Chapter 5
RNA Dynamics in the Control of Circadian Rhythm

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Abstract The circadian oscillator is based on transcription-translation feedback loops that generate 24 h oscillations in gene expression. Although circadian regulation of mRNA expression at the transcriptional level is one of the most important steps for the generation of circadian rhythms within the cell, multiple lines of evidence point to a disconnect between transcript oscillation and protein oscillation. This can be explained by regulatory RNA-binding proteins acting on the nascent transcripts to modulate their processing, export, translation and degradation rates. In this chapter we will review what is known about the different steps involved in circadian gene expression from transcription initiation to mRNA stability and translation efficiency. The role of ribonucleoprotein particles in the generation of rhythmic gene expression is only starting to be elucidated, but it is likely that they cooperate with the basal transcriptional machinery to help to maintain the precision of the clock under diverse cellular and environmental conditions.

Keywords eRNA • Chromatin modifications • Nascent-seq • RNA-seq • RNAPII • Exon array • IRES • Ribosome • PolyA tail length

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1 Introduction

To adapt to predictable daily changes in the environment, organisms developed mechanisms to anticipate these changes and respond appropriately. Central to this coordination is an intrinsic *oscillator* that generates *circadian rhythms* of behavior, physiology and metabolism. Anatomically, in mammals, the hypothalamic Suprachiasmatic Nucleus (SCN) consisting of ~20,000 neurons functions is the master circadian oscillator. However, the molecular mechanism of the circadian clock is cell autonomous and is present in almost every cell of our body. The SCN oscillator uses synaptic and diffusible factors to orchestrate rhythms in peripheral tissues in appropriate phases.

The molecular circadian oscillator is based on transcription-translation feedback loops with time-delays that generate 24-h oscillations in many of its constituents. Circadian rhythms in animals are endogenous self-sustaining ~24 h rhythms generated by the basic mechanism of cell autonomous transcriptional feedback loops conserved from flies to human. Both components and mechanisms of circadian rhythms are largely conserved in animals [1]. The transcriptional activators CLOCK and BMAL1 dimerize and activate the transcription of Period (*per*) and Cryptochrome (*cry*). The PER/CRY heterodimer, in turn, represses the transcriptional activities of CLOCK/BMAL1 [2]. In an interconnected loop, nuclear hormone receptors REV-ERB and ROR act as repressors and activators to drive rhythmic transcription of several clock components [3, 4].

The molecular circadian clock drives rhythmic transcription from a large number of genes by (a) directly binding to the respective *cis*-regulatory sites, (b) indirectly by their immediate targets that are also transcription factors, (c) by post transcriptional regulations, and (d) by functional interactions with several signaling and transcriptional regulators. In any given tissue in insects and mammals, up to 15 % of the expressed genome exhibit a circadian expression pattern, with peak levels of expression of different transcripts timed to different times of the day or night [1].

Genomics studies have shown that the steady-state levels of a large number of transcripts show daily oscillations. Immediately after transcription initiation and throughout their life cycles, RNAs are bound by a large number of proteins, some of which remain stably bound while others are subject to dynamic exchange. These complexes containing RNAs and their associated proteins constitute the ribonucleoprotein particles (RNPs). The combination of factors binding to a particular RNA and their position along the transcript determines every step of RNA regulation throughout its lifetime. Given the large number of protein coding, small RNAs, miRNAs, ncRNAs that show circadian rhythm in different tissues, and the indirect evidence for circadian rhythm in ribosome turnover, proteins that bind to these RNAs to process, transport, stabilize, translate or degrade can have a profound impact on circadian rhythms in cellular and organismal function.

Upon transcription, not all mRNAs immediately enter the translationally active pool. Some destined for a particular subcellular location travel in multi-mRNA packets or particles and are held in a quiescent state awaiting either proper subcellular localization or a signal that timing is now right to make protein [5]. Similarly it is

also conceivable that some RNAs accumulate in ribonucleoprotein bodies inside the nucleus waiting to be processed or exported until a second signal is received. This integrated model for the regulation of gene expression fits very well to the features of biological clocks, whose function is to synchronize and adapt internal biological processes to environmental stimuli. Furthermore, the time lag between nascent RNA and mRNA for many of the circadian transcripts is specific to the transcript, which suggests that some type of post-transcriptional regulation at splicing or polyadenylation must play a role to maintain such phase relationship. Posttranscriptional events can also buffer variable transcriptional output to generate robust and reproducible rhythms of mRNA expression and protein synthesis. Overall, there is ample evidence for potential roles of RBPs in circadian organization; however, there are very few RBPs with known function in the circadian function. We will review all the different levels at which gene expression shows circadian variation, from transcription initiation to mRNA stability and translation efficiency and cite specific examples of RBPs regulating circadian function.

