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Circadian timekeeping and output mechanisms in animals

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Abstract

Daily rhythms in animal behavior, physiology and metabolism are driven by cell-autonomous clocks that are synchronized by environmental cycles, but maintain ~24 hours rhythms even in the absence of environmental cues. These clocks keep time and control overt rhythms via interlocked transcriptional feedback loops, making it imperative to define the mechanisms that drive rhythmic transcription within these loops and on a genome-wide scale. Recent work identifies novel post-transcriptional and post-translational mechanisms that govern progression through these feedback loops to maintain a period of ~24 hours. Likewise, new microarray and deep sequencing studies reveal interplay among clock activators, chromatin remodeling and RNA Pol II binding to set the phase of gene transcription and drive post-transcriptional regulatory systems that may greatly increase the proportion of genes that are under clock control. Despite great progress, gaps in our understanding of how feedback loop transcriptional programs maintain ~24 hours cycles and drive overt rhythms remain.

Introduction

Organisms exposed to daily environmental cycles display diurnal rhythms in physiology, metabolism and behavior. These rhythms are generated and sustained by cell-autonomous circadian clocks, which help organisms anticipate predictable changes in the environment. They continue to operate in constant environmental conditions (i.e., free-run) with a period of about 24 hours. Genetic and molecular analysis of circadian clocks in *Drosophila* and mice revealed that the circadian timekeeping mechanism consists of interlocked transcriptional feedback loops, which drive rhythmic transcription of ‘clock genes’ that encode feedback loop components and ‘output genes’ that control physiological, metabolic and behavioral rhythms. Most clock genes are well conserved from insects to humans, and with few exceptions, play similar roles in the timekeeping mechanism.

Although transcriptional feedback loops were established as the molecular basis of circadian timekeeping more than 20 years ago [1,2], fundamental questions remain about the mechanisms by which these feedback loops sustain ~24 hours rhythm and drive rhythmic expression of output genes. Here we will review recent studies of clock protein synthesis and modifications that provide significant insight into post-transcriptional mechanisms that control feedback loop progression, and whole genome analysis of transcription, protein–DNA binding and chromatin modifications that shed new light on clock regulation of rhythmic gene expression.

The architecture of transcriptional feedback loops in animals

Transcriptional feedback loops that keep circadian time in animals have been largely derived from studies in *Drosophila* and mice. These feedback loops have recently been reviewed [3–5]; thus, we will present a sketch of their essential working parts (Figure 1). In both of these model systems, a pair of orthologous basic helix–loop–helix PER-ARNT-SIM (bHLH-PAS) transcription factors called CLOCK and BMAL1 (or its homologue NPAS2) in mammals and CLOCK (CLK) and CYCLE (CYC) in *Drosophila* form heterodimers that bind E-box regulatory elements to activate transcription of genes encoding their repressors, CRYPTOCHROME 1 and CRYPTOCHROME 2 (mCRYs) and PERIOD 1 and PERIOD 2 (mPERs) in mammals and PERIOD (PER) and TIMELESS (TIM) in *Drosophila* [6–10]. mPER–mCRY complexes in mammals and PER–TIM complexes in *Drosophila* accumulate in the cytoplasm, move into the nucleus, and then bind to and inactivate the CLOCK–BMAL1 and CLK–CYC activators, respectively, to repress transcription [11,12]. mPER–mCRY and PER–TIM are then degraded, which permits the activators to bind E-boxes and initiate the next cycle of transcription. The primary function of this ‘core’ feedback loop is to determine circadian period.

CLOCK–BMAL1 and CLK–CYC also activate a second ‘interlocked’ feedback loop that controls rhythmic expression of activator genes (e.g., *Bmal1* and *Clk*), which are transcribed in the opposite circadian phase as repressor genes (e.g., *mPers/mCrys* and *per/tim*) [13,14]. In mammals, this feedback loop is controlled by the nuclear hormone receptors Ror $\alpha/\beta/\gamma$ and RevErb α/β , which bind RevErbA/Ror-binding elements (RREs) to activate and repress *Bmal1* transcription, respectively [15,16]. In contrast, this feedback loop is controlled by the basic leucine zipper (bZIP) transcription factor VRILLE (VRI) in flies, which binds D-box elements to repress *Clk* activation by PAR Domain Protein 1 δ/ϵ (PDP1 δ/ϵ) and other uncharacterized activators [17,18]. Both PAR bZIP and nuclear hormone receptors play major roles in animal physiology and metabolism. Their role in the clock represents a conserved element through which stability and precision of the clock is tied to the metabolic state of the animal.

