Regulation of Circadian Gene Expression in Liver by Systemic Signals and Hepatocyte Oscillators

B. KORNMANN,*† O. SCHAAD,‡ H. REINKE,* C. SAINI,* AND U. SCHIBLER*

**Departments of Molecular Biology and NCCR Frontiers in Genetics, Sciences III, University of Geneva 30, CH-1211 Geneva-4, Switzerland; †Howard Hughes Medical Institute/University of California, San Francisco, Genentech Hall N316-600, San Francisco, California 94158-2517; ‡Departments of Biochemistry and NCCR Frontiers in Genetics, Sciences II, University of Geneva 30, CH-1211 Geneva-4, Switzerland*

The mammalian circadian timing system has a hierarchical structure, in that a master pacemaker located in the suprachiasmatic nuclei (SCN) coordinates slave oscillators present in virtually all body cells. In both the SCN and peripheral organs, the rhythm-generating oscillators are self-sustained and cell-autonomous, and it is likely that the molecular makeup of master and slave oscillators is nearly identical. However, due to variations in period length, the phase coherence between peripheral oscillators in intact animals must be established by daily signals emanating directly or indirectly from the SCN master clock. The synchronization of individual cellular clocks in peripheral organs is probably accomplished by immediate-early genes that interpret the cyclic systemic signals and convey this phase information to core clock components. This model predicts that circadian gene expression in peripheral organs can be influenced either by systemic signals emanating from the SCN master clock, local oscillators, or both. We developed a transgenic mouse strain in which hepatocyte clocks are only operative when the tetracycline analog doxycycline is added to the food or drinking water. The genome-wide mapping of genes whose cyclic expression in liver does not depend on functional hepatocyte oscillators unveiled putative signaling pathways that may participate in the phase entrainment of peripheral clocks.

INTRODUCTION

In mammals, virtually all aspects of physiology and behavior are influenced by the circadian clock (for review, see Gachon et al. 2004). According to knowledge gained during the past 10 years on the subject, the term "clock" is actually somewhat misleading, because nearly every body cell contains its own circadian oscillator. Thus, in organ explants or serum-shocked fibroblasts kept in tissue-culture medium, the expression of clock genes oscillates with a period length close to 24 hours (Balsalobre et al. 1998; Yamazaki et al. 2000; Nagoshi et al. 2004; Welsh et al. 2004; Yoo et al. 2004). The molecular makeup of the clockwork circuitry is probably shared by SCN neurons and cultured fibroblasts. In both, circadian oscillators function in a self-sustained and cell-autonomous manner, the phase relationship between the expression cycles of different clock genes is practically identical, and clock gene mutations elicit related phenotypes (Yagita et al. 2001; Brown et al. 2005). There is an important difference, however, when these cellular oscillators are studied in the tissue context. Although the clocks of SCN neurons are tightly coupled, those of peripheral cells do not appear to communicate with each other to any significant extent. As a consequence, neurons of organotypic SCN slice cultures maintain phase coherence, whereas oscillators of cultured peripheral cells or tissue explants rapidly desynchronize (Liu et al. 2007). Likewise, the cellular oscillators of peripheral tissues are quickly put out-of-phase in SCN-lesioned animals, suggesting that they must be phase-entrained daily by signals emanating from the SCN (Yoo et al. 2004; Guo et al. 2006). As outlined below, the SCN master pacemaker, which itself is synchronized by the photoperiod, appears to set the phase in peripheral oscillators by employing a multitude of different timing cues (zeitgebers). Elegant parabiosis studies by Bittman and coworkers suggest that at least for liver and kidney cells, some of these zeitgebers are probably blood-borne signals, such as hormones and metabolites (Guo et al. 2005). Thus, in a parabiosed pair composed of an intact and an SCN-lesioned hamster, circadian liver and kidney gene expression is synchronized in both parabiosed partners. Work by the same group also suggests that the SCN-phase entrains peripheral timekeepers on the single cell rather than the organ level, at least in liver (Guo et al. 2006). In this study, the authors experimentally scrutinized the following strong prediction: If phase coherence was maintained within the liver of SCN-lesioned animals, the livers of different SCN-lesioned animals should display vastly different ratios of mRNAs issued from antiphasically expressed genes (e.g., *Per1* and *Bmal1*). Yet, the mean values of *Per1* mRNA to *Bmal1* mRNA ratios were found to be very similar for all examined animals, and the standard deviations of these mean values were quite small. This result is only compatible with a scenario in which individual hepatocytes within an SCN-lesioned animal do not exhibit significant phase preference.

