Molecular Architecture of the Circadian Clock in Mammals

Joseph S. Takahashi

Abstract The circadian clock mechanism in animals involves an autoregulatory transcriptional feedback loop in which CLOCK and BMAL1 activate the transcription of the *Period* and *Cryptochrome* genes. The PERIOD and CRYPTOCHROME proteins then feed back and repress their own transcription by interaction with CLOCK and BMAL1. We have studied the biochemistry of the CLOCK:BMAL1 transcriptional activator complex using structural biology as well as the genomic targets of CLOCK and BMAL1 using ChIP-seq methods. We describe the dynamics of the core circadian clock transcriptional system. CLOCK and BMAL1 interact with the regulatory regions of thousands of genes. The gene network and dynamics of the system will be discussed. A mechanistic description of the core circadian clock mechanism should promote our understanding of how the circadian clock system influences behavior, physiology and behavioral disorders.

Introduction

Over the last 20 years, my laboratory has been focused on understanding the molecular mechanism of circadian clocks in mammals. We have used mouse genetics as a tool for discovery of the critical genes involved in the generation of circadian rhythms of mammals (Takahashi et al. 1994; Lowrey and Takahashi 2011). Our initial discovery of the *Clock* gene using forward genetic screens and positional cloning (Vitaterna et al. 1994; Antoch et al. 1997; King et al. 1997), and the identification of BMAL1 as the heterodimeric partner of CLOCK (Gekakis et al. 1998), led to idea that the CLOCK:BMAL1 transcriptional activator complex

J.S. Takahashi (✉)
Howard Hughes Medical Institute, University of Texas Southwestern Medical Center, Dallas, TX 75390, USA

Department of Neuroscience, University of Texas Southwestern Medical Center, Dallas, TX 75390, USA
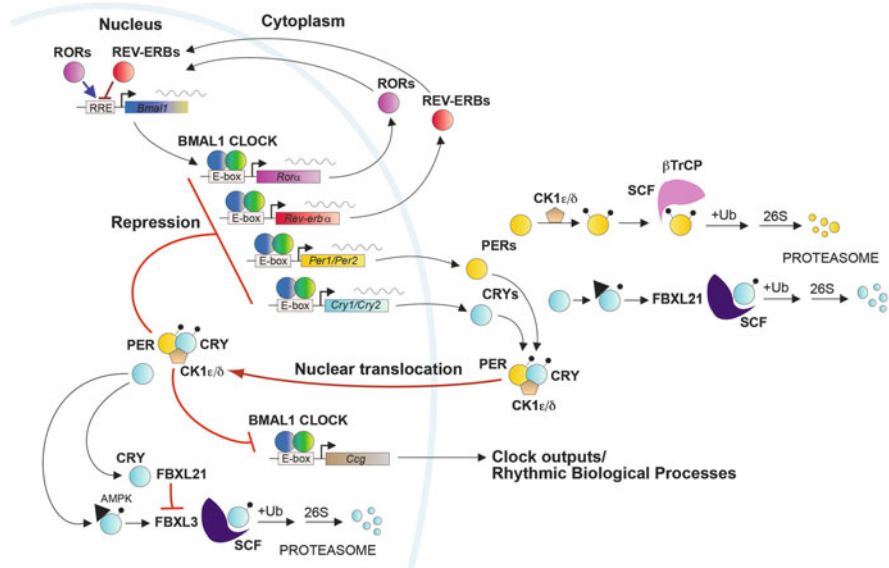e-mail: joseph.takahashi@utsouthwestern.edu