2 Circadian Regulation of Transcription Initiation

With increased understanding of the mechanism of transcription initiation, it is becoming apparent that in addition to the RNA polymerase complex, several proteins involved in chromatin modification and several RNAs, including enhancer RNAs, non-coding RNAs, and antisense RNAs, play an integral role in initiation and initiation rate. Transcription is the first step of gene expression, and it starts with the binding of the enzyme RNA polymerase II to a segment of DNA. Of particular importance for transcription initiation is the carboxy-terminal domain (CTD) of RNA polymerase II.

The RNA polymerase II CTD typically consists of up to 52 repeats of the sequence Tyr-Ser-Pro-Thr-Ser-Pro-Ser and it serves as a flexible binding scaffold for numerous nuclear factors, determined by the phosphorylation patterns on the CTD repeats. RNA polymerase II can exist in two main forms: RNAPII0, with a highly phosphorylated CTD, and RNAPIIA, with a nonphosphorylated or hypophosphorylated CTD. The phosphorylation state changes as RNAPII progresses through the transcription cycle: the initiating RNAPII is form IIA, and the elongating enzyme is form II0. The phosphorylated CTD physically links pre-mRNA processing to transcription by tethering processing factors to elongating RNAPII, e.g., 5′-end capping, 3′-end cleavage, and polyadenylation. Nearly all of our knowledge of genome-wide transcriptional and post-transcriptional regulation of circadian transcription comes from experiments done in several labs on male mouse liver. In these experiments the adult mice are fed a normal diet ad libitum and entrained to 12 h light: 12 h dark (LD) cycle for a few days. If the mice are held under LD cycle conditions during sample collection, the sampling times are represented as Zeitgeiber time (ZT), where the time of lights-on is considered ZT0. If the mice are transferred to constant darkness prior to sample collection, the timing is represented as Circadian time (CT), where CT0 roughly corresponds to the subjective time of lights-on (or equivalent to ZT0). The

data collected from different times of at least a full day is analyzed by fitting to a wave function, so that the probability values related to robustness of oscillation, peak and trough time/phase of oscillation can be derived. Detected oscillation in given molecules or their activities and the associated phase or time of peak or trough level can be used to explain potential sequence of regulatory events. Due to changes in experimental conditions and sampling frequency, the circadian rhythm parameters might be slightly different in different manuscripts. Therefore, it is relevant to compare the phase information from the same experiment.

Among several published circadian ChIP-seq experiments, the large number of histone modifications, oscillator components binding to chromatin, RNA pol binding, and transcripts from mouse liver reported in Koike *et al*. makes this study relevant to compare the timing of various steps in circadian transcription regulation. In this study CTD phosphorylation status shows a circadian variation, with RNAPIIA signal reaching its peak level at CT14.5 and the RNAPII0 signal peaking at CT0.6. The hypophosphorylated RNAPIIA peak at CT14.5 coincides closely with the peak of intron-containing or nascent transcripts at CT15.1. On the other hand, the peak of hyperphosphorylated RNAPII0 signal coincides with the peak of CRY1 occupancy at CT0.4. At this time CLOCK and BMAL1 are beginning a new cycle of binding but are transcriptionally silent, likely because CRY1 is bound at the same sites. One possible scenario is that RNAPII can be recruited by CLOCK:BMAL and that RNAPII initiation occurs but then pauses or stalls and accumulates at CT0. Alternatively, RNAPII0 occupancy at CT0 could be independent of CLOCK:BMAL1 and could reflect a peak of transcription at CT0 [6].

In addition to circadian oscillations at promoter activity, oscillations in enhancer activities are also found. Fang et al. recently identified circadian transcriptional activity at enhancer regions in mouse liver. This transcriptional activity produces cycling enhancer associated non-coding RNAs called enhancer RNAs (eRNAs; [7, 8]). The circadian eRNAs oscillate in diverse phases and each phase group is enriched in binding motives for different classes of clock transcription factors. The motif enrichment in a given eRNA group is predictive of the specific transcription factor binding. Moreover, circadian eRNAs transcription correlates and can predict rhythmic transcription of nearby genes. The authors propose that circadian transcription factors like CLOCK/BMAL and Rev-erba can bind multiples sites in the genome; however, many of these genes are bound but not controlled, due to inactive binding or long distance looping at different genes. Transcriptional activity at enhancers can be used as markers to assess where a transcription factor is actually functional and they suggest that only the genes that are expressed in phase with CLOCK/BMAL binding are true BMAL1/CLOCK targets [9].