The timing of feedback loop events during the daily environmental cycle is different in flies and mice. For example, *per* transcription in all fly tissues peaks around Zeitgeber Time (ZT) 15 (where ZT 0 is lights on and ZT 12 is lights off), whereas the mPers peak around ZT 6 in the ‘master’ brain pacemaker, called the suprachiasmatic nucleus (SCN), and 4–8 hours later in peripheral tissues [19]. This phase difference reflects the principle that light, the principal environmental cue, initially synchronizes the SCN clock, which then acts to synchronize peripheral clocks [20,21]. Light is able to synchronize SCN and *Drosophila* clocks because the accumulation of key repressor mRNAs and proteins in the core feedback loop is rate limiting; light-dependent degradation of TIM in *Drosophila* and induction of mPer1 transcription in mammals cause abrupt changes in the phase of the clock that ensure repressor levels are low in flies and high in mammals during daytime. The mechanisms that drive and interpret TIM degradation and mPer1 induction have been reviewed extensively [3,5,22]. Another essential function of these feedback loops is to drive expression of output genes that control overt rhythms, a topic we consider further below.

Mechanisms by which feedback loops maintain ~24 hours periods

The steps required for completing one cycle of the core feedback loop include activator binding to E-boxes, the transcription, RNA processing/cytoplasmic transport, translation, and nuclear localization of repressors, binding and inhibition of activators by repressors, and degradation of repressors. The time it takes to complete these steps should take much less than 24 hours, thus a net delay must be imposed to set the free-running period to ~24 hours. This ‘delay’ principle applies to *Drosophila* and mammalian systems alike, and the

regulation of feedback loop processes common to both systems are remarkably similar [3–5], thus we will focus on *Drosophila* here for brevity and note important differences between these model systems.

Several feedback loop processes are regulated at the post-translational level, including PER nuclear localization, transcriptional repression, and degradation (reviewed in [3]) (Figure 2). PER phosphorylation by SHAGGY (SGG)/glucose synthase kinase 3 (GSK3) and casein kinase II (CKII) promotes PER nuclear localization, and mutants in either kinase lengthen period [23–26]. This period lengthening suggests that SGG and CKII normally act to shorten period, indicating that this step is inherently slow and must be advanced to achieve a ~24 hours period. DOUBLE-TIME (DBT)/casein kinase I δ/ϵ (CKI) phosphorylates PER to promote transcriptional repression while decreasing PER stability in the nucleus, thus enhancing repression while limiting the time that repression can occur [27–29]. DBT/CKI, along with NEMO kinase, delays PER degradation in the nucleus by phosphorylating residues in the ‘per-short’ domain that includes the original period shortening *per^S* mutant [29–31]. Phosphorylated PER is stabilized by TIM binding, which delays PER degradation until after TIM is destroyed after dawn [32,33]. Reduced PER–TIM binding, such as in the *per^L* mutant [34], lengthens circadian period by increasing the time it takes PER to accumulate. Ultimately, PER phosphorylation by DBT/CKI at S47 forms a binding site for the E3 ubiquitin ligase SLIMB/ β -TrCP, which targets PER for degradation via the ubiquitin–proteasome pathway [29]. Processes promoted by PER phosphorylation are counterbalanced by protein phosphatases, including PP2a and PP1, which dephosphorylate PER [35,36]. Phosphorylation-dependent regulatory mechanisms that delay PER degradation in the nucleus extend transcriptional repression for many hours, thereby delaying the core feedback loop. As in flies, clock protein phosphorylation governs the same feedback loop processes in mice, including nuclear localization, transcriptional repression and degradation (reviewed in [4]). Many kinases have the same specificity and function in the mammalian feedback loop (e.g., CKI phosphorylates the mPERs to promote their degradation in the nucleus), but clock components that serve a different function in mice are targeted by different kinases (e.g., AMPK targeting mCrys) [37].

