE (CYC) activate *per* and *tim* transcription at the same time as they repress *Clk* transcription, thus controlling the opposite cycling phases of these transcripts. CLK-CYC directly bind E box elements to activate transcription, but the mechanism of CLK-CYC-dependent repression is not known. Here we show that a CLK-CYC-activated gene, *vrille* (*vri*), encodes a repressor of *Clk* transcription, thereby identifying *vri* as a key negative component of the *Clk* feedback loop in *Drosophila*'s circadian oscillator. The blue light photoreceptor encoding *cryptochrome* (*cry*) gene is also a target for VRI repression, suggesting a broader role for VRI in the rhythmic repression of output genes that cycle in phase with *Clk*.

Introduction

Circadian clocks are cell-autonomous timekeeping mechanisms found in a diverse array of organisms. The primary role of these molecular timekeepers is to maintain a ~24 hr rhythm that is both self-sustaining and able to regulate various output genes that, in turn, control daily rhythms in physiology and behavior. Though these molecular oscillators persist in constant conditions, external entraining cues such as light and heat input time-of-day and seasonal information to bring the clockworks in line with the local environment.

The *Drosophila* circadian oscillator is comprised of interlocked *per*/*tim* and *Clk* transcriptional/translational feedback loops. In the *per*/*tim* loop, two basic-helix-loop-helix-PAS [PER-ARNT-SIM] transcription factors, CLK and CYC, form heterodimers that bind canonical E box sequences (CACGTG) to activate *per* and *tim* transcription (Allada et al., 1998; Bae et al., 1998; Darlington et al., 1998; Hao et al., 1997; Rutila et al., 1998). Subsequently, PER and TIM proteins accumulate as heterodimers in the cytoplasm after a ~6 hr phosphorylation-dependent delay, then enter the nucleus (Curtin et al., 1995; Kloss et al., 1998; Martinek et al., 2001; Price et al., 1995, 1998; Saez and Young, 1996; Vosshall et al., 1994). PER and/or TIM then bind CLK-CYC, prevent activation of *per*/*tim*, and hence complete the *per*/*tim* negative feedback loop (Bae et al., 2000; Lee et al., 1998, 1999).

PER/TIM interaction with CLK-CYC has the opposite effect on *Clk* transcription because, unlike with *per*/*tim* activation, CLK-CYC somehow repress *Clk* transcription: *Clk* mRNA levels are constitutively high in Clk^{Jrk} and cyc^{01} mutants (Allada et al., 1998; Glossop et al., 1999; Rutila et al., 1998). Thus, in the *Clk* loop, CLK-CYC-dependent repression of *Clk* is relieved (derepressed) by PER/TIM sequestration of CLK-CYC (Glossop et al., 1999). This derepression will result regardless of the mode of CLK-CYC repression, i.e., whether CLK-CYC repress *Clk* directly or indirectly via activation of a repressor. Ultimately, the opposing effect of the (PER/TIM)-(CLK-CYC) interaction on *per*/*tim* and *Clk* transcription ensures that *per*/*tim* and *Clk* mRNA transcripts cycle in opposite phases of the circadian cycle (Glossop et al., 1999).

To further understand the molecular basis of the oscillator, we wanted to determine how CLK-CYC represses *Clk* transcription. A good candidate for the *Clk* repressor is the basic-zipper (b-ZIP) transcription factor *vri*. Initially isolated due to its role in the *decapentaplegic* developmental pathway (George and Terracol, 1997), *vri* was subsequently shown to be a core oscillator component in larval/adult central oscillator cells (the ventral lateral neurons; LN_vs) (Blau and Young, 1999). Although embryonic lethality of *vri*-null mutants prevents direct examination of VRIs molecular role in oscillator cells (George and Terracol, 1997), previous results are consistent with a role for VRI as a repressor of *Clk*: (1) *vri* is activated by CLK-CYC, (2) overexpression of VRI in larval LN_vs causes a reduction/absence of two *Clk*-dependent transcripts (*per* and *tim*), and (3) adult locomotor activity (an LN_v-driven output behavior) shows short period rhythms in $vri^{-/+}$ hemizygotes and long period rhythms in VRI overexpressing flies—indicative of a role for VRI as a repressor (Blau and Young, 1999).

Here we show that VRI is a transcriptional repressor that feeds back to control the rhythmic expression of *Clk* within the circadian oscillator. VRI also controls the circadian transcription of *cry*, which suggests that VRI may control the rhythmic expression of other clock outputs whose RNAs cycle in phase with *Clk*. Thus, these results show that VRI is a potent transcriptional regulator that controls rhythmic gene expression both within the core feedback loop mechanism and downstream of the circadian oscillator.

Results

VRI Protein Abundance Cycles in Opposite Phase to *Clk* mRNA

Previous work has shown that overexpression of VRI in larval oscillator cells leads to low/absent levels of *per* and *tim* mRNA (Blau and Young, 1999). The simplest explanation of this result is that VRI binds *per* and *tim* regulatory elements and represses transcription directly. However, given that *per* and *tim* share a common

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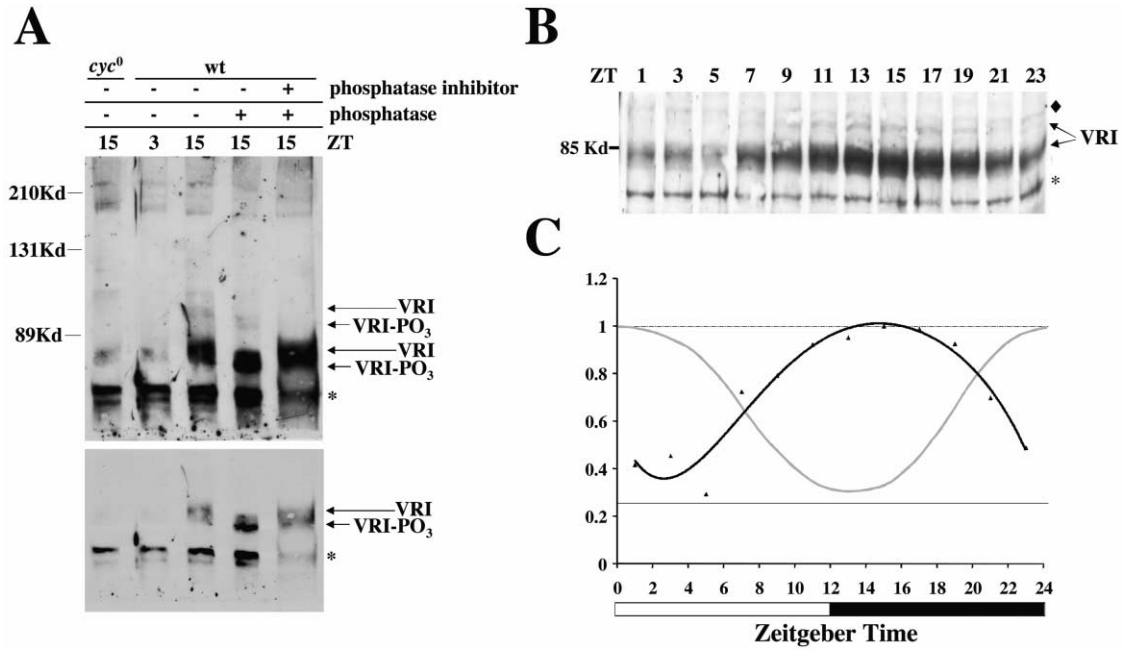

Figure 1. VRI Protein Cycles in Antiphase with *Clk* mRNA

(A) Western blot analysis of untreated, phosphatase-treated, or phosphatase plus phosphatase inhibitor-treated total proteins extracted from the heads of wild-type and *cyc*^{01} mutant flies collected at the indicated times and probed with guinea pig VRI antisera. The lower blot is a shorter exposure of the upper blot. VRI, VRI protein; VRI-PO_3, dephosphorylated VRI; asterisk, nonspecific band; ZT, Zeitgeber time.

(B) Western blot analysis of total protein extracted from heads of wild-type flies collected at 2 hr intervals during an LD 12:12 cycle and probed with guinea pig anti-VRI antiserum. Nonspecific bands are denoted as closed diamond and asterisk.

(C) The relative OD of VRI protein abundance (ratio of VRI/asterisk, closed triangle) was fit using a fifth order polynomial (thick black line) and plotted against a composite *Clk* mRNA (thick gray line) abundance curve. In *cyc*^{01} mutants, VRI protein levels are constitutively low (thin solid line) and *Clk* mRNA transcripts are constitutively high (thin dashed line).

mode of activation, by CLK-CYC, low levels of *per* and *tim* mRNA would also result if VRI repressed *Clk*. Given these alternatives, it is of paramount importance to determine the daily phase of cycling since VRI, which acts as a repressor, is predicted to cycle in the opposite phase as mRNAs of the gene(s) it represses.