The coexistence of cyclic systemic cues and local oscillators implies that circadian transcription in peripheral tissues could be regulated by two different—albeit not mutually exclusive—mechanisms. One class of rhythmically expressed genes, dubbed systemically driven genes (Kornmann et al. 2007), may be under the direct control of systemic signals, such as diurnal hormones. For example, glucocorticoid-responsive genes would be expected to be cyclically transcribed, because the plasma levels of the glucocorticoid receptor ligands cortisol or corticosterone

oscillate with a robust daily amplitude (Lejeune-Lenain et al. 1987 and references therein). The transcription of another class of genes, perhaps best described as cell-autonomously clock-controlled genes, may be driven by core components of local oscillators, such as BMAL1, CLOCK, CRYs, and PERs, or transcription factors such as the PAR basic leucine zipper (bZIP) proteins DBP, HLF, and TEF, whose circadian accumulation and/or activity depends on these factors. As argued later in this chapter, the discrimination between systemically driven and clock-controlled genes is not just a matter of semantics. The first class of genes may actually contain immediate-early genes that participate in the synchronization of peripheral clocks, and the identification of such genes may therefore provide important insight into the signaling cascades involved in this process.

FEEDING/FASTING CYCLES ARE DOMINANT ZEITGEBERS FOR CLOCKS IN LIVER AND OTHER PERIPHERAL ORGANS

Genome-wide transcriptome-mapping studies revealed that the fraction of diurnally accumulating liver transcripts amounts to 2–10%, depending on the stringency of algorithms used for the analyses of microarray hybridization data (Akhtar et al. 2002; Duffield et al. 2002; Panda et al. 2002; Storch et al. 2002; Walker and Hogenesch 2005). Many of these genes encode enzymes involved in the processing and detoxification of food components, suggesting that the temporal coordination of metabolism is a major task of hepatocyte clocks. If so, it would make sense to tune the phase of circadian liver clocks to the metabolic cycles in order to optimize the temporal coordination of catabolic and anabolic processes. Damiola et al. (2000) and Stokkan et al. (2001) therefore examined whether daily feeding/fasting cycles might serve as zeitgebers for the oscillators operative in liver and, perhaps, other peripheral tissues. To this end, food was offered only during a restricted time window outside the normal (nocturnal) activity phase, and the phase of circadian gene expression was determined. The results showed that the inversion of feeding cycles from nighttime feeding to daytime feeding provoked a complete inversion in the liver and some other tissues, such as pancreas, kidney, and heart. In contrast, feeding cycles had no significant effect on the circadian phase of the SCN master clock and a somewhat less dramatic effect on the phase of lung oscillators. In both studies, the liver was found to respond more quickly to the imposed feeding regimens than other tissues examined.

On the basis of the observations described above, we suspect that under normal circumstances, the SCN master timekeeper synchronizes clocks in peripheral tissues primarily through behavioral rhythms. In the case of liver and some other metabolically active tissues, rest/activity cycles may drive feeding rhythms, and the absorption and processing of nutrients may elicit a number of strong timing cues for peripheral oscillators. Clearly, however, the SCN may also act on peripheral cell types through more direct zeitgebers, such as body temperature rhythms (Brown et al. 2002) and cyclically secreted hormones (Balsalobre et al. 2000a,b; Le Minh et al. 2001). The phase entrainment of the liver clock by behavioral and more direct SCN outputs under normal conditions is schematically illustrated in Figure 1A (top panel). Imposed feeding rhythms can completely uncouple peripheral oscillators from the SCN master pacemaker, and the timing signals provoked by feeding cycles must therefore be dominant over more directly controlled systemic zeitgebers. This scheme makes some clear predictions that can be experimentally tested: (1) The kinetics of phase uncoupling after imposing an anticyclic feeding regimen should be slower than the phase recoupling after termination of the feeding regimen, because the feeding-related zeitgebers are only antagonistic with the "direct timing signals" during the forced-feeding schedule. Damiola et al. (2000) have examined and validated this prediction. (2) If a "direct signaling pathway" is eliminated (Fig. 1A, lower left panel), the kinetics of feeding-induced phase inversion should be faster than in the presence of all "direct signaling routes" (green curve vs. black curve in Fig. 1B), because the competitiveness of feeding-related versus direct zeitgebers is now enhanced. Again, this scenario has been examined and validated for glucocorticoids, a strong phase-shifting agent whose cyclic production and secretion are controlled by the SCN via the hypothalamus-pituitary-adrenal (HPA) axis (Le Minh et al. 2001). (3) Obliteration of a food-related zeitgeber pathway should slow down the feeding-induced phase inversion (Fig. 1A, lower right panel and red curve in Fig. 1B), because the impaired indirect timing mechanisms now suffer a fiercer competition from antagonistic timing cues directly controlled by the SCN. No food-related timing signals have yet been identified in an unambiguous manner, and this prediction could hence not yet be scrutinized. Obviously, a more detailed experimental evaluation of the model proposed in Figure 1 requires profound knowledge on the molecular makeup of the signaling pathways that participate in both behavioral (indirect) synchronization routes and directly SCN-driven phase entrainment mechanisms. Below, we propose an experimental system that hopefully will contribute to the deciphering of the various input pathways implicated in the synchronization of peripheral oscillators.