Also chromatin modifications associated with transcription initiation and elongation show circadian variations. Histone H3K4me3, H3K9ac, and H3K27ac are enriched at promoters and show robust circadian rhythms in occupancy at transcription starting sites (TSSs). Histone H3K4me1, a marker that is characteristic of enhancer sites and gene bodies, exhibits a very subtle circadian modulation. There is an antiphase rhythm in H3K4me1 and H3K4me3 occupancy at the Dbp intron1 site. Histone H3K27ac is also highly enriched at both intragenic and intergenic enhancer

sites. The elongation marks, H3K36me3 and H3K79me2, also express very low-amplitude circadian modulation. On a genome-wide level, circadian rhythms in RNAPIIA, RNAPII0, H3K4me3, H3K9ac, and H3K27ac occupancy at TSSs can be seen in all classes of expressed genes and are stronger in intron RNA cycling genes. Unexpectedly, noncycling intron expressed genes also show circadian modulation of RNAPII occupancy and histone modifications. Genome-wide analysis of the periodicity and phase of these histone marks reveals that large number of genes exhibit circadian rhythms in histone modifications. The overall number of histone modification sites does not appear to vary on a circadian basis; rather the recruitment (and initiation) of RNAPII appears to underlie the variation in the amplitude on histone marks. Circadian modulation of RNAPII occupancy and histone modifications occurs not only at promoter proximal regions but also at distal intergenic enhancer sites. Thus, circadian transcriptional regulation appears to be involved in the initial stages of RNAPII recruitment and initiation and the histone modification associated with these events to set the stage for gene expression on a global scale [6].

3 Circadian Regulation of Transcription Termination

Although most focus in circadian regulation has been on daily oscillations in transcription initiation by alternating action of activators and suppressors, recent results have indicated that transcription termination may also be rhythmic. In mammals, PER and CRY proteins accumulate, form a large nuclear complex, and associate with the dimeric transcription factor CLOCK-BMAL at *Per* and *Cry* promoters, repressing their own transcription. PER complexes include the RNA-binding protein NONO and the histone methyltransferase WDR5 [10], and they function in part by recruiting a SIN3 histone deacetylase complex to clock gene promoters [11]. In addition to known PER-associated proteins, Padmanabahn et al. identified the RNA helicases DDX5, DHX9 and SETX in mouse PER complexes. DDX5 and DHX9 function in transcription and pre-mRNA processing [12]. Both are associated with elongating RNA polymerase II [13] and are components of the 3′ transcriptional termination complex [14]. After cleavage of the nascent transcript, unwinding of the RNA-DNA duplex by SETX at the 3′ termination site permits the XRN2 nuclease to degrade the downstream 3′ RNA and thereby release the polymerase [15]. PER complex inhibits SETX activity, blocking subsequent processing by XRN2 and thus blocking transcription termination. Inhibition of termination reduces the rate of transcription, and as a consequence during the negative feedback, RNA polymerase II accumulates at the 5′ site as well as at the 3′ site of *Per* and *Cry* genes [16]. The mammalian PER complex has at least two actions in circadian feedback. It represses transcription by recruiting a SIN3 hystone deacetylase complex to clock gene promoters [11], and it inhibits termination by antagonizing the action of SETX at the 3′ termination site. Both processes contribute to circadian Per gene repression, but one or the other could predominate at different target genes [16].

4 Evidence for the Relevance of Posttranscriptional Events on Circadian Gene Expression

Although circadian regulation of mRNA expression at the transcriptional level is one of the important steps for circadian rhythms in cellular function, multiple lines of evidence point to a disconnect between transcript oscillation and protein oscillation, which can be explained by regulatory RNA-binding proteins acting on the transcripts. The transcription rate itself varies significantly in cells from different tissues [17], yet the free-running endogenous circadian oscillator shows remarkable stability in period length among tissue types. Thus it is likely that the oscillator possesses a mechanism that allows compensating for differences in transcription rates. Indeed, partial inhibition of transcription by a-amanitin treatment in mouse fibroblasts reduces RNA PolII dependent transcription rate by up to threefolds, but doesn't stop cell-autonomous oscillator. The circadian oscillator as measured by translated protein continues to oscillate, albeit with dampened amplitude and slightly shorter periodicity [18]. Similarly, in *Drosophila*, constitutively high mRNA expression of a circadian clock component does not stop the clock; rather, the translated protein level continues to oscillate. These results suggest a posttranscriptional mechanism potentially involving mRNA-binding proteins that can support translational rhythm even when transcriptional oscillation is blunted or inhibited [18, 19].

Furthermore a systematic analysis of the mammalian "circadian proteome" revealed that up to 20 % of soluble proteins in mouse liver are subject to circadian control; however, almost half of the cycling proteins lack a corresponding cycling transcript, further supporting the hypothesis that posttranscriptional mechanisms play a significant role in mammalian circadian rhythms [20, 21]. Interestingly, human red blood cells, which have no nucleus (or DNA) and therefore cannot perform transcription, display robust, temperature-entrainable and temperature-compensated circadian rhythms in peroxiredoxin redox cycles, consistent with the presence of a circadian clock within these cells despite the lack of ability to make new RNA [22].