Polyclonal antisera were generated against full-length VRI and used for Western blot analysis. In wild-type flies, two VRI-specific bands are detected: a weak band of ~98 kDa and a strong broad band of ~82–89 kDa (Figure 1A). Phosphatase treatment reduces the molecular weight of the weak band to ~95 kDa and collapses the strong broad band to a tight band of ~80 kDa, which matches the predicted molecular weight of VRI. As expected, VRI levels are low in *cyc*^{01} mutant flies since *vri* mRNA expression is CLK-CYC dependent. In addition, VRI levels are lower at ZT 3 than at ZT 15 in wild-type flies, suggesting that VRI levels cycle. These results demonstrate that our antisera detect VRI, that VRI is a phosphoprotein, and that VRI levels are lower in the light phase than in the dark phase.

To more precisely determine the phase of VRI cycling, we measured VRI in wild-type flies collected every 2 hr in LD. VRI levels peak during the early night (ZT 13–17) and reach trough levels during the early day (ZT 01–05; Figure 1B). A similar cycling phase for VRI persists in constant darkness (data not shown). This cycling profile closely follows that of *per*, *tim*, and *vri* mRNA transcripts and is opposite that of *Clk* mRNA, which peaks between ZT 22 and 04 and falls to low levels between ZT 10 and 16 (Figure 1C). VRI protein therefore cycles in opposite phase to *Clk* mRNA, which supports a role for VRI as the *Clk* repressor.

Circadian Regulatory Sequences from *Clk* Contain Multiple E4BP4-Consensus Binding Sites

Transcription from the *Clk* gene is initiated at two sites: a minor site, which has been detected only through 5′ RACE and RT-PCR, and a major start site that accounts for the vast majority of *Clk* transcription in heads (Allada et al., 1998; Bae et al., 1998; Darlington et al., 1998). Based on the available *Clk* cDNAs, the minor transcript includes an additional 5′ exon plus the entire major transcript due to the removal of a ~5 kb intron between the exons initiated at the minor (first) and major (second) transcription start sites (Figure 2A). To identify a region that mediates *Clk* circadian transcription, a genomic DNA fragment from −8000 to +40 (the major *Clk* transcription start = +1) was used to drive Gal4 mRNA transcription in transgenic flies containing a functional clock. This fragment mediated rhythmic Gal4 mRNA transcription having the same phase and amplitude as *Clk* transcripts in wild-type flies, thus demonstrating that all the necessary circadian regulatory elements are present (Figure 2B).

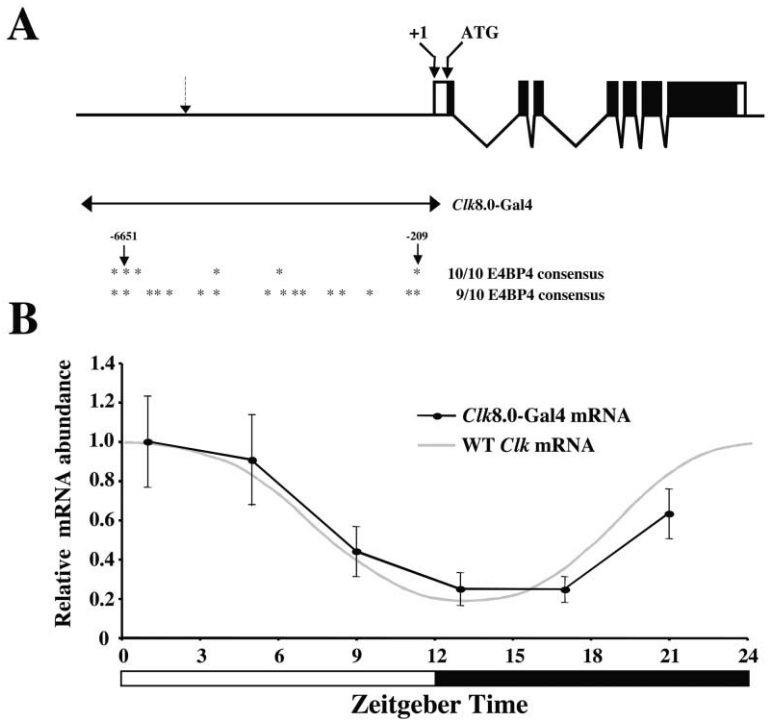

Figure 2. The *Clk* Regulatory Region Contains Multiple E4BP4-Consensus Sites

(A) Schematic of the *Clk* genomic locus. +1, the major transcription start site; ATG, start of translation; dashed arrow, minor transcription start site; white boxes, untranslated exon from major transcript; black boxes, translated exons; bent lines, introns; straight lines, untranscribed sequence. The arrow-tipped line represents *Clk* genomic sequences contained in the *Clk*8.0-Gal4 transgene. Asterisks show regions that have high similarity (9/10 or 10/10 nucleotide identity) with the consensus E4BP4 binding site in either orientation. Numbers above asterisks are based on the location of the +1 sense residue of the 3′ half site in relation to the major transcription start site and denote two sites used for EMSA analysis. (B) An 8.0 kb *Clk* genomic region contains regulatory elements sufficient for circadian transcription. Quantification of Gal4 mRNA levels from wild-type flies containing the *Clk*8.0-Gal4 transgene. RNase protection assays were performed on head mRNA from flies collected at the indicated times under LD conditions. The peak Gal4 mRNA level (black line) was normalized to 1.0. Gal4 mRNA levels are based on six independent experiments using three independent lines and plotted along with a composite *Clk* mRNA curve (gray line) from wild-type flies (Williams and Sehgal, 2001). White and black boxes represent times when lights were on or off, respectively.

For VRI to be a direct repressor of *Clk*, the 8.0 kb *Clk* genomic fragment should contain binding sites for VRI. The consensus binding site for VRI has not been determined; however, the basic (DNA binding) domain shares >85% homology with that of mammalian E4BP4, and hence, VRI should bind similar DNA sequences as E4BP4 (George and Terracol, 1997). The optimal binding site for E4BP4 has been determined as 5′-(A/G)TTAC: (A/G)T(A/C)A(A/T/C)-3′ (Cowell et al., 1992). A search for E4BP4-consensus sites at the *Clk* locus identified multiple sites on both DNA strands upstream of the major transcription start site (Figure 2A). This density of E4BP4 sites is much higher than predicted by random chance, where 10/10 sites should be present every ~22,000 bp and 9/10 sites would be present every ~8,000 bp on average. Searches of the *per* and *tim* loci (i.e., 4 kb upstream through intron 1) identified no 10/10 or 9/10 E4BP4 sites (not shown), which is in line with the number of sites predicted by random chance. The lack of E4BP4 binding sites and the phase of VRI cycling are inconsistent with VRI directly repressing *per* and *tim*, but do support the possibility that VRI directly represses *Clk*.

VRI Binds E4BP4-Consensus Sites from *Clk*

Having identified E4BP4/VRI sites at the *Clk* locus, we used electrophoretic mobility shift assays (EMSAs) to determine if VRI can form protein-DNA complexes with these *Clk*-derived sequences. For the two sites analyzed in this way (−6651*Clk* and −209*Clk*), shifted complexes were observed when VRI was incubated with wild-type probe, but did not bind probes with mutated E4BP4 sites (Figure 3A). Consistent with this, VRI complexes were effectively competed by wild-type, but not mutant, E4BP4 sites. VRI protein that lacked the basic domain (VRIΔb) did not form a shifted complex when used in conjunction with the wild-type probes, a result indistinguishable from unprimed reticulocyte lysate controls. Further, a strong shifted complex was also observed when purified, baculovirus-expressed VRI was incubated with wild-type, but not mutant, −6651*Clk* probe (Figure 3B). Addition of anti-VRI antiserum, but not preimmune serum, to the reaction completely blocked the mobility shift due to purified VRI. VRI antiserum also blocked binding of rabbit reticulocyte lysate-generated VRI to the −6651*Clk* and −209*Clk* probes (data not shown). Taken together, these data demonstrate that VRI specifically binds to consensus E4BP4 binding sequences and that the basic domain of VRI is essential for binding.

Additional mutants of the −6651*Clk* site were generated to further address the specificity of VRI binding (Figure 3C). GC or AT content of the mutant binding site did not affect nonspecific binding of VRI to the probe, as no shifted complex was observed for either mutation. Further, both half sites are required for high-affinity binding by VRI, as mutation of one of the half sites dramatically reduced the shifted complex. The specificity of VRI for the E4BP4 site contained within the probe is apparent because a single base change (T-G) at the −4 position of the −6651*Clk* probe almost completely eliminated the shifted complex. Similar results were obtained when we challenged radio-labeled wild-type probe with an excess of nonlabeled competitor (data not shown).