Feeding rhythms consist of an absorptive phase, during which food is ingested and used to build energy stores such as glycogen granules and fat droplets, and a postabsorptive phase, during which the energy stores are used. The metabolism is obviously quite different during these two phases, and the question thus arises of whether the most efficient molecular zeitgebers for peripheral clocks are produced during the absorptive or the postabsorptive phase. At least for rodents, the latter may be more important than the former for the synchronization of hepatocyte clocks. This conclusion is based on the temporal analysis of hepatic gene expression in mice and voles subjected to ultradian and circadian feeding regimens, respectively (van der Veen et al. 2006). Mice display a strongly circadian behavior, and as nocturnal animals, they consume about 80% of their food during the dark phase when fed ad libitum. When mice received only 75% of the normal calorie intake as portions offered

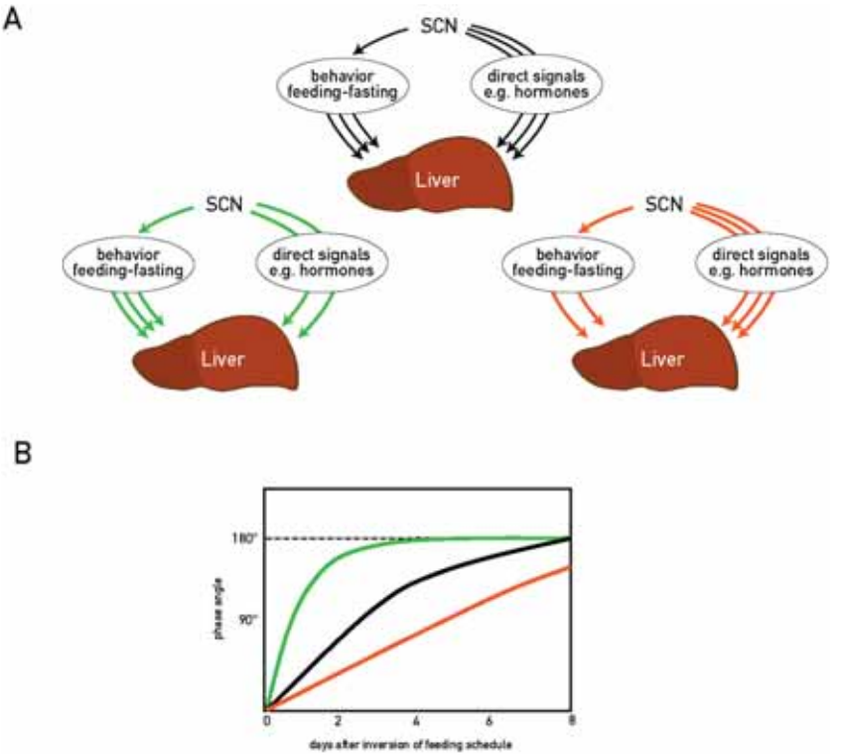

Figure 1. Direct and indirect phase entrainment routes for hepatocyte oscillators. The SCN synchronizes cellular circadian oscillators in liver (and other peripheral tissues) indirectly through circadian behavior (feeding rhythms) and more directly via controlling cyclic hormone secretion or via establishing body temperature oscillations. The scheme presented in *A* arbitrarily assumes that both routes employ three signaling pathways (*top scheme*). When an inverted feeding regimen is imposed on such animals (rats or mice), the kinetics of phase inversion follows the hypothetical black curve in *B*. Feeding inversion does not affect the phase of the SCN and thus the phase of direct zeitgeber cues. Therefore, after inverting the phase of the feeding/fasting cycles, the direct and indirect synchronization signals are in conflict. The phase inversion kinetics in liver depends on the competition between direct and indirect zeitgeber signals. If one (or more) of the direct signaling pathways are inactivated, the food-regimen-induced phase inversion is expected to be accelerated (*left scheme in A*, *green curve in B*). Conversely, if a feeding-dependent pathway is impaired, the kinetics of food-regimen-induced phase inversion is expected to be slowed down (*right scheme in A*, *red curve in B*).