Phosphorylation is not the only post-translational modification of PER. Rhythmic glycosylation of cytosolic PER at S and T residues with O-linked N-acetylglucosamine (O-GlcNAc), which peaks around mid-night (e.g., ZT16–ZT20), acts to enhance PER stability and delay nuclear entry [38**]. Blocking the enzyme which O-GlcNAcylates PER, called O-GlcNAc transferase (OGT), shortens circadian period, which implies that PER O-GlcNAcylation imposes a delay in the core loop, perhaps by competing with phosphorylation-dependent PER nuclear entry and degradation. It is not clear how the delay in cytosolic PER accumulation (which generates the lag between *per* mRNA and protein accumulation) is controlled. Although a novel phosphorylation-dependent destabilization of cytosolic PER could generate such a lag, growing evidence indicates that this lag is mediated by regulated PER translation. Recent work shows that TWENTY-FOUR (TYF) is targeted to *per* mRNA via ATAXIN 2 (ATX2) RNA binding protein to form a complex with POLY-A BINDING PROTEIN (PABP) and promote PER translation [39**,40*,41**]. Since the role of this complex is to promote PER translation, it suggests that the lag in PER accumulation arises because *per* mRNA is difficult to translate or translation is repressed via a separate mechanism. Regulation of PER translation by TYF/ATX2/PABP complexes occurs only in brain pacemaker neurons [39**,40*,41**], suggesting that other mechanisms regulate the lag in PER accumulation in peripheral tissues.

As PER enters the nucleus, it binds to CLK–CYC and promotes CLK phosphorylation, transcriptional repression, and the release of CLK–CYC from E-boxes (reviewed in [3]). Although DBT/CKI plays a non-catalytic role in targeting CLK for phosphorylation, neither

the kinases nor phosphorylation sites that inhibit CLK transcription and/or DNA binding have been identified. In contrast to CLK phosphorylation, CLK ubiquitylation peaks when CLK–CYC transcriptional activity is maximal from ZT10-14 [42*]. This rhythm in ubiquitylation is mediated by UBIQUITIN SPECIFIC PROTEASE 8 (USP8), which deubiquitylates CLK to downregulate CLK–CYC activity from ~ZT18-ZT4, thereby reinforcing PER-dependent repression [42*]. Once PER is degraded, CLK–CYC transcription is reactivated coincident with an increase in ubiquitylated CLK and a reduction in phosphorylated CLK, but the extent to which these processes contribute to delays that set the ~24 hours circadian period is not known. The increase in ubiquitylated CLK could be mediated by the Circadian TRIP (CTRP) E3 ubiquitin ligase [43], ortholog of TRIP12 in mammals, whereas the reduction in phosphorylated CLK could result from CLK dephosphorylation or new CLK synthesis.

CLOCK phosphorylation correlates with mPER–mCRY binding and increased CLOCK phosphorylation [44], suggesting that similar mechanisms operate on mammals. However, BMAL1 phosphorylation, acetylation, sumoylation and ubiquitylation also control CLOCK–BMAL1 transcriptional activity [4], thus adding regulatory complexity compared to *Drosophila*, where CYC appears to be a permissive rather than an instructional factor [3]. Importantly, factors that mediate the post-translational modification of clock components are modulated by other signaling pathways and have other targets. Therefore, these steps also form nodes connecting the core clock with different signaling pathways. For example, fasting induced activation of AMPK in the mouse liver promotes mCRY degradation, thereby constituting a mechanism that integrates energy sensing with the core clock [37].

Regulation of rhythmic outputs via transcriptional feedback loops

Components of both the core and the interlocked loops are transcription regulators, so their action on other loci is the first step in generating overt rhythms. Here we will discuss how microarray and deep-sequencing approaches have revealed the extent of tissue-specific rhythms in chromatin state, factor binding, transcription and transcript abundance, and allude to novel post-transcriptional gene regulatory mechanisms. Since CLK–CYC and CLOCK–BMAL1 directly or indirectly initiate all circadian transcription in flies and mice, respectively, we will focus on these core regulators.

A genome-wide analysis of CLK, PER and RNA polymerase II (Pol II) binding in fly heads revealed CLK binding rhythms at >800 CLK target sites that peak at ~ZT14, followed by PER binding ~6 hours later [45*]. Only ~30% of rhythmically bound CLK targets showed rhythmic Pol II binding, but many of these genes were not previously detected as producing cycling mRNAs, likely because they represent a single RNA isoform and/or may have a limited expression pattern. Often the Pol II binding rhythm was not synchronous with that of CLK, implying that CLK–CYC binding can drive rhythmic expression in different circadian phases. Analysis of nascent and processed transcripts revealed rhythms in RNA editing, RNA splice variants, and non-coding RNAs that mediate ribosome biogenesis [46,47]. Important issues that arise from these studies are how CLK–CYC is targeted to specific genes and isoforms in different tissues, how the phase of Pol II binding is determined once activators bind, and how the clock regulates mRNA cycling at the post-transcriptional level.