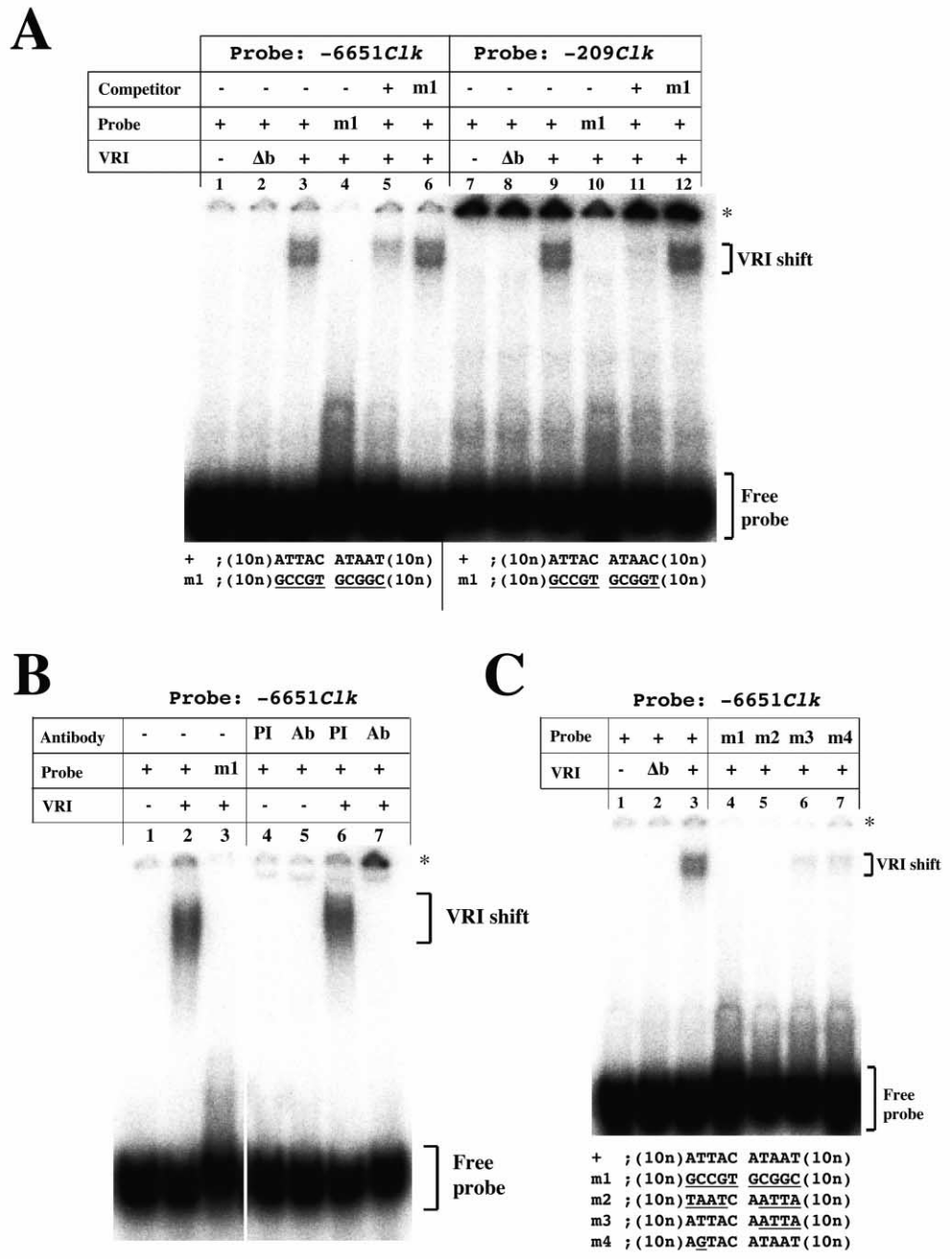

Figure 3. VRI Binds E4BP4-Consensus Sequences from the *Clk* Locus

EMSAs showing VRI-specific binding of E4BP4-consensus sites from the *Clk* locus. Each set of experiments was repeated at least three times with similar results.

(A) The proteins used were wild-type VRI generated in rabbit reticulocyte lysates (+); basic domain-deleted version of VRI generated in rabbit reticulocyte lysate (Δb); and unprimed lysate (−). The probes were wild-type (+) or mutant (m1) −6651*Clk* and wild-type (+) or mutant (m1) −209*Clk*. Competition experiments were done with >100-fold excess of unlabeled wild-type (+) or mutant (m1) −6651*Clk*, wild-type (+) or mutant (m1) −209*Clk*, or no competitor (−). VRI shift, VRI shifted product; Free probe, unbound probe; asterisk, probe remaining in the well.

(B) EMSAs showing VRI antisera block binding of VRI. Baculovirus-generated VRI was purified and used in EMSAs with the −6651*Clk* probe. Ab, VRI antiserum; PI, preimmune serum. Symbols are as described in (A).

(C) EMSAs defining sites important for VRI binding. Three additional mutants (m2, m3, and m4) were generated in the context of the −6651*Clk* site to use as probes for VRI binding. Symbols are as described in (A).

To obtain a rough consensus for VRI and to determine which *Clk* sequences contain the highest-affinity targets, we competed the −6651*Clk* and −209*Clk* radiolabeled probes with nonlabeled oligonucleotides from other natural E4BP4 target sites at the *Clk* locus (see Figure 2) and with oligonucleotides containing single-

point mutations at each base throughout the E4BP4 consensus in the context of the −6651*Clk* probe (data not shown). From this crude analysis, we found little difference in VRI binding between different natural E4BP4 target sites; however, this result is not surprising since these sequences were initially isolated due to their similarity with the E4BP4-consensus. With the mutant probes, VRI was sensitive to the same nucleotide changes found to be important for E4BP4 binding (Cowell et al., 1992), and thus the VRI consensus is essentially identical to that of E4BP4. Like many other b-ZIP proteins, E4BP4 binds as a homodimer to control transcription (Cowell et al., 1992; Hurst, 1994). The necessity of both half-sites for VRI binding, the near-perfect palindromic nature of the VRI binding site, and the similarity in sequence to the E4BP4 binding site suggest that VRI binds as a homodimer.

Overexpression of VRI Causes a Reduction in *Clk* mRNA Levels

Having determined that VRI is able to bind *Clk* sequences in vitro and that VRI protein is present in heads at times when *Clk* is being repressed, we wanted to determine if VRI represses *Clk* in vivo. Overexpression of VRI leads to low levels of *per* and *tim* expression in larvae and long period rhythms or arrhythmicity in adults (Blau and Young, 1999), consistent with our hypothesis that VRI represses *Clk* transcription. To determine the effect of VRI overexpression on *Clk* expression in vivo, we overexpressed *vri* in a wild-type background using *tim*-Gal4 driver (Stanewsky et al., 1998), UAS-Gal4 sustainer (Hassan et al., 2000), and UAS-*vri* responder transgenes (Blau and Young, 1999), and we monitored VRI protein and *Clk* mRNA levels. These triply transgenic flies showed long period (26 hr–28 hr) or arrhythmic behavioral activity (data not shown) and produced constant high levels of VRI that were several fold higher than the wild-type VRI peak value (Figure 4A). The high levels of VRI in these flies reduced *Clk* mRNA levels considerably; peak *Clk* mRNA levels were reduced to only ~50% the wild-type peak value, whereas trough levels were essentially the same as those of wild-type flies (Figure 4A). Given that the wild-type *Clk* mRNA cycling amplitude is approximately 4-fold (i.e., 25% to 100% peak levels), VRI overexpression represses *Clk* activation by about two-thirds (i.e., 25% to 50% the wild-type peak value). Although the amplitude of *Clk* cycling is reduced to ~2-fold, *Clk* mRNA still cycles with normal phase in these flies under LD conditions.

Since an increase in VRI strongly represses *Clk*, it was of interest to determine whether a loss of *vri* function would increase *Clk* mRNA levels. Since homozygous *vri* mutants are embryonic lethals (George and Terracol, 1997), we employed loss of function vri^2 mutant/+ heterozygotes, which have behavioral rhythms ~1 hr shorter than wild-type flies (Blau and Young, 1999). VRI levels in vri^2/+ flies were not obviously lower than those in wild-type flies (Figure 4A). However, *Clk* mRNA levels in vri^2/+ flies tended to be higher than those in wild-type flies and were significantly higher than those in wild-type at ZT21. Moreover, *Clk* mRNA levels in vri^2/+ flies were also significantly higher than *Clk* mRNA in flies overexpressing VRI at ZT1, ZT5, ZT17, and ZT21 (Figure 4A). Thus, a reduction in VRI levels leads to an increase in *Clk* mRNA levels, mainly during the mid to late evening. This result is consistent with VRI acting as a repressor of *Clk* transcription.

Although these results are consistent with VRI acting to repress *Clk* transcription, it is possible that VRI may not repress *Clk* directly in these flies, but rather through other genes within the feedback loop. For instance, if VRI were directly repressing *per* and/or *tim*, the reduced levels of PER and TIM would indirectly feed back to reduce the levels of *Clk* expression (Glossop and Hardin, 2002). Since it is not possible to distinguish among these possibilities in the presence of a functional feedback mechanism, we designed an experiment in which oscillator-independent action of VRI on *Clk* transcription could be tested in vivo.