at 150-minute intervals (ultradian feeding regimen), they eagerly ingested each meal immediately after it was provided by the feeding machine. This ultradian feeding regimen had little influence (a small phase advance) on the amplitude and magnitude of clock gene expression in the liver, suggesting that more direct SCN-controlled timing cues synchronize hepatocyte clocks when mice are feeding throughout the day (van der Veen et al. 2006). Common voles (*Microtus arvalis*) normally forage and feed at 150-minute intervals and hence are considered to be ultradian animals (Gerkema et al. 1990; Gerkema and van der Leest 1991). Under these conditions, circadian clock gene expression was found to be flat at intermediate levels in liver and kidney, suggesting that the cells of peripheral organs are not synchronized when voles feed at their normal ultradian schedule (van der Veen et al. 2006). However, when 8-hour daily fasting periods were imposed upon these animals, their livers and kidneys displayed robustly circadian clock gene expression. The timing signals provoked by the fasting periods in laboratory rodents remain to be identified, but it is tempting to speculate that fibroblast growth factor 21 (FGF-21) is one of them (see below).

MANY CHEMICAL AND PHYSICAL SIGNALS CAN SYNCHRONIZE CIRCADIAN OSCILLATORS IN CULTURED FIBROBLASTS

Cultured fibroblasts offer a simple experimental system to demonstrate the efficacy of body temperature rhythms as circadian timing cues. Originally, we thought that such low-amplitude temperature oscillations would sustain but not establish the synchronization of fibroblast oscillators (Brown et al. 2002). However, by reexamining the impact of body temperature rhythms on the phase entrainment with more sensitive techniques during extended time periods, we realized that a substantial fraction of fibroblasts can be synchronized by such temperature oscillations (C. Saini and U. Schibler, unpubl.).

Although fibroblast clocks are synchronized when implanted under the skin in the neck region of mice, it turned out to be difficult to use them as an experimental system for the characterization of chemical signals. Obviously, serum contains a myriad of growth factors, cytokines, metabolites, and hormones, and as shown in Figure 2A, experiments performed in several laboratories have indicated that many signaling pathways can syn-