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Fig. 1 Model of the circadian clock in mammals. CLOCK and BMAL1 act as master transcription factors to regulate: (1) the *Per* and *Cry* genes in the core feedback loop of the clock; (2) the REV-ERB/ROR feedback loop regulating *Bmal1* transcription; and (3) thousands of target genes that are clock outputs. The stability of the PER and CRY proteins is tightly regulated by E3 ubiquitin ligases in both the cytoplasm and nucleus that determine circadian period (Adapted from Mohawk et al. 2012 and Yoo et al. 2013)

was upstream of the *Period* and *Cryptochrome* genes, whose gene products then repressed CLOCK:BMAL1 to form an autoregulatory transcriptional feedback loop (Lowrey and Takahashi 2000). Since the identification of these "core circadian clock genes" (i.e., *Clock*, *Bmal1*, *Per1*, *Per2*, *Cry1* and *Cry2*), additional feedback loops driven by CLOCK:BMAL1, such as the loop involving *Rev-erbα* to repress *Bmal1* transcription, have been described (Preitner et al. 2002). In addition, the regulation of the stability of the PER and CRY proteins by specific E3 ubiquitin ligase complexes has been found to be important for determining the periodicity of the circadian oscillation (Busino et al. 2007; Gallego and Virshup 2007; Siepka et al. 2007; Meng et al. 2008; Yoo et al. 2013). Together, this work has led to a description of a model of the circadian clock in mammals (Fig. 1).

With the discovery and cloning of clock genes came the realization that their expression was ubiquitous (Lowrey and Takahashi 2004). We now accept that clock genes are housekeeping genes and are expressed in essentially all cells. What was perhaps even more surprising was the observation made using circadian gene reporter technology that essentially every peripheral organ system and tissue has the capacity to express autonomous circadian rhythms (Yoo et al. 2004). Thus the ubiquitous expression of clock genes is a reflection of the ubiquitous capacity of most tissues and cells to express circadian oscillations. These distributed circadian oscillators are cell autonomous and can function independently of the central clock

located in the suprachiasmatic nucleus (SCN) (Nagoshi et al. 2004; Welsh et al. 2004; Yoo et al. 2004). The realization that the body is composed of a multitude of cell-autonomous clocks has raised a number of questions concerning the organization of the clock system and the role of the SCN clock in "circadian organization." Elsewhere, we have also explored the role of the SCN as a master pacemaker to synchronize peripheral oscillators (Yoo et al. 2004; Hong et al. 2007; Kornmann et al. 2007; Buhr et al. 2010; Hughes et al. 2012), as well as the role of intercellular coupling in the robustness of the SCN oscillator (Liu et al. 2007; Buhr et al. 2010; Ko et al. 2010; Welsh et al. 2010).

Structural Biology of Clock Proteins

Despite our general knowledge of clock components and their interactions, the biochemical mechanisms of circadian clock proteins and how they function within the circadian feedback loop are largely unknown. For example, many coding mutations have been described for mammalian clock proteins but, at a macroscopic level, we have little hope of understanding how they exert their phenotypic effects without a deeper understanding of their molecular mechanism. For these reasons, we have turned to structural biology to understand circadian proteins at an atomic level of resolution. Recently, we have solved the three-dimensional structure of the CLOCK:BMAL1 heterodimeric transcriptional activator complex using X-ray crystallography (Huang et al. 2012). The CLOCK:BMAL1 structure reveals an asymmetric heterodimer in which the bHLH, PAS-A and PAS-B domains of each subunit interact with their complementary domains but do so in an unexpected manner (Fig. 2). The PAS-A domains dimerize via symmetrical interactions involving α-helical domains (that are N-terminal to the canonical PAS fold) that pack against the β-sheet surfaces of the PAS-A domains (Fig. 3a). In contrast, the PAS-B domains dimerize in an asymmetric, head-to-tail fashion so that the β-sheet surface of BMAL1 interacts with the α-helical surface of CLOCK (Fig. 3b). A conserved BMAL1 Trp427 residue on an H-I loop (connecting the Hβ and Iβ strands) inserts into a hydrophobic pocket on the α-helical surface of CLOCK that resembles the co-factor binding pocket in other PAS proteins. Interestingly, a Trp residue is also conserved on the H-I loops of CLOCK, PER1 and PER2 PAS domains, suggesting that an aromatic residue inserting into the PAS receiver pocket may represent a common motif for PAS domain interactions (Crane 2012).