Heat-Inducible Expression of VRI in cyc^{01} Mutants Causes a Reduction in *Clk* mRNA Levels

In the nonfunctional Clk^{Jrk} and cyc^{01} mutants, the levels of *Clk* mRNA are at their peak at all times of the circadian cycle (Glossop et al., 1999). This result implies that *Clk* is consitutively activated at all times of the circadian cycle and that CLK-CYC-dependent rhythms in *Clk* transcription occur through rhythmic repression. Since *per* and *tim* activation is also eliminated in Clk^{Jrk} and cyc^{01} flies (Allada et al., 1998; Rutila et al., 1998), we can test the ability of VRI to directly repress *Clk* expression in the absence of a functional feedback loop, thus eliminating indirect effects of VRI through *per* and *tim*.

To overexpress VRI, we generated several independent lines of flies carrying a heat-inducible *vri* transgene. These transgenes were crossed into cyc^{01} mutant flies and used to determine if *vri* induction reduced the level of *Clk* mRNA (Figure 4B). High levels of VRI were produced in heads by 3 hr after either 30 min or 15 min of heat induction and dropped to baseline levels by 24 hr. Importantly, heat induction had no affect on VRI levels in heat-induced cyc^{01} flies. After a 30 min heat induction, *Clk* mRNA levels were reduced about 3-fold compared to non-heat-induced hs-*vri*;cyc^{01} or heat-induced cyc^{01} controls. In addition, *Clk* mRNA levels were maximally repressed by 3 hr after the 30 min heat induction ended, then recovered to ~60% of the initial (i.e., non-heat-induced) *Clk* levels by 8 hr after heat induction. After a 15 min heat induction, *Clk* mRNA levels progressively decreased to ~40% of the non-heat-induced hs-*vri*;cyc^{01} or heat-induced cyc^{01} controls, then returned to the initial non-heat-induced *Clk* levels by 24 hr after heat induction. These results demonstrate that the feedback loop is not required for VRI to repress *Clk* expression, which indicates that VRI directly represses *Clk*. Taken together with the VRI overexpression results in wild-type flies, the phase of VRI cycling, and the ability of VRI to bind target sequences within the *Clk* circadian regulatory region, the VRI heat induction results in cyc^{01} flies suggest that VRI mediates *Clk* mRNA cycling via daily repression of *Clk* transcription within the core of the interlocked feedback loops.

VRI Binds E4BP4-Consensus Sites from the *cry* Locus and Represses *cry* Transcription In Vivo

VRI-dependent repression of *Clk* leads to *Clk* mRNA cycling that peaks in the late night/early morning, which

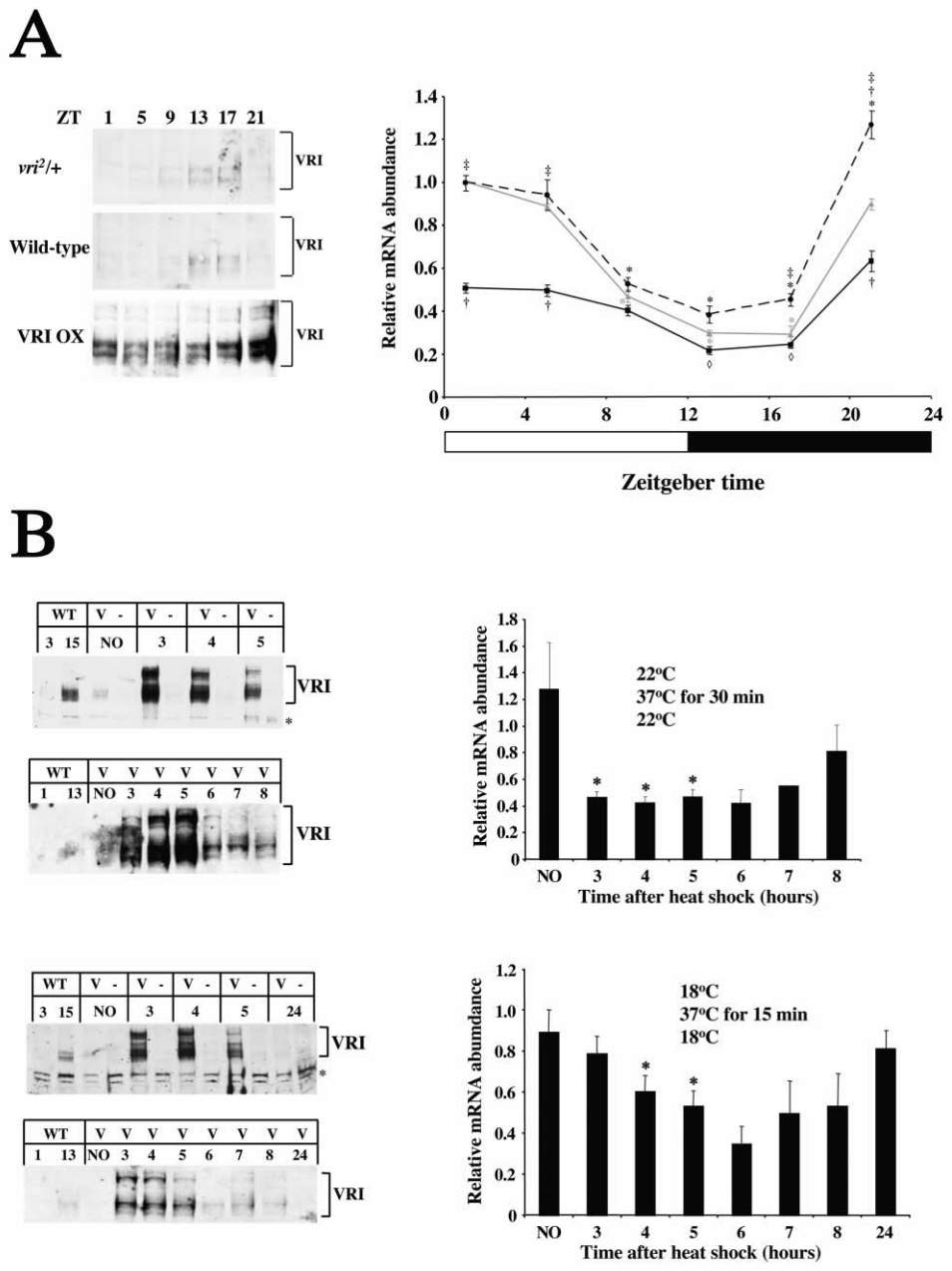

Figure 4. Overexpression of VRI In Vivo Causes a Reduction in *Clk* mRNA Levels

(A) Overexpression of VRI in wild-type flies. Wild-type flies, flies overexpressing VRI (VRI OX), and heterozygous *vri* mutant flies ($vri^2/+$) were entrained for at least 3 days to an LD 12:12 cycle and collected at the indicated time points. Western blots showing relative VRI levels (left) and quantified ribonuclease protection assays (RPAs) showing the relative levels of *Clk* mRNA (*Clk*/*rp49* ratio ± SEM) plotted against time of day (ZT) (right) are shown. Black line, *Clk* mRNA levels in flies overexpressing VRI; gray line, *Clk* mRNA in wild-type flies; dashed line, *Clk* mRNA in $vri^2/+$ flies. RNA levels for each genotype are relative to the *Clk* mRNA peak at ZT 01 in wild-type flies, which was set to 1.0. Black asterisk, *Clk* mRNA values that are significantly different ($p < 0.0004$) from those at ZT 01 in $vri^2/+$ flies; gray asterisk, *Clk* mRNA values that are significantly different ($p < 0.0002$) from those at ZT 01 in wild-type flies; open diamond, *Clk* mRNA values that are significantly different ($p < 0.0002$) from those at ZT 01 in VRI OX flies; dagger, *Clk* mRNA values in VRI OX and $vri^2/+$ flies that are significantly different ($p < 0.0002$) than the values for wild-type flies at the same time point; double dagger, *Clk* mRNA values in $vri^2/+$ flies that are significantly different ($p < 0.02$) than the values for VRI OX flies at the same time point. The data represent six independent experiments for VRI OX flies, five independent experiments for wild-type, and four independent experiments for heterozygous *vri* mutant flies.

(B) Overexpression of VRI in cyc^{01} flies. cyc^{01} flies containing a heat-inducible *vri* transgene (hs-*vri*) and cyc^{01} controls were reared in constant light (LL) at 22°C, heat shocked at 37°C for 30 min, and allowed to recover at 22°C in LL (upper Western blots and RPA quantification) or reared in constant light at 18°C, heat shocked at 37°C for 15 min, and allowed to recover at 18°C in LL (lower Western blots and RPA quantification). hs-*vri*;cyc^{01} (V) or cyc^{01} (−) flies were collected before heat induction (NO) or 3 hr, 4 hr, 5 hr, 6 hr, 7 hr, 8 hr, or 24 hr after heat

is antiphase to that of *per*, *tim*, and *vri* mRNA cycling. In addition to these core feedback loop components, many additional genes are under clock control (Claridge-Chang et al., 2001; Lin et al., 2002; McDonald and Rosbash, 2001; Ueda et al., 2002). Since a subset of these clock-controlled genes are transcribed in phase with *Clk*, it is possible that some or all of these genes are regulated via VRI-dependent repression. The *cryptochrome* (*cry*) gene, which encodes a blue light photoreceptor (Egan et al., 1999; Emery et al., 1998; Ishikawa et al., 1999; Stanewsky et al., 1998), is a good candidate for VRI-dependent repression because *cry* mRNA cycles in phase with that of *Clk*, and *cry* mRNA is at peak levels in cyc^{01} or Clk^{Jrk} mutants (Emery et al., 1998).