In mammals, global transcriptional regulation by components of the core loop also bear considerable similarity to that in insects (Figure 3). Integrative analyses of the dynamic chromatin environment and transcript abundance in mouse liver have revealed five major phases of circadian transcription [48**,49*,50**]. Maximum levels of CLOCK–BMAL1 complex along with p300 are detected around circadian time 8 (CT8, where CT12 corresponds to activity onset in constant darkness) when H3K9ac and H3K4me1 levels also

peak. This marks the transcription activation phase, immediately followed by active transcription for the next 4–5 hours leading to maximum nascent transcript levels at ~CT14-16. Binding of mPER and mCRY repressors then marks the repression phase until ~CT22, after which accumulation of Ser5 phosphorylated Pol II marks the poised state of transcription initiation until ~CT2. Transcriptional de-repression must occur between CT2 and CT6 for the next round of activation to start. Robustly oscillating transcripts show coordinated rhythms in histone modifications and recruitment of clock components to their proximal regulatory sites [51**]. Beyond this generalized schema, locus specific regulation might produce transcription rhythms having different phases or magnitudes. These genomic studies also identified circadian oscillations in antisense RNA, non-coding RNAs, miRNA, RNA processing factors, and in ribosome biogenesis, thus offering mechanisms for generation of circadian rhythms at the post-transcriptional level [48**,50**,51**,52].

Recent deep-sequencing studies have produced some clues for tissue specific transcript oscillations. Transcriptionally silent loci show characteristic DNA and histone modification marks of silent transcription and a near absence of active marks. The expressed transcripts span several orders of dynamic range and roughly correlate with activation marks from proximal regulatory sites. The vast majority of cycling transcripts show oscillations with a peak to trough ratio of <10 fold; a small portion of the large transcript dynamic range. At the trough there are still detectable transcript levels and the loci are not completely devoid of activation chromatin marks [49*,51**]. This implies that tissue specific factors likely mark loci for basal transcription and clock components generate transcript oscillations. Such a dual mode of regulation likely explains the tissue specific nature of circadian outputs.

While these deep-sequencing approaches have revealed genome-wide rhythms in transcriptional regulation, some cautionary notes should be mentioned. As was seen earlier with micro-array studies, rhythmic transcript sets from the same organ (even those identified by the same groups) rarely overlap by >50%. Antibody quality, wet lab methods, and data analysis methods complicate these experiments. Hence, the lack of overlap between any two parameters may not be entirely due to biological differences. For example, statistical tests showed only a fraction of promoters with H3K4me3 oscillations also showing robust H3K27Ac rhythms, while visualization revealed a larger overlap [51**]. The peak phases of rhythmic H3K4me3 and H3K9Ac in one study were coincident while another study found them ~10 hours apart [48**,51**]. These discrepancies underscore the value of validating chromatin marks, factor binding and mRNA levels before detailed studies of individual genes commence.

Conclusions

Recent studies in *Drosophila* and mice have provided new insights into the nature of delays within the core feedback loop that generate a 24 hours period and the regulation of global rhythms in gene expression required for circadian timekeeping and driving overt rhythms. Although phosphorylation promotes nuclear localization and delays degradation of the PER and mPER repressors in the nucleus, new data in *Drosophila* show that O-GlcNAcylation of PER delays its nuclear localization and enhances its stability, possibly by competing with PER phosphorylation. CLK deubiquitylation by USP8 reinforces transcriptional repression by PER complexes, whereas CLK ubiquitylation and decreased phosphorylation may be involved in shifting CLK to a transcriptionally active state. TYF-ATX2-PABP complexes promote PER translation in brain pacemaker neurons, which suggests that inefficient PER translation accounts for the lag in cytoplasmic PER accumulation. Despite these advances in defining delays in the core loop, we do not know how PER complexes inhibit CLK-CYC, how CLK-CYC transcriptional activity is reactivated, the basis of inefficient PER translation in pacemaker neurons, whether translational regulation delays PER accumulation

in other tissues, and the extent to which delays in the mammalian core loop are regulated by the same mechanisms.

Genome-wide analysis of transcript dynamics in their chromatin context has revealed novel gene regulatory mechanisms at the transcriptional, post-transcriptional and translational levels. CLK–CYC and CLOCK–BMAL1 activator binding promotes Pol II binding at different phases, indicating that additional factors regulate the phase of transcription. CLK–CYC and CLOCK–BMAL1 also regulate specific output genes in different tissues, which suggests that they combine with tissue-specific activators that permit circadian transcription of certain output genes. Rhythmic transcription extends to non-coding RNAs and enzymes that regulate gene expression at the post-transcriptional and translational levels. Given the potential for circadian regulation of gene expression at many different levels, it is likely that a much larger proportion of genes are under clock control than previously thought. However, this number will be hard to determine given the technical and biological variability.