More recently, additional evidence underlined the importance of posttranscriptional regulation in circadian gene expression. Through high-throughput sequencing or Nascent-Seq and RNA-Seq, Rodriguez at al. identified 136 robust "nascent cyclers" and 237 robust mRNA cyclers in fly heads. Despite a highly significant overlap, most genes in the two data sets are distinct. They propose a model in which transcriptionally active genes can be organized in four groups: genes with both robust nascent and robust mRNA cycling, genes with robust nascent RNA cycling but little or no mRNA cycling, genes with robust mRNA cycling with weak nascent RNA oscillations and genes with robust mRNA cycling but even weaker, and perhaps no, nascent RNA cycling [23].

Menet et al. conducted a similar study in mouse liver. Although many genes are rhythmically transcribed in mouse liver (~15 % of all detected genes), only 42 % of these show mRNA oscillation. More importantly, about 70 % of the genes that exhibit rhythmic mRNA expression do not show transcriptional rhythms, suggesting that posttranscriptional regulation plays a major role in defining the rhythmic mRNA landscape. Also the way in which CLOCK:BMAL1 regulates the transcription of its

target genes differs from what would be expected. Although CLOCK:BMAL target genes are significantly enriched for rhythmic transcribed genes, there is a large discrepancy between the phases of rhythmic BMAL1 DNA binding and those of rhythmic transcription. This is because BMAL1 binding is essentially uniform at ZT3-5, whereas the transcription peaks are much more broadly distributed. The dramatic, genome-wide disconnect between the phases of rhythmic CLOCK:BMAL1 DNA binding and rhythmic target gene transcription suggests that other transcription factors and/or mechanisms collaborate with CLOCK:BMAL1 binding and are critical to determine the phase of clock gene [24].

Another way of assessing the presence of posttranscriptional regulation in circadian gene expression is to sequence the transcriptome and consider the intron signal as a representation of pre-mRNA expression or nascent transcription and the exon signal as a representation of mRNA expression, which can reflect not only transcriptional activity but also posttranscriptional processing events. Koike and collegues quantified intron and exon signals for cycling transcripts in mouse liver and found 1371 intron and 2037 exon RNA cycling transcripts. The intron cycling transcripts are clustered, whereas the exon cycling transcripts have three peak phases. Only 458 genes are in common, and this set of common genes is enriched for known circadian clock genes and high-amplitude cycling target genes reported previously. The phases of the common intron and exon cycling transcripts are correlated, suggesting that transcriptional cycles primarily drive these mRNA rhythms. In the intron-cycling/exon non-cycling class, the cycling pre-mRNA transcripts are clustered at the same phase as the overall intron cycling class, but the steady-state mRNAs are likely to have long half-lives, which would dampen oscillation generated at the transcriptional level. By contrast, in the intron not cycling/exon cycling class, the phases are widely distributed as seen in the overall exon cycling class, and these rhythms likely arise from posttranscriptional regulatory processes such as circadian changes in RNA splicing, polyadenylation, or mRNA stability [6].

5 Circadian Regulation of Alternative Splicing

Alternative splicing is a regulated process during gene expression that results in a single gene coding for multiple proteins. In this process, particular exons of a gene may be included within, or excluded from, the final, processed messenger RNA (mRNA). Alternative splicing is of particular importance amongst the posttranscriptional processes that regulate gene expression, as it allows the human genome to direct the synthesis of many more proteins than would be expected from its 20,000 protein-coding genes.

Alternative splicing is widespread in mammalian genes, affecting approximately 95 % and 80 % of multi-exon genes in humans and mice, respectively [25, 26]. Moreover, alternative splicing is highly regulated by the activity, abundance and binding position of various splicing factors and heterogeneous nuclear ribonucleoproteins, and by the kinetics of transcription elongation and chromatin modifications [27–29].

Using an Affymetrix exon array, McGlincy and collegues demonstrated that the circadian clock regulates alternative splicing in the mouse. 55 exon-probesets from 47 genes where identified to have significant circadian variation. The 47 genes were enriched in pathways representing the circadian clock itself, drug detoxification, caffeine and retinol metabolism and the peroxisome proliferator-activated receptor (PPAR) signaling pathway. The circadian regulation of alternative splicing is tissue dependent, in terms of both phase and amplitude. For some of the exons identified the temporal relationship between alternative splicing and transcript level expression was preserved across tissues, suggesting that these two processes may be coupled in these particular cases. Fasting conditions modulate circadian alternative splicing in an exon dependent manner, but they also modulate the temporal relationship between circadian alternative splicing and circadian mRNA abundance in a gene-dependent manner. Moreover, the alternative splicing of the identified exons is under the control of the local liver clock [30].