A search of the *cry* locus revealed the presence of numerous VRI binding sites that have 10/10 and 9/10 base identity with the E4BP4 DNA binding consensus (Figure 5A). A *cry*-gal4 transgene containing ~4.5 kb of *cry* upstream sequence and the first intron is expressed in several groups of clock neurons in the adult brain (Figure 5A; Emery et al., 2000b). To determine whether *cry* transcriptional regulatory elements are present in this transgene, we tested whether Gal4 reporter gene mRNA cycled in abundance. Indeed, *cry*-driven Gal4 mRNA cycled with a similar amplitude but an earlier phase, compared to endogenous *cry* mRNA (Figure 5B), indicating that circadian regulatory elements are present in this genomic region. Oligonucleotide probes containing three of the E4BP4 sites were generated for EMSA analysis. To determine which binding sites from *cry* were bound as strongly as those from *Clk*, competition experiments were done using 5 ng of unlabeled E4BP4 binding sites, since this amount of −6651 *Clk* led to a 50% reduction in shifted product. E4BP4 binding sites from the *cry* locus competed as well (2 and 3) or better (1) than −6651*Clk* (Figure 5C), demonstrating that these sites are efficiently bound by VRI.

Since VRI binds to E4BP4-like sites at the *cry* locus, we reasoned that overexpressed VRI would repress *cry* transcription. VRI was overexpressed in a wild-type background using *tim*-Gal4 driver, UAS-Gal4 sustainer, and UAS-*vri* responder transgenes as before, and *cry* mRNA levels were monitored under LD conditions. Under these conditions, the phase of *cry* mRNA cycling was unaltered, but cycling amplitude was reduced because peak *cry* mRNA levels were 50% lower while trough levels were unchanged (Figure 5D). Thus, as with *Clk* mRNA, VRI repressed *cry* mRNA at times when VRI is not normally present, but did not further repress *cry* at times when VRI repression is already maximal, indicating that VRI represses *cry* transcription.

Discussion

VRI as a Bridge between the Interlocked Feedback Loops of the *Drosophila* Oscillator

Here we demonstrate that the b-ZIP transcription factor VRI feeds back to control circadian transcription of *Clk* within the oscillator mechanism. Previous results showed that CLK-CYC heterodimers activate *per* and *tim* transcription at the same time that they repress *Clk* transcription, thus providing a bidirectional switch that mediates the opposing cycling phases of these transcripts within the circadian oscillator (Glossop et al., 1999). Since there are no canonical (CACGTG) E boxes within the region known to mediate *Clk* mRNA cycling (N.R.J.G. and P.E.H., unpublished observations), the simplest interpretation of these results is that CLK-CYC also activate an intermediate that feeds back to repress *Clk* transcription. We have developed a model to explain this regulatory mechanism, which proposes that VRI functions to repress *Clk* transcription. Positive drive by CLK-CYC and negative drive by PER and TIM confer *vri* mRNA and protein rhythms, and rhythms in VRI accumulation in turn mediate the rhythmic repression of *Clk* (Figure 6).

Several lines of evidence support this model. First, *vri* transcription is activated by CLK-CYC via E box elements in its upstream sequence (Blau and Young, 1999), showing that *vri* could act as a CLK-CYC-dependent intermediate factor. Second, VRI overexpression leads to the repression of *per* and *tim* (Blau and Young, 1999). Since *per* and *tim* both rely upon *Clk* for their activation, VRI-dependent inhibition of *Clk* could readily account for their coordinate repression. That VRI acts as a repressor within the oscillator mechanism is further supported by genetic analysis: VRI overexpression leads to long period activity rhythms (via reductions in *per* and *tim* expression), and reduced *vri* copy number leads to short period activity rhythms (presumably through increases in *per* and *tim* expression) (Blau and Young, 1999). Third, *vri* mRNA and protein cycle in the same phase. With this phase relationship, VRI accumulates to high levels as *Clk* mRNA drops to low levels. After a substantial (~6 hr) delay, PER and TIM inhibit CLK-CYC activation of *vri*, consequently reducing VRI to low levels as *Clk* mRNA accumulates to high levels. Once PER and TIM are degraded, the next cycle of *vri* expression is initiated (Figure 6B). Fourth, VRI binds to sequence elements within the *Clk* circadian control region in vitro, which suggests that VRI action is direct. Fifth, VRI overexpression reduces *Clk* mRNA levels in vivo. This VRI-dependent repression preferentially affects the peak levels of *Clk* mRNA in wild-type animals, which suggests that normal peak levels of VRI are maximally active since

induction and used to determine the levels of VRI. Wild-type (wt) flies were collected at ZT 3 and ZT 15 to compare levels of VRI. Two independent VRI time courses are shown for each heat shock regime. Total head mRNA was subjected to RPA using *Clk* and *rp49* probes to determine relative levels of *Clk* mRNA. Quantification of *Clk* mRNA levels is shown for heat-induced hs-*vri*;cyc^{01} flies normalized to the level of *Clk* mRNA in heat-shocked cyc^{01} flies at each time point. In the 30 min heat shock regime, the no, 3 hr, 4 hr, and 5 hr time points represent three independent experiments (error bars = SEM), and the 6 hr, 7 hr, and 8 hr time points represent two independent experiments (error bars = range). In the 15 min heat shock regime, the no. 3 hr, 4 hr, and 5 hr time points represent three independent experiments (error bars = SEM) and the 6 hr, 7 hr, and 8 hr time points represent two independent experiments (error bars = range). Asterisk, *Clk* mRNA values for the time points that could be statistically analyzed (i.e., no, 3 hr, 4 hr, and 5 hr for the 30 min heat shock and no, 3 hr, 4 hr, 5 hr, and 24 hr for the 15 min heat shock) that were significantly different ($p < 0.02$) than the non-heat-induced controls.

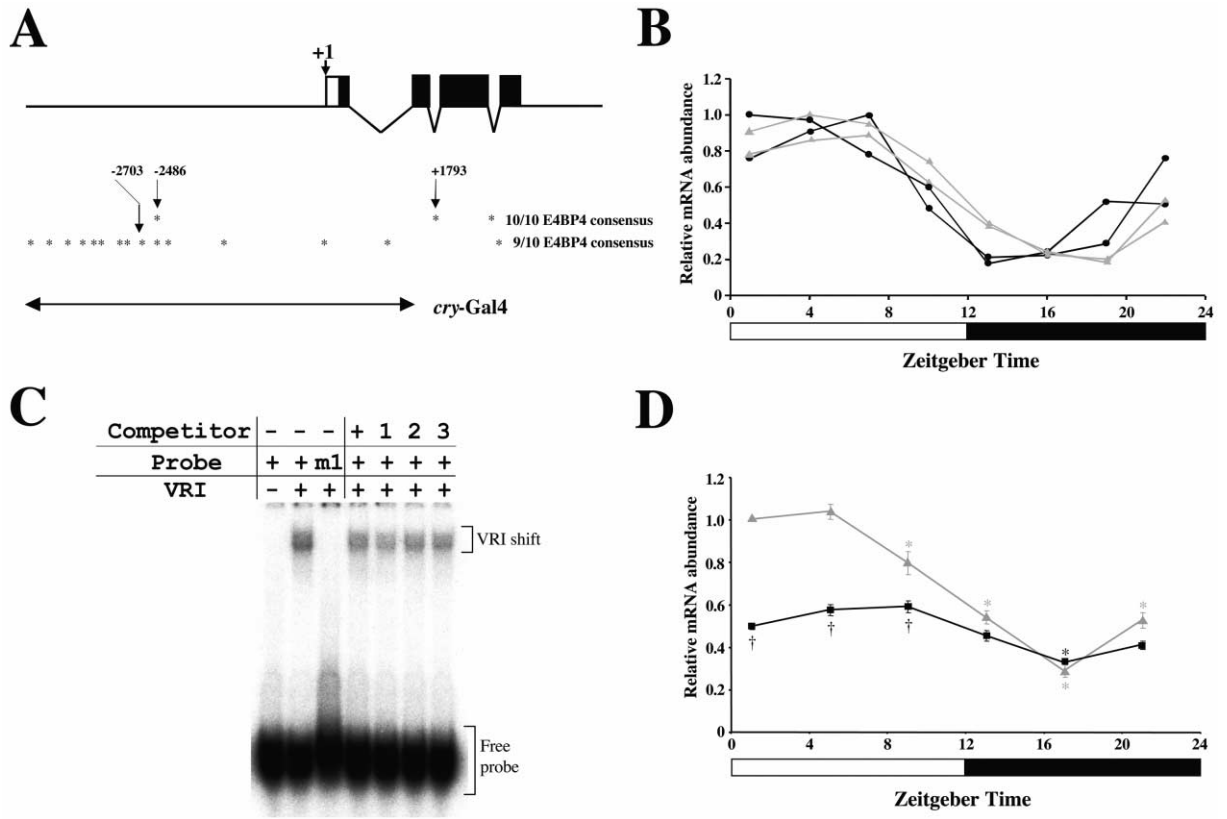

Figure 5. VRI Mediates *cry* mRNA Cycling

(A) Schematic of the *cry* genomic region. Symbols are as described in Figure 2A, except the arrow-tipped line represents *cry*Gal4 (Emery et al., 2000b).