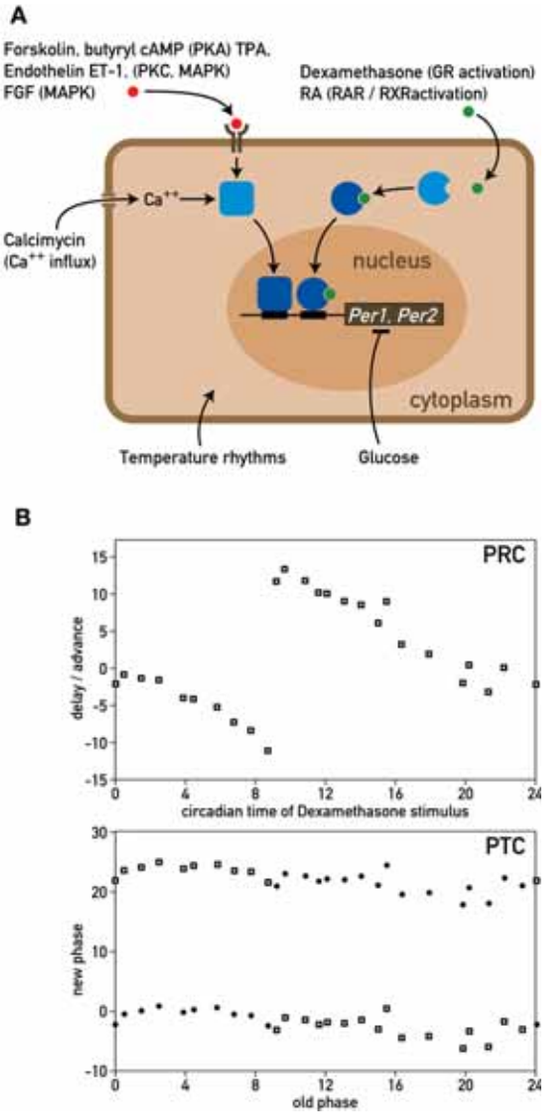

Figure 2. Many signaling pathways can synchronize the circadian oscillators of cultured fibroblasts. (*A*) The synchronization of cultured fibroblasts can be accomplished by a short treatment of cells with chemicals activating a variety of known signaling pathways, metabolites, and artificially imposed body temperature rhythms. All of these entrainment pathways probably involve the up- or down-regulation of *Per1* and *Per2*, and some of them act through the activation of various protein kinases (given in parentheses) and transcription factors (e.g., CREB, *blue square*). Ligand-bound nuclear receptors, such as the glucocorticoid receptor, may directly bind to hormone-responsive DNA response elements within the promoter and enhancer regions of *Per* genes. (*B*) Phase-response curve (PRC, *upper panel*) and phase transition curve (PTC, *lower panel*) of dexamethasone-treated NIH-3T3 cells expressing luciferase from a *Bmal1* promoter. Dexamethasone pulses were applied to parallel cultures approximately every hour. The resulting phase shifts in the daily luminescence cycles were plotted against circadian time. The data presented in the PRC are represented as a phase-transition curve (PTC), in which the new phase is plotted against the old phase. *Black dots* and *open squares* can be considered as phase-shifts measured on 2 consecutive days. For simplicity, all points are plotted on both days. Note that all new phases are nearly identical irrespective of when cells were treated with dexamethasone. Hence, the slope of the PTC is near zero, and the corresponding phase-response curve is called type-zero PRC. (*B*, Reprinted, with permission, from Nagoshi et al. 2004 [© Elsevier].)

chronize circadian oscillators in cultured fibroblasts (Gachon et al. 2004). Moreover, fibroblast clocks exhibit a so-called type-zero phase-response curve (PRC) (Nagoshi et al. 2004). When they are synchronized using a serum shock and then phase-shifted by a short pulse of dexamethasone at different times during the day, the slope of the phase transition curve (PTC), in which the new phase is plotted against the old phase, is close to zero (see PRC and PTC in Fig. 2B). This exemplifies the high sensitivity of fibroblast oscillators to phase-shifting cues and explains why they can readily be synchronized. However, these phase-shifting properties also render it difficult to measure the effect of, say, a blood-borne zeitgeber signal in a dose-dependent manner.

Given these difficulties, we have decided to design experimental strategies aimed at the identification of physiological zeitgebers in live animals. One such approach is presented in the next section.

A MOUSE WITH A CONDITIONALLY ACTIVE LIVER CLOCK

In principle, circadian gene expression in peripheral tissues can be regulated by systemic cues and/or local oscillators. Systemic cues (e.g., hormones) are interpreted by their sensors (i.e., hormone receptors), and this leads to the activation of immediate-early genes. If the blood-borne systemic signaling component oscillates during the day and if its average concentration is equal or lower than the dissociation equilibrium constant (K_D) determining its interaction with its receptor, the transcription rates of the signal-dependent immediate-early gene should also oscillate. Moreover, if an immediate-early (or early) gene controlled by this pathway was itself a cock component (such as PER1 or PER2), its system-controlled cyclic expression might influence the phase of the cycling signaling component. Therefore, the separation of system-driven and oscillator-dependent immediate-early (or early) genes would possibly provide insight into the molecular signaling pathways involved in the phase entrainment of peripheral clocks.

