

Minireview

Molecular orchestration of the hepatic circadian symphony

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Abstract

The circadian clock determines the rhythmic expression of many different genes throughout a 24-hour period. A recent study investigating the circadian regulation of liver proteins reveals multiple levels of regulation, including transcriptional, post-transcriptional and post-translational mechanisms.

Circadian rhythms are recurring physiological processes in plants, animals, fungi and cyanobacteria with a period of about a day (Latin: *circa diem*), and probably evolved to optimize an organism's energy expenditure and uptake [1]. In animals, with which we are concerned here, circadian rhythms provide the ability to predict recurring events such as food availability and emergence of predators. Every cell contains a circadian oscillator made up of transcriptional and post-transcriptional autoregulatory feedback loops driving the rhythmic expression of particular genes and proteins [1]. The specialization of cells to fulfill designated tasks such as storing energy, digesting food, neutralizing toxic substances and producing movement, has led to the formation of organs such as the liver, kidneys and brain, and tissues such as muscle and adipose tissue, in which the individual cellular clocks are synchronized to each other to generate an organ- or tissue-specific circadian time [2]. The 24-hour rhythms are different in the various organs, but they maintain a fixed phase relationship to each other. The circadian system is therefore made up of individual tissue and organ clocks, all in stable phase relationships with each other.

Because the beginning of the day and daylength change throughout the year, animals need to adapt their clocks to bring them back to a stable phase relationship with the environment. The most dominant cue for this synchronization is light, although food, especially when it is available in restricted amounts, can also serve as a synchronizing signal. The brain, which is directly connected to the light-sensing

eye, and the liver, which is the main metabolic sensor, are therefore central in the optimal adjustment of the output of the animal's circadian system to adapt to environmental changes [3,4]. Temporal information encoded by the circadian clocks within organs is only of use if it is translated into physiologically meaningful events. This is achieved through coupling of the clocks to pathways, such as metabolic pathways, which thus become the 'hands' of the clock or its output [5].

To study this output, transcriptional profiling of multiple tissues using microarrays has revealed many genes that are expressed in a circadian fashion [6,7]. Each tissue exhibits its own distinct pattern of phase distribution of circadian transcripts [6,7-11] and only a few of those transcripts are common to more than one tissue [6], reflecting the different demands on specific organs. These experiments gave the first insights into how metabolism is coordinated in particular tissues, which is of importance because a disruption of the circadian system is associated with metabolic disease [12,13]. In a paper published recently in *Current Biology*, Reddy and colleagues [14] have gone a step further, investigating the relevance of the microarray results by determining the circadian patterns of protein expression in the liver, the body's central metabolic clearing house.

Circadian expression of liver proteins

Reddy *et al.* [14] examined the temporal expression patterns of proteins in the soluble fraction of the liver using

two-dimensional difference gel electrophoresis (2D-DIGE). They prepared liver extracts at 4-hour intervals over a 24-hour period and reliably detected 642 protein spots across the matrix of gel analyses. The relatively small number of proteins is due to the extraction method, which caused the loss of small and low-abundance proteins as well as membrane proteins. Of the proteins consistently detected, 60 exhibited highly significant circadian variation and 135 significant variation, corresponding respectively to 10% and 20% of known soluble liver proteins identified in this experiment. This is an interesting number, because the circadian transcriptome detected by microarray experiments has been estimated to contain only 5-10% of the total number of genes that are expressed in a circadian manner [6,7,9,10]. This indicates that there are other levels of circadian regulation of gene expression besides transcription. Hence, Reddy *et al.* [14] found more proteins to be expressed in a circadian fashion than previous microarray experiments would suggest.

The identity of the spots exhibiting highly significant circadian rhythms was determined by peptide mass fingerprinting (matrix-assisted laser desorption-ionisation - time-of-flight, or MALDI-TOF mass spectrometry) or liquid chromatography followed by tandem mass spectrometry (LC-MS/MS); more than half the spots were identified as known proteins. Interestingly, several isoforms of the same proteins were detected and therefore only about three-quarters of the identified proteins corresponded to distinct genes. Circadian rhythmicity of expression was then verified for those proteins for which antibodies were available. Immunoblotting revealed that most of the rhythmic proteins (80%) peaked at night and the remaining 20% during the day. This is consistent with the notion that the liver is providing energy during the activity period (night in mice) and is also active during the resting period (day in mice) for detoxification and regeneration. Because only soluble liver proteins were investigated, the proteins found were predominantly enzymes. These enzymes are associated with vital liver functions such as carbohydrate metabolism, the urea cycle, detoxification, the metabolism of reactive oxygen species and apoptosis.

Because rhythmic protein abundance in the liver is probably dependent on the local molecular clockwork, Reddy *et al.* [14] investigated whether genetic disruption of normal circadian patterning affected the rhythmic expression of three of the circadian proteins identified: the metabolic enzymes aldolase, arginase and catalase. They found that the rhythmic expression of these proteins was indeed altered in mice carrying mutations in the genes *Per2* and *Clock*, which are part of the core clock mechanism that governs the transcription of circadian genes. These findings indicate that the expression of aldolase, arginase and catalase is probably regulated downstream of the transcriptional core clock mechanism and hence they represent hands (output) of the circadian clock. It appears, therefore,

that the proper expression of these circadian metabolic proteins is dependent on an intact molecular clock. An interesting observation in the *Per2* mutant animals was that not only the expression patterns of circadian proteins were altered but also those of non-circadian proteins, thus hinting at a function of the *Per2* protein in regulating gene and protein expression outside the core clock mechanism.