(B) A 6.0 kb *cry* genomic region contains regulatory elements sufficient for circadian transcription. Quantification of Gal4 (black line) or *cry* (gray line) mRNA levels from wild-type flies containing the *cry*Gal4 transgene (Emery et al., 2000b). RNase protection assays were performed on head mRNA from flies collected at the indicated times under LD conditions. The peak Gal4 and *cry* mRNA levels were normalized to 1.0. White and black boxes represent times when lights were on or off, respectively. Two independent experiments are shown.

(C) VRI binds E4BP4-consensus sites in the *cry* promoter. EMSA of competitions using 0.5 pmol of −6651*Clk* (+) or *cry* sites 1, 2, or 3. Symbols are as described in Figure 3.

(D) Overexpression of VRI reduces *cry* mRNA in vivo. Flies were entrained and collected as in Figure 4A. RPAs were performed and plotted as in Figure 4A, except *cry* and *rp49* probes were used. Black line, *cry* mRNA levels in flies overexpressing VRI; gray line, *cry* mRNA in wild-type flies. RNA levels are relative to *cry* mRNA at ZT 01 in wild-type flies, which was set to 1.0. Gray asterisk, *cry* mRNA values that are significantly different ($p < 0.0006$) from those at ZT 01 in wild-type flies; black asterisk, *cry* mRNA values that are significantly different ($p < 0.03$) from those at ZT 01 in VRI overexpression flies; dagger, *cry* mRNA values in VRI overexpression flies that are significantly different ($p < 0.0005$) than the values for wild-type flies at the same time point. The data represent six independent experiments for the VRI overexpression flies and five independent experiments for wild-type flies.

additional VRI cannot further reduce trough levels of *Clk*. VRI also represses the peak levels of *Clk* mRNA present in *cyc*^{01} animals. Since *cyc*^{01} flies lack a functional feedback mechanism due to the absence of CYC, PER, TIM, and VRI, this result indicates that VRI represses *Clk* transcription directly in vivo rather than through other components of the feedback loop. The repression of *Clk* by VRI indicates that rhythmic *Clk* transcription occurs through the circadian repression of *Clk*. This is different than the situation with *per*, *tim*, and *vri*, where circadian transcription is mediated by rhythmic CLK-CYC-dependent activation and PER-TIM-dependent repression.

VRI overexpression in wild-type or *cyc*^{01} flies produces more VRI than the wild-type peak, yet *Clk* and *cry* mRNAs are not fully repressed to wild-type trough levels. The inability of high VRI levels to fully repress *Clk* and *cry* suggests that VRI may act in concert with another factor to repress transcription (Figure 6A). Constant high levels of VRI do not fully repress *Clk* and *cry* expression in wild-type flies during the late evening and early morning. This temporal difference in the ability of VRI to repress implies that another repressor is present in limiting amounts at these times of the circadian cycle, i.e., a second rhythmically expressed repressor. Likewise, the inability of high levels of VRI to repress *Clk* completely in *cyc*^{01} flies suggests that the complimentary repressor is only present in limiting amounts in this genotype. Given that VRI is a bZIP transcription factor, it is tempting to speculate that VRI forms a heterodimer with another bZIP transcription factor to fully repress *Clk* and *cry* transcription. However, no such factor has been identified and it is also possible that another *Clk* and *cry* repressor acts independently of VRI. Although VRI

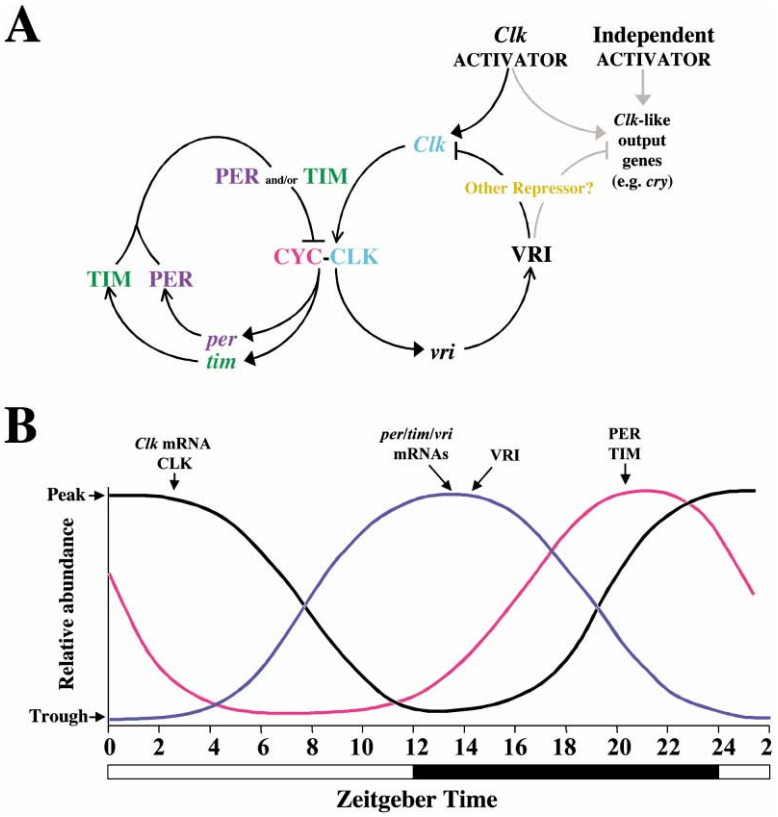

Figure 6. Model of the Interlocked Feedback Loop Mechanism in *Drosophila*

(A) The *per/tim* loop (left) and *dClk* loop (right) are shown. In the *per/tim* loop, CLK-CYC heterodimers activate *per* and *tim* transcription via E boxes in the *per* and *tim* promoters. PER and/or TIM then bind CLK-CYC, repressing this activation and thus completing the *per/tim* negative feedback loop. In the *Clk* loop, CLK-CYC heterodimers activate *vri* transcription via E boxes in the *vri* promoter. VRI then binds VRI boxes in the *Clk* promoter and represses transcription, possibly in concert with a second repressor, thus completing the *Clk* negative feedback loop. PER/TIM positive regulation of *Clk* occurs indirectly by preventing CLK-CYC from activating *vri*. VRI also rhythmically represses VRI box-containing output genes whose mRNA transcripts cycle in phase with *Clk* (e.g., *cry*). Such genes may share a common activator with *Clk,* or they may be activated by independent factor(s). Filled arrows, transcriptional activation; open arrows, translation; bars, repression; gray arrow, output activation; gray bar, output repression.

(B) Phases of clock gene product cycling. *per*, *tim*, and *vri* are activated by CLK-CYC in the early morning (ZT 04; blue line) and reach peak levels in the early evening (ZT 14). As *per/tim/vri* mRNA levels rise, *Clk* mRNA levels fall (black line) due to repression by rising levels of VRI (ZT 04, blue line). Unlike VRI, PER and TIM don't start accumulating until ZT 12 and reach high levels by ZT 18. Rising levels of PER-TIM bind CLK-CYC and prevent activation of *per*, *tim*, and *vri*, hence, *per/tim/vri* transcripts begin to decline during the early/mid-night (ZT 16; blue line). Declining levels of *vri* mRNA and protein allow *Clk* transcription to resume (ZT 18; black line). CLK-CYC dimers then begin to accumulate but are sequestered by PER/TIM during the late night/early morning (ZT 21–ZT 02, pink line), thus preventing premature *per/tim/vri* activation and *Clk* repression. When PER/TIM falls to trough levels (ZT 04; pink line), CLK-CYC is released and activates *per*, *tim*, and *vri*, thereby starting the next cycle. Open bar, lights on; closed bar, lights off.

alone cannot fully repress *Clk*, the ability of VRI to repress *Clk* activation by two-thirds indicates that VRI is the major *Clk* repressor.