The structure of CLOCK:BMAL1 represents a starting point for understanding at an atomic level the mechanism driving the mammalian circadian clock. Many of the previously identified mutations on CLOCK and BMAL1 can be mapped onto the structure and, for example, predict regions of interaction of CLOCK with the CRY proteins (Huang et al. 2012). The crystal structures for the PAS-A/PAS-B domains of the mammalian PERIOD proteins (Hennig et al. 2009; Kucera et al. 2012), for the photolyase homology domains of the mammalian CRY1 (Czarna et al. 2013) and CRY2 (Xing et al. 2013) proteins, and for the CRY2/PER2-CRY binding domain complex (Nangle et al. 2014) beg the question of how
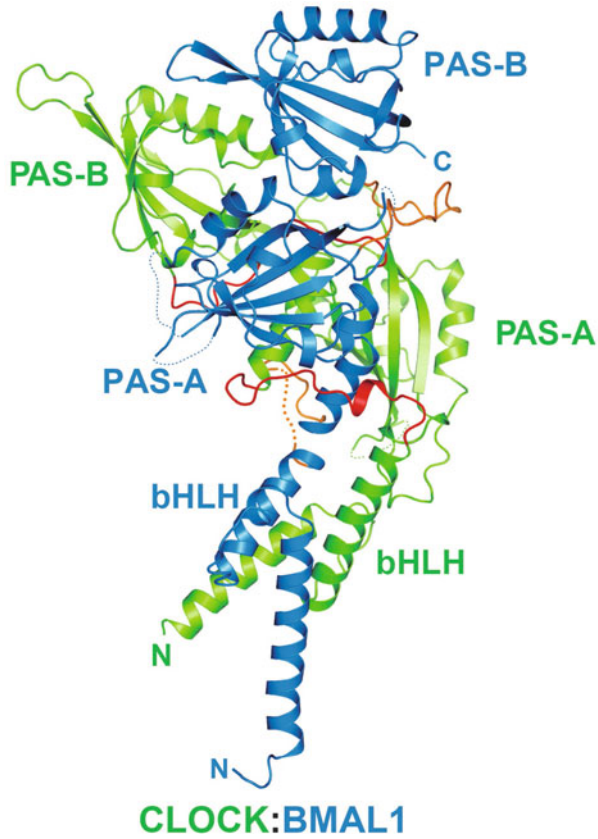
Fig. 2 CLOCK:BMAL1 structure showing bHLH, PAS-A and PAS-B domains. Linker regions shown in *red* or *orange* (From Huang et al. 2012)
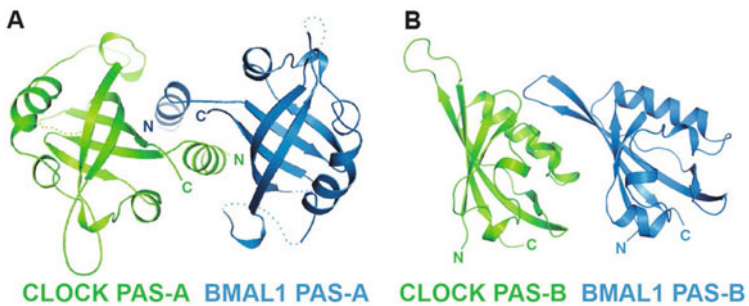

Fig. 3 PAS domains of CLOCK:BMAL1. (**a**) PAS-A interactions shown looking down the axis of the complex. (**b**) PAS-B interactions shown from a *side view* (From Huang et al. 2012)

PER and CRY interact with CLOCK:BMAL1 to repress their function. Because the native CLOCK:BMAL1/PER:CRY quaternary complexes are megadalton in size and involve other interacting proteins, and because important domains of these proteins are flexible, the solution of these complexes likely will require a combination of crystallography, NMR, and cryo electron microscopy methods in future work.

Transcriptional Architecture and Chromatin Dynamics of the Clock

To define the *cis*-acting targets of the core circadian transcriptional regulators, we used chromatin immunoprecipitation followed by sequencing (ChIP-seq) to locate DNA binding sites for BMAL1, CLOCK, NPAS2, PER1, PER2, CRY1 and CRY2 in vivo in murine liver at six times during the circadian cycle. Figure 4 shows a browser view of the *Dbp* locus, a major target gene of CLOCK-BMAL1 (Ripperger and Schibler 2006). The activators BMAL1, CLOCK and NPAS2 bind in a cyclic manner between CT0 and CT12 (CT = circadian time; CT0 is the beginning of the subjective day; CT12 is the beginning of the subjective night) at three locations in the promoter, intron 1 and intron 2. PER1, PER2 and CRY2 bind the same sites with an opposite phase at CT12-20. CRY1 exhibits a third pattern that peaks at CT0.