Post-transcriptional and post-translational mechanisms of regulating circadian output

Reddy *et al.* [14] found that certain genes yielded multiple rhythmically expressed protein isoforms. In the case of aldehyde dehydrogenase (*Aldh2*), the different isoforms were expressed synchronously, but those of carbamoyl phosphate synthase (*Cps1*), the rate-limiting enzyme for the urea cycle, were expressed in different temporal patterns. Because the different isoforms for *Aldh2* and *Cps1* are each translated from a single gene transcript these findings show that post-transcriptional control is a significant component of the temporal coordination of metabolism.

Proteins can also be modified by phosphorylation and other post-translational mechanisms. That such mechanisms can be under temporal control is illustrated by the differential phasing found by Reddy *et al.* [14] of the phosphorylated and unphosphorylated forms of peroxiredoxin 6, an antioxidant protein. That post-translational modifications are relevant for circadian rhythms has been demonstrated *in vivo* in several cases. In cyanobacteria, for example, phosphorylation and dephosphorylation can generate a robust self-sustained circadian rhythm involving three proteins. This rhythm is independent of transcription and translation [15,16]. In the fungus *Neurospora crassa*, a rhythmic phosphorylation of the white collar complex of proteins is necessary to drive the clock mechanism [17]; phosphorylation of this complex represses gene expression, dephosphorylation activates it [18]. In humans, a mutation affecting the phosphorylation of the *Per2* protein affects the phasing of the circadian clock and individuals with the mutation display advanced sleep phase syndrome [19]. SUMOylation, another type of post-translational modification, has also been suggested to be involved in circadian clock control [20].

Divergence between the circadian transcriptome and proteome

The core clock mechanism is viewed as a transcriptional autoregulatory feedback loop with the proteins *Bmal1* and *Clock* as the transcriptional activators. Therefore the assumption at present is that rhythmically expressed proteins are the result of transcriptional programs. Reddy *et al.* [14] find, however, that there is a marked dissociation between the circadian transcriptome and proteome. That is, rhythmic abundance of mRNA can be advanced, synchronous or delayed with respect to the rhythmic protein. Furthermore, not all

rhythmically expressed proteins have their corresponding mRNA expressed in a 24-hour rhythm, underlining the importance of post-transcriptional mechanisms regulating circadian coordination. To relate the differences in mRNA and protein expression Reddy *et al.* [14] looked *in silico* to determine whether known circadian promoter elements (E-boxes, D-elements and ROREs) could be predictors of rhythmically expressed genes. They found no strict correlation, indicating that promoter activity of known circadian elements is unlikely to explain the observed variation in mRNA rhythmicity. Instead, mechanisms involving RNA-binding proteins regulating RNA processing could explain this variance. A mechanism for post-transcriptional control of circadian-related mRNA has been proposed in the frog *Xenopus laevis* via the protein nocturnin, which acts as a de-adenylase [21], and antisense RNA is involved in regulating circadian clock function in *N. crassa* [22]. These findings highlight the fact that post-transcriptional mechanisms exert significant control over the circadian proteome.

Taken together with other work, the study by Reddy *et al.* [14] shows that although transcriptional control can account for circadian proteomic oscillations, an extensive component of the circadian coordination of liver physiology depends on post-transcriptional and post-translational mechanisms. It provides a general paradigm for combining transcriptomic and proteomic approaches to the analysis of complex and dynamic metabolic regulation. An unprecedented degree of temporal regulation over gene and protein expression was found, emphasizing the global influence of the circadian mechanism on transcriptome and proteome, and thereby providing insights into the control of hepatic physiology. Although the results do not explain how the complex and dynamic hepatic transcriptome and proteome mechanistically work together, the study provides a framework for further studies.

The approach by Reddy *et al.* [14] reveals specific relationships between metabolic pathways in the liver. However, each tissue appears to have a different set of oscillating genes [9,11]. Therefore, one can speculate that in the whole organism, every gene is expressed in a circadian manner, some genes in the liver, others in the kidney, and even others in the heart. In keeping with our opening remarks, this makes sense, because different tasks and demands on the various organs make the organism function optimally. As a consequence, the physiology of complex biological systems such as animals and humans should be studied from the viewpoint of circadian oscillations. The systems biology view of metabolic regulation put forward in the study of Reddy *et al.* [14] uncovers the tip of an iceberg of genome-wide temporally regulated gene and protein expression. In the future, an integrated understanding of systems biology approaches will result in new insights into how complex systems such as metabolism and the brain interact with the circadian system and the environment [2,23]. This should lead to an integrated

view of treatment for diseases that are caused by systemic malfunction rather than by single molecular alterations in a pathway.

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