At least 15 splicing factors where shown to be robustly cycling in the mouse liver. They include well characterized regulators of alternative splicing (*Srsf3*, *Srsf5*, *Tra2b* and *Khdrbs1*(*Sam68*)), a component of the U2 snRNP (*Sf3b1*), two RNA helicases (*Ddx46* (also component of U2 snRNP) and *Dhx9*), three hnRNP proteins better known for their roles regulating RNA stability and translation (*Hnrnpdl*, *Cirbp* (hnRNP-A18) and *Pcbp2* (hnRNP E2)), and six other proteins with less well characterized roles in RNA processing (*Gtl3*, *Rbms1*, *Thoc3*, *Pcbp4* and *Topors*). Some of these circadian splicing factors were under the control of the local liver clock, while others are likely rhythmic in response to systemic rhythmic cues. Some of the known exon targets of these splicing factors where previously identified to be cycling exons. The discovery of robustly circadian splicing factors, and the fact that a number of their previously characterized target exons are circadian, provide candidates for further study into the molecular mechanisms regulating circadian exons and other posttranscriptional processes [30].

Recently, Preußner et al. demonstrated that the rhythmic alternative splicing of the mRNA encoding U2-auxiliary-factor 26 (U2AF26) contributes to the regulation of Period 1 stability. More in detail they found that U2AF26 undergoes circadian alternative splicing of exons 6 and 7 in peripheral clocks (U2AFΔE67) and that the splicing switch generates a shift in the mRNA reading frame. Skipping of U2AF26 exons 6 and 7 generates a domain with homology to *Drosophila* TIM and enables cytoplasmic, circadian expression of the U2AF26ΔE67 isoform. Furthermore, U2AF26ΔE67 interacts with PER1 and induces its proteasomal degradation; this limits the light induced increase of PER1 and it's proposed as buffering mechanism against sudden light changes [31].

6 Circadian Polyadenylation

The addition of a poly(A) tail to a primary transcript RNA is known as RNA polyadenylation. In nuclear polyadenylation, a poly(A) tail is added to an RNA at the end of transcription. The poly(A) tail consists of multiple adenosine monophosphates; in other words, it is a stretch of RNA that has only adenine bases. In eukaryotes, polyadenylation

is part of the process that produces mature messenger RNA (mRNA) for translation. The poly(A) tail protects the mRNA molecule from enzymatic degradation in the cytoplasm and aids in transcription termination, export of the mRNA from the nucleus, and translation [32]. Almost all eukaryotic mRNAs are polyadenylated [33]. The tail is shortened over time, and, when it is short enough, the mRNA is enzymatically degraded [32]. Regulation of poly(A) tail length is traditionally considered to be unidirectional, going from long to short. However more recent evidence has demonstrated that the ultimate poly(A) tail length is determined by a balance between concomitant deadenylation and polyadenylation, and this balance is controlled in a highly regulated and mRNA-specific manner. In some cases mRNAs with short poly(A) tails can be stored for later activation by re-polyadenylation in the cytosol [34].

Some evidences suggest that poly(A) tail length regulation may take part in controlling circadian-regulated rhythmic gene expression. The deadenylase nocturnin (NOC) removes poly(A) tails from its target RNAs and this process is thought to control target RNA expression by either enhancing RNA degradation or silencing translation. NOC shows rhythmic expression in many tissues such as spleen, kidney and heart in mice with peak levels at the time of light offset. This rhythmicity has been shown to be particularly robust in liver. The mouse NOC gene (*mNoc*) is expressed in a broad range of tissues and in multiple brain regions including suprachiasmatic nucleus and pineal gland. The widespread expression and rhythmicity of *mNoc* mRNA parallels the widespread expression of other circadian clock genes in mammalian tissues, and suggests that NOC plays an important role in clock function or as a circadian clock effector [35]. *mNoc* is also an immediate early gene, and its expression is acutely induced by stimuli such as serum and 12-O-tetradecanoyl-phorbol-13-acetate (TPA) in cultured cells. Remarkably, *mNoc* is the unique deadenylase induced by serum shock. Thus NOC may act in turning off the expression of genes that are required to be silenced as a response to extracellular signals [36].