The mechanism of VRI repression is not known, but could be achieved in two different ways. VRI could bind to its target sequences and directly affect chromatin structure or the basal transcriptional machinery to effect repression (i.e., active repression) (Johnson, 1995; Maldonado et al., 1999). Alternatively, VRI may simply bind the same consensus sequence(s) as the *Clk* activator, thus repressing *Clk* by blocking activator-DNA interactions (passive repression) (Johnson, 1995; Maldonado et al., 1999). Distinguishing among these possibilities will be important to determine whether transcriptional repression can be completely separated from transcriptional activation, which would further set this mechanism apart from that controlling *per*, *tim*, and *vri* mRNA rhythms.

With the identification of VRI as a repressor of *Clk* transcription, it is apparent that CLK and CYC function to activate a set of repressors that act on different targets at different times in the circadian cycle (Figure 6B). Activation of *vri* by CLK-CYC leads to the immediate production of VRI, which represses *Clk* transcription and consequently reduces the levels of CLK (and necessarily CLK-CYC). Activation of *per* and *tim* by CLK-CYC leads to the delayed accumulation of PER-TIM heterodimers. This delayed PER-TIM accumulation allows VRI to repress *Clk* from midday to early evening (ZT4 to ZT16) and inhibits the ability of newly generated CLK to activate *per* and *tim* expression until early morning (ZT4). This difference in accumulation between VRI and PER/TIM is therefore critical for controlling the opposite cycling phases of *Clk* and *per/tim/vri* within the interlocked feedback loop mechanism.

The Function of VRI-Dependent Repression

Previous analyses have shown that *vri* overexpression can slow down or stop circadian oscillator function (Blau and Young, 1999). Since *vri* mutants are embryonic lethals (George and Terracol, 1997), their effect on circadian clock function cannot be tested in adults. However, reducing the dosage of *vri* leads to period shortening of 0.5–1 hr (Blau and Young, 1999) and modest increases in the level of *Clk* mRNA toward the end of the dark phase (Figure 4A). If VRI-dependent repression of *Clk* were eliminated altogether, it would probably not severely perturb the oscillator, since feedback via PER and TIM could maintain oscillator function. Indeed, increasing *Clk* mRNA to high levels at inappropriate times using the *per* or *tim* promoters had little effect on oscillator function because posttranscriptional mechanisms appear to maintain proper CLK cycling (Kim et al., 2002). However, increased *Clk* mRNA levels are not without effect since light-induced behavioral activity is prolonged in *per*-driven *Clk* flies (Kim et al., 2002), and the

period is reduced by ~1 hr in *tim*-driven *Clk* transgenic flies (F.S.N., N.R.J.G., and P.E.H., unpublished results). Thus, although *vri* is an integral component of the circadian oscillator mechanism, it is not required for oscillator function per se.

In addition to its function within the interlocked feedback loop, *vri* also controls rhythmic transcription downstream of the oscillator. Control of *cry* mRNA cycling is particularly interesting because *cry* encodes a photoreceptor that also serves to input light information to the oscillator (Emery et al., 1998, 2000a, 2000b; Stanewsky et al., 1998). CRY levels change with respect to light intensity rather than *cry* mRNA abundance (Emery et al., 1998; Lin et al., 2001), which indicates that the clock controls *cry* mRNA cycling but not CRY levels. The classification of CRY as a clock input is complicated by its light-independent role, which is required for circadian oscillator function in certain tissues (Ivanchenko et al., 2001; Krishnan et al., 2001). In constant darkness, CRY levels are constitutively high, but *cry* mRNA continues to cycle. This uncoupling of *cry* mRNA and protein levels indicates that while *cry* mRNA cycling is a clock output, it appears to be irrelevant to CRY's light-dependent or light-independent functions.

There are a number of mRNAs that are regulated similarly to *cry* and *Clk* based on microarray studies (Claridge-Chang et al., 2001; Lin et al., 2002; McDonald and Rosbash, 2001; Ueda et al., 2002). For instance, *low density lipoprotein receptor* (*ldlr*), *immune deficiency* (*imd*), and *Dipeptidase B* (*Dip-B*) mRNAs cycle in phase with *Clk* and *cry* and are constitutively high in Clk^{Jrk} flies (Claridge-Chang et al., 2001; McDonald and Rosbash, 2001). These genes are likely targets for VRI-dependent repression, and preliminary sequence analysis indicates that multiple 10/10 and 9/10 E4BP4-like sequences are present within and upstream of these genes (N.R.J.G. and P.E.H., unpublished observations). Transcripts that cycle in phase with *Clk* and *cry* are not necessarily regulated by VRI. For instance, *takeout* (*to*) mRNA cycles in phase with *Clk* and *cry*, but *to* mRNA is constitutively low in Clk^{Jrk} mutant flies (Claridge-Chang et al., 2001; McDonald and Rosbash, 2001; So et al., 2000; Ueda et al., 2002). This suggests that *Clk*-dependent activation can also mediate cycling in phase with *Clk* and *cry* mRNAs—antiphase to mRNAs that are directly activated by *Clk* (e.g., *per*, *tim*, and *vri*). *Clk* therefore directly or indirectly activates rhythmic transcription both within and downstream of the circadian timekeeping mechanism.

Interlocked Feedback Loop Mechanisms in Flies and Mammals

The mammalian circadian oscillator is also comprised of interacting transcriptional/translational feedback loops that share many components with their *Drosophila* counterparts (Glossop and Hardin, 2002; Reppert and Weaver, 2001; Young and Kay, 2001). In mammals, the *mPer/mCry* feedback loop is analogous to the *per/tim* feedback loop in flies in that CLOCK and BMAL1 (a CYC homolog) activate transcription of the *mPers* (i.e., *mPer1*, *mPer2*, and *mPer3*) and *mCrys* (i.e., *mCry1* and *mCry2*), which feed back to inhibit CLOCK-BMAL1 activation. Likewise, the mammalian *Bmal1* loop is analogous to the *Clk* loop in flies: BMAL1 and CLOCK lead to the repression of *Bmal1* transcription and the mPERs and mCRYs activate *Bmal1* transcription. As in flies, CLOCK-BMAL1-dependent activation occurs via E box binding (Gekakis et al., 1998; Jin et al., 1999), but CLOCK-BMAL1-dependent repression has not been characterized.

A possible candidate for the *Bmal1* repressor is *E4bp4*, the mammalian homolog of *vri* (Mitsui et al., 2001). E4BP4 is a transcriptional repressor that is expressed in the central suprachiasmatic nucleus (SCN) clock as well as peripheral clock tissues (Cowell et al., 1992; Mitsui et al., 2001). *E4bp4* mRNA and protein are rhythmically expressed in these tissues, but their levels peak at the same time *Bmal1* mRNA levels peak (Mitsui et al., 2001), indicating that E4BP4 does not directly repress *Bmal1* transcription. Indeed, E4BP4 is thought to act antagonistically with other PAR domain transcription factors (e.g., DBP, TEF, and HLF) to increase the amplitude of *mPer1* and clock output genes (Mitsui et al., 2001). Recent work demonstrates that the orphan nuclear receptor REV-ERBα is the mammalian analog of *Drosophila* VRI: *Rev-erb*α is activated by CLOCK and BMAL1, and REV-ERBα feeds back to repress *Bmal1* expression (Preitner et al., 2002). Thus, REV-ERBα mediates rhythmic expression of *Bmal1* in antiphase to the *mPers*, *mCrys*, and *Rev-erb*α, just as VRI mediates *Clk* expression in antiphase to *per*, *tim*, and *vri*. Such a switch in the function of integral feedback loop components is not unprecedented as TIM functions to promote PER stability and nuclear localization in *Drosophila*, whereas the mCRYs carry out these functions in mice (Glossop and Hardin, 2002; Reppert and Weaver, 2001; Young and Kay, 2001).

Although some components of interlocked feedback loop oscillators have changed through evolution, the mechanism has been remarkably well conserved. In both flies and mammals, the positive bHLH-PAS factors (i.e., CLK-CYC and CLOCK-BMAL1) activate a repressor, which then feeds back to mediate rhythmic expression of one of the positive factors (i.e., *Clk* in flies and *Bmal1* in mammals). Although rhythmic transcription of positive factors is not predicted to be necessary for oscillator function in flies and is not required for function of the mammalian oscillator (Preitner et al., 2002), the conservation of interlocked feedback loops suggests that they are important for function of the circadian clock. This view is supported by findings in *Neurospora* that suggest that the interlocked feedback loop mechanism is important for the robustness and periodicity of the circadian clock (Cheng et al., 2001).