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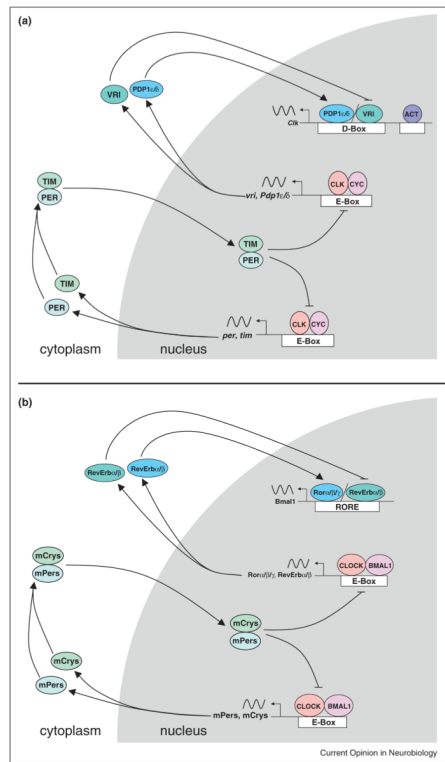
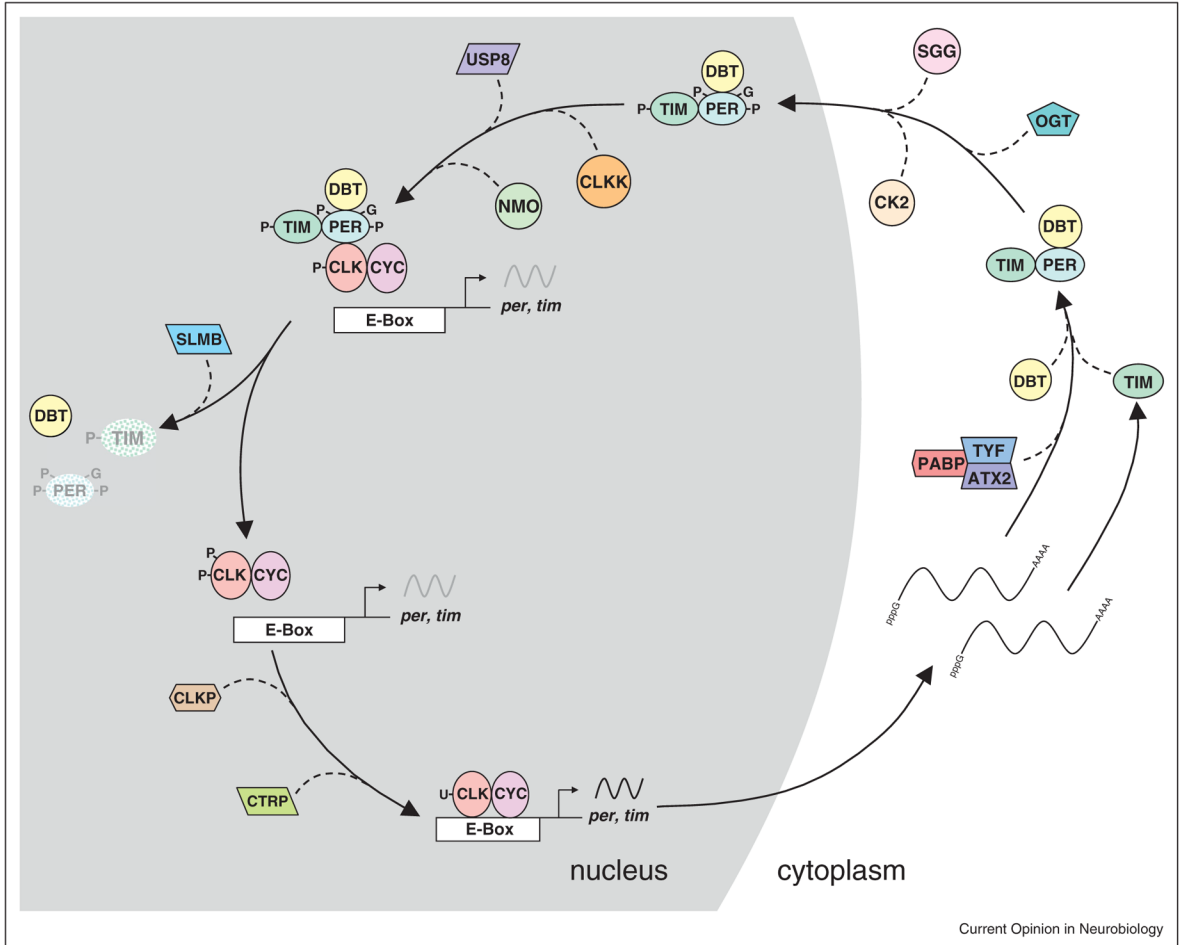