We thus attempted to design a transgenic mouse model that would allow us to discriminate between system- and oscillator-driven genes in hepatocytes. For this purpose, we established a mouse in which local hepatocyte oscillators can be switched off and on at will by providing or not the tetracycline analog doxycycline (Dox) in the food or drinking water (Kornmann et al. 2007). In this system, a rat REV-ERBα (rREV)-encoding cDNA expression vector was brought under the control of a liver-specifically expressed Tet activator that can only bind to its operators within the rREV cDNA transgene promoter in the absence of Dox (Tet-off system). Under these conditions, overproduced rREV occupies its RORE cognate elements within the *Bmal1* promoter and thereby represses *Bmal1* transcription. As BMAL1 is indispensable for circadian oscillator function, the hepatocyte oscillators are not operational in the absence of Dox. If Dox is added to the food (or drinking water), the Tet activator changes conformation and loses its affinity for the Tet operators within the rREV transgene promoter. Hence, rREV no

longer accumulates, *Bmal1* transcription is up-regulated, and hepatocyte oscillators become functional (Fig. 3A). An *mPer2::luc* reporter allele, engineered by Takahashi and colleagues by inserting a fire fly luciferase open reading frame into the resident *mPer2* locus by homologous recombination (Yoo et al. 2004), was crossed into the Tet-activator/rREV double transgenic mice. This allowed us to record circadian luminescence cycles in tissue explants in real time. As shown by the data presented in Figure 3B (right panels), liver slices from Tet-activator/rREV/ *mPer2::luc* triple transgenic mice displayed circadian luminescence cycles only when Dox was added to the culture medium. As expected, Dox treatment had little effect on circadian *mPer2::luc* expression in control mice without the Tet-activator and rREV transgenes (Fig. 3B, left panels). Importantly, the phases of *mPer2-luc*-dependent luminescence cycles were nearly identical in *mPer2::luc* control mice and Tet-activator/rREV/*mPer2::luc* mice when these animals received Dox for 48 hours before they were sacrificed (Fig. 3B, bottom panels). However, Dox-activated liver explants from untreated Tet-activator/ rREV/*mPer2::luc* mice produced luminescence cycles with a delayed phase (Fig. 3B, middle panels). Presumably, the decay of transgene-encoded rREV repressor and the accumulation of BMAL1 to appropriate levels lasted several hours. All in all, these experiments provided proof of principle for the Dox dependence of circadian oscillator function in Tet-activator/rREV/ *mPer2::luc* mice, and, by inference, in Tet-activator/ rREV mice.

SYSTEMICALLY AND OSCILLATOR-DRIVEN GENES

Encouraged by these results, we performed genome-wide transcriptome-profiling experiments in mice that did or did not receive Dox as a food accompaniment. To this end, untreated and Dox-treated mice were sacrificed every 4 hours during 2 entire days, and the liver RNAs from these animals were hybridized to 430 2.0 Affymetrix oligonucleotide arrays representing the majority of protein-coding genes. Circadian transcripts were identified by applying data analysis procedures that use relatively stringent algorithms (see legend to Fig. 4). The circadian transcripts identified in the Dox-treated mice are expected to be products of both system-dependent and oscillator-dependent genes. The class of genes encoding these mRNAs contained core clock genes (e.g., *mPer1, mPer2, Bmal1, mCry1, Dec1, Dec2, Rorγ*, *Rev-erbα*, and *Rev-erbβ*) and clock-controlled genes (e.g., *Dbp, E4bp4, Tef,* and *Hlf*). In addition and in keeping with related studies, many genes encoding enzymes involved in the metabolism of proteins, carbohydrates, lipids, cholesterol, bile acids, and xenobiotics were found to be cyclically expressed. Given the high stringency of the algorithms we used to extract circadian genes from the Affymetrix microarray data, our analysis revealed somewhat fewer genes (350 genes represented by 470 feature sets) than other studies (Panda et al. 2002; Storch et al. 2002).

The genes identified in mice not receiving Dox as a food supplement are expected to be issued from system-driven, oscillator-independent genes (Fig. 3C). This inter-