In genome-wide analysis, CLOCK and BMAL1 bind to over 4600 and 5900 sites, respectively, corresponding to ~3000 unique genes (Koike et al. 2012). The repressors CRY1 and CRY2 bind to significantly more sites, and many thousands of these sites are independent of CLOCK:BMAL1 and reveal DNA binding motifs for nuclear receptors (Koike et al. 2012), including the glucocorticoid receptor consistent with recent work (Lamia et al. 2011). To examine functional readouts, we used whole transcriptome RNA-seq to profile cycling genes in the liver using samples taken every 4 h over 48 h (Koike et al. 2012). Using the intron RNA signal as a proxy for pre-mRNA, we found ~1300 cycling genes and, surprisingly, they were clustered in time with a peak at CT15 (Fig. 5). To explore the possible origins of the global rhythms in nascent transcription, we analyzed the genome-wide occupancy of RNA polymerase II (RNAPII) as a function of the circadian cycle. The large subunit of RNAPII contains a C-terminal domain (CTD) that is modified at various stages of transcription (Sims et al. 2004; Fuda et al. 2009). RNAPII is recruited into the pre-initiation complex with a hypophosphorylated CTD that is recognized by the 8WG16 antibody (Jones et al. 2004). Again to our surprise, we found that RNAPII-8WG16 occupancy was highly circadian across the genome in the liver, with a peak at CT14.5, which preceded the intron RNA peak by 0.5 h (Fig. 5). Initiation of RNAPII involve phosphorylation on serine 5 (Ser5P) on the CTD of RNAPII and is recognized by the 3E8 antibody (Chapman et al. 2007). We found that RNAPII-Ser5P occupancy was also circadian, with over 13,000 sites that were significant for cycling. The timing of RNAPII-Ser5P peaked at CT0 and coincided with the peak of CRY1. At this time we found an association of CRY1, CLOCK,
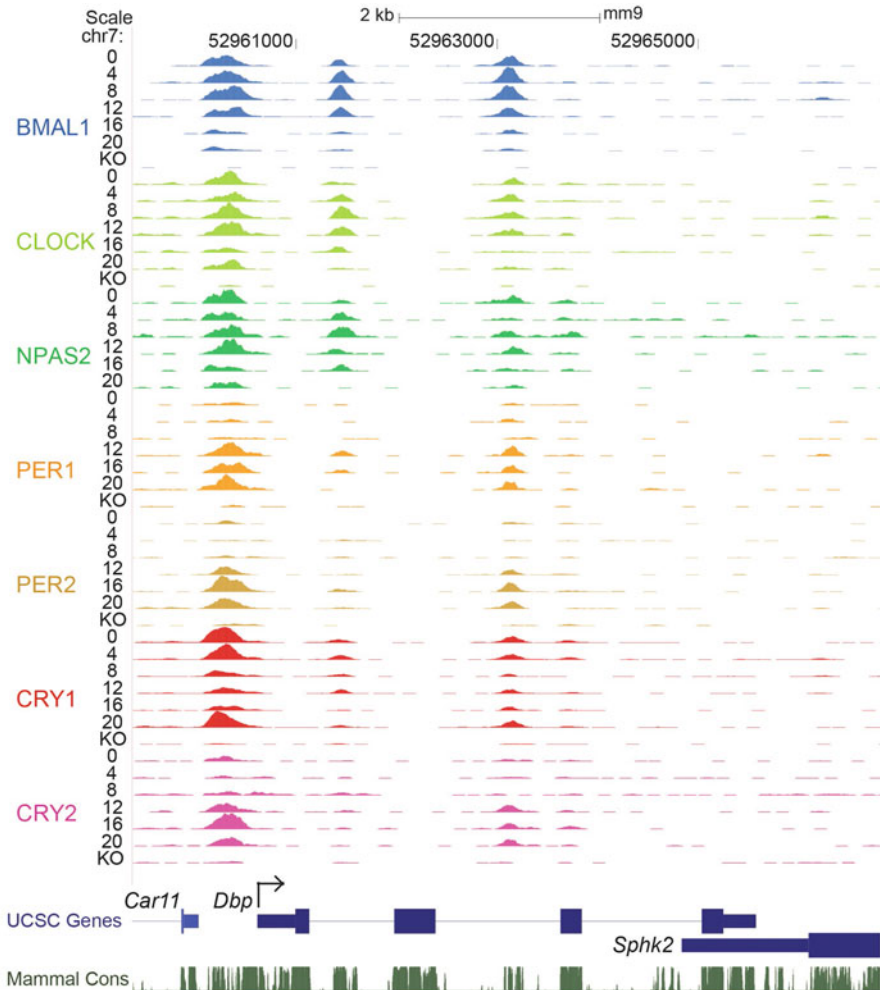
Fig. 4 UCSC genome browser view of ChIP-seq profiles of circadian transcription factors at the *Dbp* gene at six circadian times of day. BMAL1 (*blue*), CLOCK (*green*), NPAS2 (*dark green*), PER1 (*orange*), PER2 (*gold*), CRY1 (*red*), CRY2 (*pink*). 0, 4, 8, 12, 16, 20 CT (h). *KO* knockout. (From Koike et al. 2012)