More recently, it has been shown that 2.3 % of all expressed mRNA exhibit statistically significant rhythmicity in the poly(A) tail length (i.e. the ratio between the "long tail" and the "short tail" fraction of an RNA). The "poly(A) rhythmic" (PAR) mRNAs include mRNAs with peak tail lengths at all phases of the daily cycle but with significantly higher numbers of mRNAs with peak long/short ratios during the night. Based on the pre-mRNA and steady-state mRNA profiles, the PAR mRNAs can be categorized into three classes: Class I PAR mRNAs (49.2 %) are rhythmic in their poly(A) tail length and pre-mRNA and steady-state mRNA levels, class II PAR mRNAs (32.3 %) are rhythmic in their poly(A) tail length and pre-mRNA expression but not in steady-state mRNA levels, and class III PAR mRNAs (18.5 %) are rhythmic in their poly(A) tail length rhythms but not in pre-RNA or steady-state mRNA levels. There are significant differences in mRNA half-lives among the different PAR classes; class III mRNAs are the most stable, followed by class II mRNAs, with class I mRNAs being the least stable [37].

The rhythmic poly(A) lengths of both class I and II mRNAs reflect nuclear polyadenylation (likely by the canonical poly(A) polymerase α), coordinated with rhythmic transcription during classical 3′ end processing. The defining characteristics of these two classes are the differences in the steady-state mRNA rhythmicity and mRNA

stability, suggesting that the lack of rhythmicity in the class II PARs reflects the longer half-lives of these mRNAs. The mechanism of poly(A) rhythmicity in both class I and II mRNAs results from the addition of long tails following rhythmic synthesis and subsequent deadenylation that does not cause immediate decay. This delay in decay is more pronounced in the class II mRNAs and results in the arrhythmic steady-state levels. It appears that class I/II PAR mRNAs can exist in short-tailed states and that rhythmic control of poly(A) tail length is somehow correlated with delayed accumulation of steady-state mRNA and may be part of a regulatory mechanism to regulate the timing of mRNA/protein rhythmicity [37].

Class III mRNAs exhibit robust rhythmicity in their poly(A) tail length, yet are not rhythmically transcribed and have longer half-lives. Thus, class III PARs must employ transcription-independent mechanisms to control their rhythmic poly(A) tail lengths. Peak distribution analysis of class III PAR mRNAs revealed that >80 % had their longest poly(A) tails during the day, which is distinct from the nighttime poly(A) rhythmic profile of class I and many of the class II mRNAs. The poly(A) rhythms of class III mRNAs are likely to be controlled by rhythmic cytoplasmic polyadenylation. Indeed, the steady-state mRNA level of several putative cytoplasmic polyadenylation machinery components in the liver, including *Cpeb2*, *Cpeb4*, *Parn*, and *Gld2*, are rhythmically expressed with phases similar to the majority of the class III PAR mRNAs, peaking in the early day [37].

In mouse liver rhythmic poly(A) tail lengths correlate strongly with the ultimate circadian protein expression profiles, with the protein peaking ~4–8 h after the time of the longest poly(A) tail. Therefore poly(A) tail rhythms can generate rhythmic protein levels even when there is no rhythm in the steady-state mRNA levels [37].

7 Regulation at Translation Initiation and Ribosome Biogenesis

Many oscillating proteins in the mouse liver are encoded by constantly expressed mRNAs and among the rhythmically expressed genes in the liver, there are several genes encoding proteins involved in mRNA translation, including components of the translation pre-initiation complex [38]. The circadian clock controls the transcription of translation initiation factors as well as the rhythmic activation of signaling pathways involved in their regulation. Initiation in eukaryotes requires at least ten proteins, which are designated eIFs (eukaryotic initiation factors). The mRNAs of most of the factors involved in translation initiation are rhythmically expressed with a period of 24 h. There isn't a significant variation in protein abundance, but these factors undergo strong rhythmic phosphorylation [38]. The initiation factors eIF4E and eIF4G, in association with eIF-4A and eIF-4B, are involved in binding the mRNA and bringing it to the 40S ribosomal subunit. eIF4E, which recognizes the 5′ cap of the mRNA, is mostly phosphorylated during the day, with a peak at the

end of the light period (ZT6-12). eIF4G, eIF4B and 4E-BP, and ribosomal protein (RP) S6 (RPS6) are mainly phosphorylated during the night, which is, in the case of nocturnal animals like rodents, the period when the animals are active and consume food. Phosphorylation of these factors is well characterized and involves different signaling pathways whose reported activity perfectly correlates with the observed phosphorylation rhythm [38]. eIF4E is phosphorylated by the extracellular signal-regulated protein kinase (ERK)/mitogen activated protein kinase (MAPK)-interacting kinase (MNK) pathway, which is most active during the day, at the time when eIF4E reaches its maximum phosphorylation. On the other hand, eIF4G, eIF4B, 4E-BP1, and RPS6 are mainly phosphorylated by the target of rapamycin (TOR) complex 1 (TORC1), which is activated during the night at the time when the phosphorylation of these proteins reaches its maximum level. It has been shown that mTOR, its partner Raptor, as well as its regulating kinase Map3k4, are also rhythmically expressed, thus potentially further contributing to the rhythmic activation of TORC1. The rhythmic phosphorylation of 4E-BP1 results in the release eIF4F, allowing its binding to the mRNA and the initiation of translation [38].