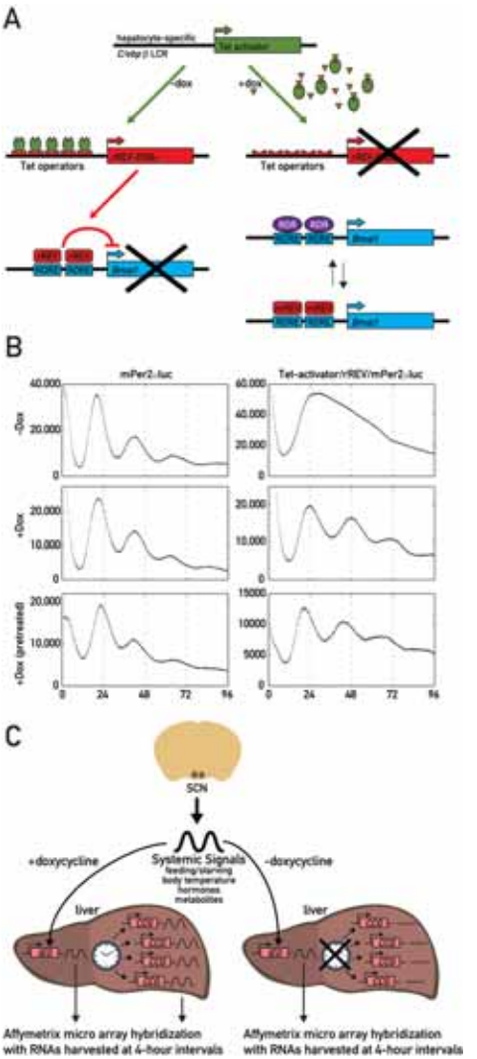

Figure 3. Systemic and oscillator-driven gene expression in transgenic mice with conditionally active liver clocks. (*A*) Hepatocyte oscillators can be arrested by the overexpression of REV-ERBα (rREV for rat REV-ERBα). Hepatocyte-specific, Dox-dependent expression of HA-REV-ERBα was achieved by placing a 5′-HA-tagged REV-ERBα cDNA transgene under the control of seven tetracycline responsive elements (TREs). In the liver of mice expressing the Tet-responsive *trans*-activator from the hepatocyte-specific *C/ebp*β-*LAP* locus control region (LCR), rREV-ERBα transcription is constitutively repressed in the absence of the tetracycline analog dox (Tet-off system). The constitutively overexpressed rREV repressor now effectively competes throughout the day with ROR activators for the occupancy at RORE elements. This leads to an attenuation of circadian oscillator function, because *Bmal1* is required for circadian rhythm generation. In the presence of Dox, the rREV-ERBα is silent, and circadian *Bmal1* transcription is regulated like in wild-type mice through alternating binding of ROR activators and endogenous murine REV-ERBα/REV-ERBβ repressors (mREV) to RORE elements. (*B*) Liver slices from *mPer2::luc* (*left*) and Tet-activator/rREV/*mPer2::luc* (*right*) were cultured in luciferin-containing medium in the absence (–Dox) or presence of 10 ng/μl doxycycline (+Dox). Luminescence was recorded using photomultiplier tubes. –Dox and +Dox samples are from the same animal, +Dox (pretreated) samples are from mice that have received two intraperitoneal injections of Dox 48 and 24 hours before being sacrificed. (*C*) A genome-wide search for liver clock-driven and systemically regulated gene (which comprise "immediate-early genes," IEG). Note that only systemically driven genes continue to be expressed in a diurnal fashion in the absence of Dox.

pretation should, however, be qualified by some notes of caution. In our Tet-activator/rREV mice, the Tet-activator transgene is expressed specifically in hepatocytes. Although these make up the majority of the liver mass, about 10–20% of the liver RNA is probably derived from Kupffer cells, bile duct cells, endothelial cells, and liver fibroblasts. In these cells, *Bmal1* accumulation is supposedly normal, even in the absence of Dox. Furthermore, even hepatocytes of untreated animals may still accumulate low levels of BMAL1, and these might be sufficient for the residual transcription of those CLOCK-BMAL1 target genes that contain very high-affinity E-box-binding motifs. To limit the number of such "false positives" in the identification of systemically driven transcripts, we selected mRNAs whose average expression levels were similar in untreated and Dox-treated animals (ratio –Dox/+Dox between 0.7 and 1.5). These mRNAs constituted the majority (60%) of liver transcripts whose expression remained rhythmic in Dox-free mice. The 61 genes encoding such transcripts were represented by 79 feature sets and are displayed in Table 1 and in the phase map of Figure 4B (right panels). As expected, virtually all of these transcripts also accumulate in a rhythmic fashion in the livers of Dox-treated mice. Interestingly, the systemically driven genes can be grouped into functional categories (Table 1; Fig. 4B,C). The genes of two categories fall into defined phase clusters. Genes involved in cholesterol synthesis are maximally expressed between ZT17 and ZT20, and genes encoding chaperones (heat shock proteins) are maximally expressed between ZT18 and ZT00.