BMAL1 and RNAPII-Ser5P binding sites, suggesting that CLOCK:BMAL1 could recruit and initiate RNAPII but CRY1 repressed the complex leading to a "poised" state.

Given the genome-wide circadian rhythms of RNAPII occupancy, we assessed chromatin states associated with transcription initiation and elongation during the circadian cycle. Figure 6 shows a browser view of six histone modifications that are characteristic of promoters, enhancers and transcription elongation (Kim et al. 2005; Barski et al. 2007; Guenther et al. 2007; Li et al. 2007; Creyghton
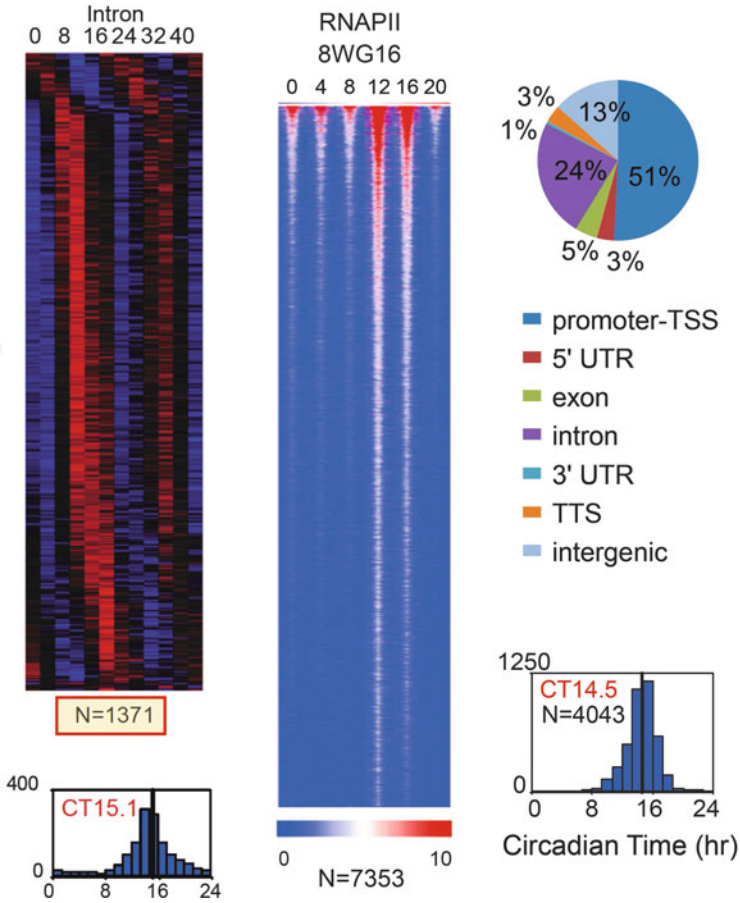
Fig. 5 Heatmap views of cycling intron RNA genes (*left*) and RNAPII-8WG16 occupancy (*right*). More than 4000 peaks had significant circadian RNAPII binding (From Koike et al. 2012)