Experimental Procedures

Fly Stocks and Generation of Transgenic Flies

Wild-type flies were the Canton-S strain. The $w;+;cyc^{01}$ flies were derived from the original cyc^{01} mutant strain (Rutila et al., 1998), and the vri^{2} mutant is a null allele of *vri* (George and Terracol, 1997). The *tim*Gal4, UASGal4, UAS*vri*, and *cry*Gal4 transgenic fly strains have been described previously (Blau and Young, 1999; Emery et al., 1998, 2000b; Hassan et al., 2000; Kaneko and Hall, 2000). To generate the 8.0*Clk*-Gal4 transgene, a 8.0 kb *Clk* genomic DNA fragment was amplified from BAC clone RPCI 98 5.K.6 (BACPAC Resources Center) by polymerase chain reaction (PCR) using sense 5′-GCGGTACCCTCAAGCCCGTGCCCCAA-3′ and antisense 5′-GCG

GATCCGTTCAGGTAATGTTCACGC-3′ primers and ligated upstream of the Gal4 coding sequence in pGATB (Brand and Perrimon, 1993) at *KpnI* and *BamHI* sites (underlined). A *Clk*8.0Gal4 fragment was excised by *KpnI* and *NotI* digestion and ligated into pCaSper4, which was then used for microinjection. To generate the hs-*vri* construct, *vri* coding sequences were amplified from a *vri*-EST clone (GenBank accession number AI404327) with sense 5′-TCTGCG GCCGCACACAAAAACAATG-3′ and antisense 5′-TGCTCTAGA ACGCGGCTAC-3′ primers. After *Not1* and *XbaI* digestion, the ~3.6 kb fragment was cloned into pCaSpeR-hs (Pirotta, 1988), sequenced to verify integrity, and used for microinjection.

*Clk*8.0Gal4 and hs-*vri* plasmids were injected into *yw* embryos, and transgenic flies were generated by P element-mediated transformation (Rubin and Spradling, 1982). *Clk* 8.0Gal4 transgenes (n = 3) were crossed into isogenized *w*;+;+ flies, and hs-*vri* transgenes on chromosome 2 (n = 2) were crossed into a *w*;+;*cyc*^{01} background. Overexpression of VRI in a wild-type background was achieved by crossing *w*;UAS-Gal4;*tim*Gal4 and *w*;+;UAS*vri*/TM6B flies to generate *w*;UAS-Gal4/+;*tim*Gal4/UAS*vri* and *w*;UAS-Gal4/+;*tim*Gal4/TM6B (control) progeny. Two independent *tim*Gal4 transgenes were used (16 and 67; Kaneko and Hall, 2000) in conjunction with two independent UAS*vri* transgenes (2 and 3; Blau and Young, 1999). In four separate experiments, control progeny showed indistinguishable *Clk* and *cry* mRNA rhythms compared to wild-type flies; thus, we used wild-type flies as a control for purposes of plotting the graphs.

Electrophoretic Mobility Shift Assays

Single-stranded sense oligonucleotides (150 ng) were end-labeled with [γ-^{32}P]ATP, gel purified, annealed to their respective complement (150 ng), and diluted to a final concentration of 5000 cpm/μl. Sense strands for probes are as follows: −6651*Clk* +, 5′-tgaagaaat tATTACATAATtctatatgaa-3′; −6651*Clk* m1, 5′-tgaagaaattGCCGTG CGGCtctatatgaa-3′; −6651*Clk* m2, 5′-tgaagaaattTAATCAATTAtcta tatgaa-3′; −6651*Clk* m3, 5′-tgaagaaattATTACAATTAtctatatgaa-3′; −6651*Clk* m4, 5′-tgaagaaattAGTACATAATtctatatgaa-3′; −209*Clk* +, 5′-agcttattcaATTACATAACctggcgataa-3′; −209*Clk* m1, 5′-agcttatt caGCCGTGCGGTctggcgataa-3′; *cry* 1 (−2703*cry*), 5′-gtaaattaatATG ATATAATgaccaaatag-3′; *cry* 2 (+1793*cry*), 5′-atactaattaTTTACAT CAAattatatttt-3′; *cry* 3 (−2486*cry*), 5′-cagcctgccaATCATGTAAT gtagcgaagc-3′.

For each oligonucleotide, the E4BP4 binding site is capitalized and mutations are underlined.

To generate plasmids for transcription/translation of VRI and VRIΔb in the rabbit reticulocyte system, we amplified *vri* coding sequence (including the polyadenylation sequence) from a *vri* EST clone (GenBank accession number AI404327). The wild-type VRI fragment was amplified using a sense 5′-GAGGTACCAATTTGTTC GTTCTAACAAC-3′ *vri* primer and antisense 5′-TGCTCTAGAACGC GGCTAC-3′ vector primer and inserted into pBSIIKS with *KpnI* and *XbaI* (underlined), thus forming pBSIIKS-*vri*+. To generate a basic-domain deleted variant, we cloned two fragments into pBSIIKS. A 5′ *vri* fragment was amplified using the sense primer above and an antisense 5′-CTTCTCCGGCGATCCCAG-3′ *vri* primer, digested with *KpnI* and *EcoRI* (a natural *EcoRI* site is immediately 5′ to the basic domain), and ligated into pBSIIKS to generate pBSIIKS-*vri*5′. A 3′ *vri* fragment was amplified using sense 5′-GCGAATTCTACAATGA CATGGTT-3′ *vri* and antisense 5′- TGCTCTAGAACGCGGCTAC-3′ vector primers, digested with *EcoRI* and *XbaI*, and ligated into pBSIIKS-*vri*5′ to generate pBSIIKS-*vri*Δb, which lacks amino acids 233–261.

Binding reactions were performed at 30°C with a final volume of 25% reticulocyte lysate-generated proteins (2.5 μl in 10 μl final volume). Lysate proteins were incubated for 30 min in binding buffer (10 mM HEPES, 0.8 mM EDTA, 0.4 mM DTT, 20 mM KCl, 4% Glycerol; v/v: final concentrations) before addition of nonspecific DNA competitor (1 μg salmon testes DNA; Sigma). After a 10 min incubation, radio-labeled probe (5000 cpm) or a premix of labeled probe and nonlabeled competitor (5 ng, to reduce the VRI-dependent shift 50%, or 100 ng, to virtually/completely eliminate the VRI-dependent shift) were added and incubated for a further 20 min. Reactions were run in 0.5× TBE buffer on a 6% native polyacrylamide gel. Quantification of band intensities was done using a Fuji phosphoimager and Image Gauge V3.0 software. Pure baculovirus-generated VRI (see below) was resuspended in dH_2O and binding reactions performed as for reticulocyte-generated VRI except 2 μg of salmon testes DNA was added as a nonspecific competitor. Guinea pig anti-VRI antibodies and preimmune serum (0.5 μl) were added post-probe and incubated for a further 10 min.

Ribonuclease Protection Assays

Ribonuclease protection assays (RPAs) were performed as described previously (Hardin et al., 1990). The RPA probe used to measure *Clk* mRNA levels was previously described (Bae et al., 1998), as was the *cry* RPA probe (Emery et al., 1998). The Gal4 RPA probe was generated by inserting a 489 bp *XhoI*-*XbaI* fragment from the *S. cerevesiae* Gal4 coding sequence into pBSIIKS, linearizing with *ClaI*, and transcribing with T7 RNA polymerase. This probe protects a 268 bp RNA fragment.

Statistical Analysis

Two-way ANOVA was used to analyze the effects of time of day, genotype, and their interactions for experiments in which *Clk* or *cry* mRNA was measured in wild-type flies overexpressing VRI. Post hoc analyses of individual treatment groups were then performed using Tukey HSD for unequal N. One-way ANOVA was used to analyze *Clk* mRNA levels upon heat induction of VRI in *cyc*^{01} flies. Post hoc analysis was performed using LSD. Statistical analyses were carried out with STATISTICA software (StatSoft; Tulsa).

VRI Antibody Production and Western Blotting

The coding region for VRI was amplified from a *vri* EST clone (GenBank accession number AI404327) using sense (5′-CAAAACATTAT GTCGATTGTC-3′) and antisense (5′-CTCCGCCAAGATCATCTT-3′) primers, cloned into pBlueBac4.5/V5-His-TOPO vector (Invitrogen), and sequenced to verify that there were no amino acid changes. After recombination into Bac-N-Blue viral DNA, the Bac-N-Blue VRI recombinants were used to transfect Sf9 insect cells and produce VRI protein. VRI was purified by running transfected Sf9 cell protein extracts over a HiTrap copper column (Amersham) under denaturing conditions and eluted through increasing concentrations of imidazol. Purified protein was probed with anti-HIS antibody (Sigma) on Western blots and stained using Coomassie blue (BioRad) on SDS gels, and a single band consistent with the predicted molecular weight of VRI (~85 kDa) was detected. Purified VRI was dialysed through a urea gradient at 4°C, lyophilized, and quantified. Purified VRI was then injected into guinea pigs for antibody production (Cocalico Biological, Inc.).

1.0 OD (595 nm) of total protein from fly heads was run on a 7.5% polacrylamide gel for each sample. VRI antisera were used at a dilution of 1:5,000 or 1:10,000 with horseradish peroxidase-conjugated goat anti-guinea pig secondary antibody diluted 1:1,000 (Sigma). ECL detection was used (Amersham). Films were scanned and VRI levels were quantified as the ratio of VRI to a nonspecific band (asterisk in Figure 1) using Fuji Image Gauge V 3.0. Dephosphorylation experiments were carried out as previously described (Kim et al., 2002).

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