The polysomal RNA fraction (RNA sub-fraction composed mainly of actively translated RNAs) in mouse liver also follows a diurnal cycle, showing that a rhythmic translation does occur in this tissue. Approximately 2 % of the expressed genes are translated with a rhythm that is not explained by rhythmic mRNA abundance as in most cases the total mRNA levels are constant. Among translationally regulated genes, 70 % were found in the polysomal fraction during the same time interval, starting at ZT8 before the onset of the mouse feeding period and finishing at the end of the dark period [38].

The circadian clock was shown to regulate also ribosome biogenesis by influencing the transcription of ribosomal protein (RP) mRNAs and ribosomal RNAs (rRNAs). RPs show a rhythmic abundance with highest expression during the night. Pre-mRNA accumulation of several RPs exhibits a rhythmic pattern too, with a peak at ZT8, just before the activation of their translation. As for rRNA transcription, the synthesis of the ribosome constituent precursor 45S rRNA (containing 28S, 5.8S and 18S rRNAs) is rhythmic and synchronized with RP mRNAs transcription, indicating that all elements involved in ribosome biogenesis are transcribed in concert and coordinated with the feeding period. In mammals rRNA transcription is highly regulated by the upstream binding factor (UBF). Not surprisingly UBF1 is rhythmically expressed in mouse liver too, at both mRNA and protein levels, in phase with RP mRNAs and rRNA transcription. Mice devoid of a functional circadian clock lose the rhythmic activation of TORC1 and ERK signaling pathways, and the rhythmic expression of UBF1. In addition these animals show lack of synchrony and coordination of 45S rRNA and RP pre-mRNA transcription, highlighting the crucial role of the circadian clock in this mechanism. Ribosomal protein synthesis in eukaryotes is a major metabolic activity that involves hundreds of individual reactions; this energy-consuming process has to be confined to a time when energy and nutrients are available in sufficient amounts, which, in the case of rodents, is during the night [38].

8 RNA-binding Proteins Regulating mRNA Stability and Translational Efficiency Are Important for Oscillation of Core Clock Components

The number of RNA-binding proteins and those with RNA-binding motifs encoded by the human genome is remarkable. As an example a single type of RNA-binding domain, the RNA recognition motif (RRM), is represented in nearly 500 different human genes. RNA-binding proteins (RBPs) couple transcription and subsequent post-transcriptional steps by interacting with their target transcripts. Even though some RBPs bind to common elements present in almost every mRNA in a sequence-independent and nonspecific manner, the majority of RNA-binding factors target particular structures or sequences present in some RNAs but not others [39]. The posttranscriptional events involving multiple mRNAs must be highly coordinated and RBPs, including export proteins, provide coordinating functions at all steps along the posttranscriptional regulatory chains. RBPs allow the mRNA molecules to interface with other intracellular machineries mediating their splicing, transport, stabilization or degradation, localization, or translation into protein, as well as the response to stimuli. Indeed individual mRNAs contain binding sites for different RBPs and can respond to a wide range on inputs, so that their expression can be adjusted to changing environmental conditions. RBPs are thus the leading actors of an intricate regulatory network, which is equally complex as that controlling initial RNA synthesis. Because RBPs can bind to more than one RNA with sequence specificity, the existence of a “posttranscriptional operon” has been postulated whose function is to expand the regulatory plasticity of our relatively “small” genome. In fact the expression of proteins with common functional themes or subcellular distributions is coordinated by large-scale regulatory networks operating at the mRNP level. The final outcome of protein synthesis is thus an mRNP-driven process that responds dynamically to the environment and cellular growth conditions [5, 39].

In particular the posttranscriptional regulation of mRNA stability and translational efficiency are often mediated by cis elements in mRNAs that interact with RNA-binding proteins and/or microRNAs. In most cases, these cis elements reside in the 3′ untranslated region (UTR), and several 3′ UTR motifs have been identified that are critical for mRNA splicing, transport, stability, localization, and translation.

The 3′UTR-dependent mRNA decay is involved in the regulation of circadian oscillation of Period 2 (*per2*) mRNA. In particular the polypyrimidine tract-binding protein (PTB), also known as heterogeneous nuclear ribonucleoprotein I (hnRNP), binds to *per2* 3′UTR and has an mRNA destabilizing activity. Indeed the cytoplasmic PTB expression pattern is reciprocal with *per2* mRNA oscillation and depletion of PTB with RNAi results in *per2* mRNA stabilization [40]. A similar study reported that the 3′UTR is also important for the mRNA stability of another core clock component, mouse cryptochrome 1 (*cry1*). The 3′UTR of *cry1* contains a destabilizing cis-acting element that contributes to the stability of *cry1* mRNA. The binding of hnRNP D to *cry1* 3′UTR is responsible for the rapid decay of *cry1* mRNA during its declining phase and modulates *cry1* circadian rhythm [41].