Figure 1. Interlocked feedback loops that keep circadian time. Genetic architecture of the core and interlocked feedback loops of *Drosophila* (a) and mice (b). Gene, protein and regulatory element names are as defined in the text. Sinusoidal lines represent rhythmic mRNAs; arrows depict the synthesis, assembly and/or localization of clock proteins; blocked line denotes repression; gray background indicates events in the nucleus; white background indicates events in the cytoplasm.



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Figure 2. Regulatory events in the core loop of *Drosophila* that control 24 hours periodicity. Gene, protein and regulatory element names are as defined in the text. CLKK represents kinases that phosphorylate CLK; CLKP denotes phosphatases that dephosphorylate CLK; stippled proteins indicate degradation; black sinusoidal lines represent active transcription; gray sinusoidal lines represent repressed transcription; arrows depict the synthesis, assembly and/or localization of clock proteins; dashed lines denote the action of regulatory proteins; lines marked with pppG and AAAA depict mature mRNAs; P depicts phosphorylation, G indicates O-GlcNAcylation; U represents ubiquitylation; gray background indicates events in the nucleus; white background indicates events in the cytoplasm.

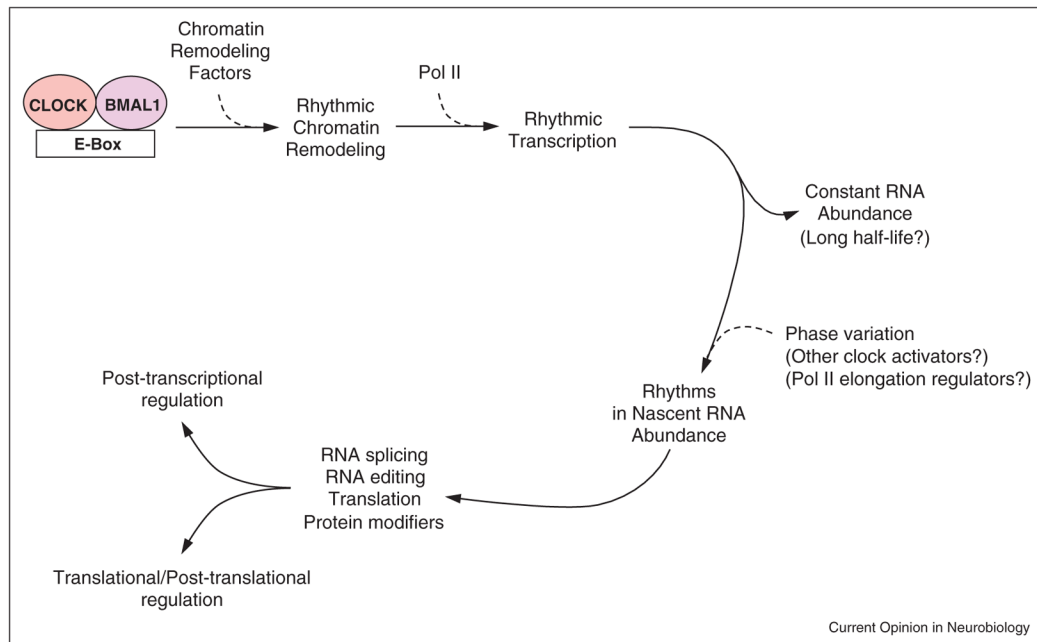


Figure 3.

Transcriptional regulation of circadian gene expression. Protein and regulatory element names are as defined in the text. Arrows, sequence of events triggered by CLOCK–BMAL1 binding to E-boxes; dashed lines, influence of transcription factors; chromatin remodeling factors, factors that alter chromatin structure; phase variation, regulation of nascent RNA cycling phase; parentheses, possible explanation for phenomena. See text for detailed description.