et al. 2010; Ong and Corces 2011; Rada-Iglesias et al. 2011). Histone H3K4me3, H3K9ac and H3K27ac are enriched at promoters and show robust circadian rhythms in occupancy at the *Dbp* gene. When examined across the genome, we found that circadian rhythms in RNAPII occupancy as well as histone H3K4me3, H3K9ac and H3K27ac modifications occurred in the majority of expressed genes, even in cases where cycling RNA could not be detected. Thus a third surprise in this work was the observation that chromatin states were being modulated in a circadian manner across the genome in the liver.

What accounts for these genome-wide circadian rhythms in RNAPII occupancy and histone modifications? Examination of the relationship between circadian transcription factor occupancy and gene expression shows that approximately 90 % of genes bound by these factors are expressed whereas only 1–5 % of unexpressed genes are similarly bound (Koike et al. 2012). These results
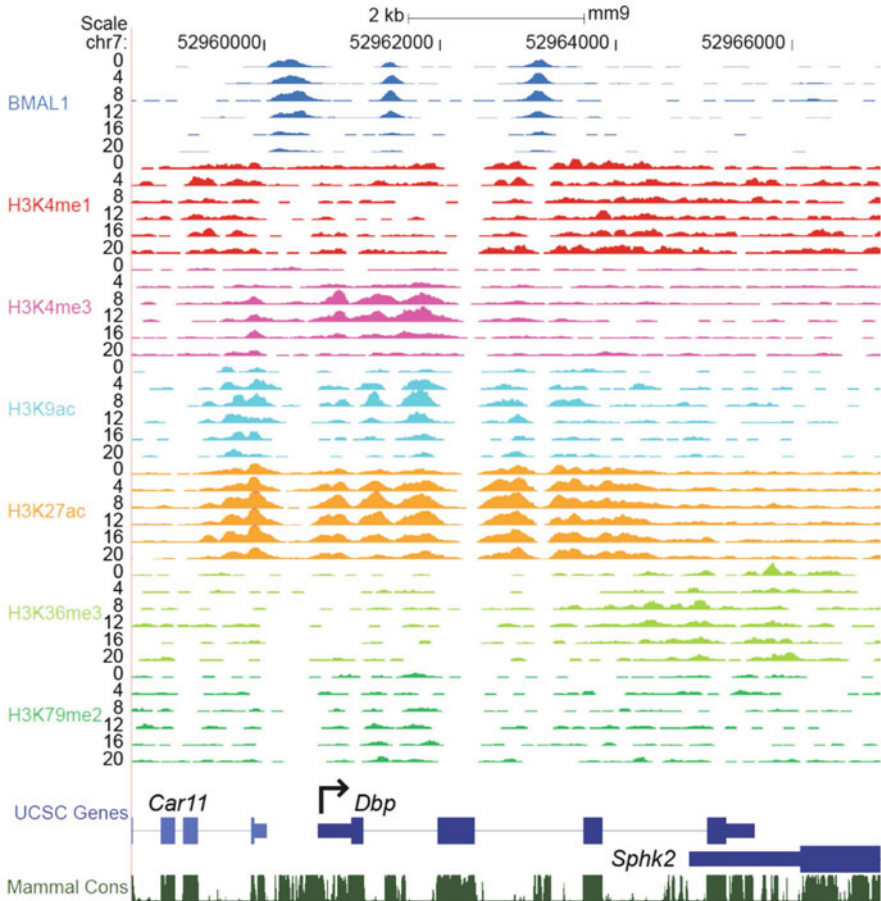
Fig. 6 UCSC genome browser view of histone methylation and acetylation at the *Dbp* gene. BMAL1 (*blue*), H3K4me1 (*red*), H3K4me3 (*pink*), H3K9ac (*aqua*), H3K27ac (*orange*), H3K36me3 (*green*), H3K79me2 (*dark green*) (From Koike et al. 2012)