Also, the stability of mouse Period3 (*per3*) is dramatically changed in a circadian phase-dependent manner. In the case of (*per3*), the control of its circadian mRNA stability requires the cooperative function of both the 5′ and 3′ UTRs. Several studies reported that mRNA stability can be regulated by the 5′UTR, a mechanism called translational regulation-coupled mRNA decay. In such cases translational inhibition causes mRNA stabilization. Similarly hnRNP Q binds to both 5′ and 3′UTR in the *per3* mRNA and not only reduces the translation efficiency but also increases the mRNA stability. *per3* mRNA decay is connected to its translation kinetics and the central region of *per3* 5′UTR is responsible for coupling of translation and mRNA decay. The binding of hnRNP Q of *per3* 5′UTR is phase dependent and maintains robust mRNA oscillation [42].

Mouse LARK, another RBP, has been shown to activate the posttranscriptional expression of the mouse period1 (*per1*) mRNA. A strong circadian cycling of the LARK protein is observed in the suprachiasmatic nuclei with a phase similar to that of PER1, although the level of the *lark* transcripts are not rhythmic. LARK protein binds directly to a cis-element in the 3′ UTR of the *per1* mRNA and causes increased PER1 protein levels, by activating *per1* mRNA translation. Alterations of *lark* expression in cycling cells causes significant changes in circadian period, with *lark* knockdown by siRNA resulting in a shorter circadian period, and *lark* overexpression resulting in a lengthened period [43].

Many studies have shown that mammalian cells utilize internal ribosome entry site (IRES)-mediated translation for rapid adaptation to certain environments, such as chemotoxic stress [44], mitosis [45] and apoptosis [46], and generally under conditions when cap-dependent translation is compromised. For IRES-mediated translation, proteins known as IRES *trans*-acting factors (ITAFs) must recognize IRES elements in a structure or sequence-dependent manner. Recent evidences suggest that IRES-mediated translation might be one of the mechanisms regulating the protein oscillation of key clock components like Rev-erb α. Also known as Nr1d1, Rev-erb α was identified as a regulator of lipid metabolism [47]. It also plays an important role in the maintenance of circadian timing in brain and liver tissue [48, 49] and it is a well-known transcriptional repressor in the positive limb of circadian transcription [3, 50]. Kim and colleagues have demonstrated that hnRNP Q and PTB modulate mRev-erb α IRES-mediated translation. Knockdown of hnRNP Q and PTB leads to the alteration of the mRNA levels of several clock genes, thus posttranscriptional regulation by hnRNP Q and PTB is necessary to maintain the circadian feedback loop [7, 8].

9 Conclusion

There is increasing evidence that the RBPs play an important role in homeostatic control of the periodicity of circadian oscillator and its output regulation. RBP mediated regulation of various steps in the transcription, RNA processing and RNA half-life helps maintain the precision of the clock under diverse cellular and environmental conditions. Circadian regulation of cellular physiology and metabolism is mediated by

daily oscillations in the steadystate levels of nascent RNA, mRNA, and protein levels. However, large fraction of oscillating proteins or mRNA does not exhibit a correlated rhythm in nascent RNAs, which suggests that post-transcriptional regulation involving RBPs is most likely involved. Since many RBPs bind and regulate the location, transport, translation of a large number of target RNAs, a rhythmic level of a given RBP likely helps temporally coordinate the function of the target RNAs. Another challenge in circadian regulation is the newly recognized role of eating pattern and nutrition quality in the daily oscillations of RNA and proteins. In rodent liver, the circadian transcriptome in peripheral organs appears to be heavily determined by the nutrition quality and time of eating. This implies that the nutrition information encoded in several metabolites might affect the circadian transcriptome by both transcriptional and post-transcriptional mechanisms and RBPs will likely play an important role in integrating nutrition status with the endogenous circadian oscillator function.

Much of the evidence for the roles of RBPs in circadian regulation is indirect. Although genome-wide transcriptome studies have shown circadian rhythms in the mRNA levels of several RBPs, whether the RBP proteins and their cellular localization are also circadian is yet to be determined. Similarly, as the target RNAs for many of the RBPs are discovered, informatics approaches to integrate these findings with circadian transcriptome datasets will begin to explain post-transcriptional mechanisms of circadian regulation. Overall, the area of investigation on how RBPs are involved in the circadian regulation is a nascent field with plenty of opportunities for discoveries and mechanistic insight.

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