demonstrate that gene expression per se rather than rhythmic gene expression is tightly correlated with circadian transcription factor binding. Rhythmic circadian transcription factor occupancy in turn could then be responsible for RNAPII recruitment and initiation on a genome-wide basis, which would then lead to the global rhythmic histone modifications seen here. Thus, circadian transcriptional regulators appear to be involved in the initial stages of RNAPII recruitment and initiation and the histone modifications associated with these events to set the stage for gene expression on a global scale, but additional control steps must then determine the ultimate transcriptional outputs from these sites.

In summary, we have defined the *cis*-regulatory network of the entire core circadian transcriptional regulatory loop on a genome scale and found a highly stereotyped, time-dependent pattern of core transcription factor binding, RNAPII
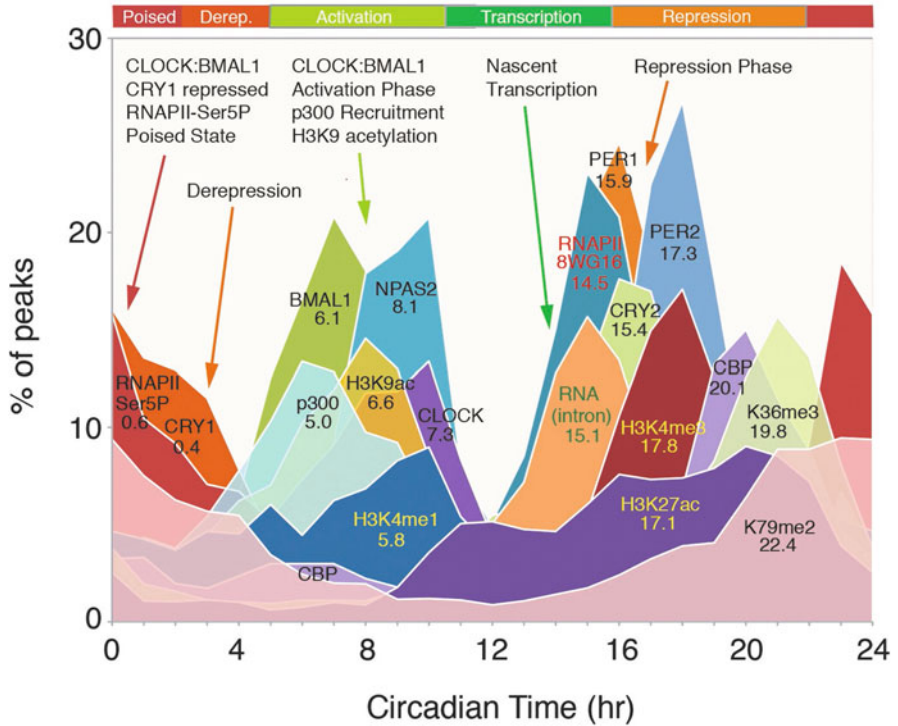
Fig. 7 Circadian transcriptional landscape in the liver. Histograms show the phase distributions of each factor as a function of time of day. *Derep* derepression (From Koike et al. 2012)

occupancy, RNA expression and chromatin states (Fig. 7). We defined three distinctive phases of the circadian cycle: (1) a poised phase in which CLOCK: BMAL1 and CRY1 bind to E-box sites in a transcriptionally silent state associated with RNAPII-Ser5P; (2) a temporally coordinated transcriptional activation phase in which RNAPII and p300 recruitment, pre-mRNA transcript expression, and H3K9ac, H3K4me3 and H3K27ac occupancy oscillate; and (3) a repression phase in which PER1, PER2 and CRY2 occupancy peaks. Circadian modulation of RNAPII recruitment and chromatin remodeling occurs on a genome-wide scale far greater than that seen previously by gene expression profiling. Thus, the circadian clock in the liver modulates the occupancy of RNAPII across the genome, leading at least in part to genome-wide circadian modulation of chromatin states that, in turn, poise the genome for transcription on a daily basis to act in concert with the daily metabolic demands of the organism.

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