As nocturnal animals, mice are active between ZT12 and ZT00 and hence consume most of their food during this time period. Increased hepatic cholesterol synthesis between ZT17 and ZT20 may be required to replenish the cholesterol converted to bile acids. Indeed, *Cyp7a1* mRNA, encoding the rate-limiting enzyme of bile acid synthesis, is maximally expressed at ZT10, shortly before the cholesterol synthesis is expected to reach maximal rates (G. Le Martelot and U. Schibler, unpubl.). Conceivably, the conversion of cholesterol to bile acids reduces membrane cholesterol levels in the endoplasmic reticulum, and this triggers the activation of SREBP, the transcription factor regulating most of the cholesterol-related genes listed in Table 1 as a consequence of cholesterol depletion. In keeping with these conjectures, the proteolytic activation of SREBP has been found to follow a robustly diurnal pattern (Brewer et al. 2005; G. Le Martelot and U. Schibler, unpubl.).

Heat shock proteins (HSPs) participate in the response to proteotoxic stress, caused by elevated temperature and/or chemical insults (Burel et al. 1992; Voellmy 1996). As chaperones, HSPs bind to and refold denatured cellular proteins or target ubiquitin ligases to damaged proteins, so that these can be recognized and degraded by the proteasome (Hirsch et al. 2006). Heat shock transcription factor 1 (HSF1) is a major sensor of elevated temperature and noxious chemicals, such as reactive oxygen species (ROS) (Liu et al. 1996; Voellmy and Boellmann 2007). It normally is sequestered in an inert cytoplasmic protein complex, consisting of HSP90 and small cochaperones. Upon exposing cells to a heat stress or treating them with certain chemicals, denatured proteins compete with HSF1 for binding to HSP90. As a consequence, HSF1 gets released from chaperones, becomes phosphorylated by various protein kinases, migrates to the nucleus, and binds as a homotrimer to heat shock elements (HSEs) within the promoters of multiple HSPs. In a second step, the *trans*-activation capacity of HSF1 becomes further stimulated through phosphorylation by calcium/calmodulin-dependent kinase 2β (CAMK2β), which leads to the transcription of HSF1 target genes (Holmberg et al. 2001). Of note, *Camk2*β mRNA was also found among the systemically driven transcripts, and the phase of *Camk2*β expression is compatible with its function in HSF1 activation (see Table 1).

PUTATIVE CANDIDATE SIGNALING PATHWAYS INVOLVED IN THE PHASE ENTRAINMENT OF PERIPHERAL CLOCKS

As pointed out earlier in this chapter, some of the systemically driven genes may participate in the synchronization of peripheral clocks. Of note, the hepatic expression of *mPer2*, a bona fide component of the molecular circadian oscillator, follows a robust diurnal rhythm in the absence of Dox in the animal but not in tissue explants (Kornmann et al. 2007). We hence surmise that systemic cues drive its rhythmic transcription in the absence of naturally occurring BMAL1 levels. Although we have not yet unambiguously identified the molecular mechanisms responsible for the systemic regulation of *mPer2* expression, based on several observations, we suspect that HSF1 may participate in this endeavor: (1) The cyclic accumulation of *mPer2* mRNA, like *Hsp* mRNAs, reach maximal levels when body temperature is highest, (2) in liver and lung explants of *mPer2::luc* mice, mPer2-LUC expression is transiently increased by a heat shock, (3) the circadian expression of clock gene expression in cultured fibroblasts can be synchronized by body temperature rhythms, and (4) during this process, the expression of *mPer2* is synchronized more rapidly than that of other clock genes (C. Saini and U. Schibler, unpubl.). We wish to reiterate, however, that temperature cycles may not be the only cues leading to a rhythmic activation of HSF1. For example, both feeding and redox potential have been reported to stimulate the activity of HSF1 (Ahn and Thiele 2003; Katsuki et al. 2004). These findings are of particular interest, given the dominant role of feeding/fasting cycles in the synchronization of oscillators in liver and other peripheral tissues.

Fibroblast growth factor 21 (*Fgf-21*) is another systemically driven gene that aroused our interest. The expression of FGF-21 has recently been shown to be controlled by peroxisome proliferator-activated receptor α (PPARα) through fasting (Badman et al. 2007; Inagaki et al. 2007; Lundasen et al. 2007). Indeed, both the expression and the activity of PPARα are subject to diurnal regulation (Waring 1970; Lemberger et al. 1996; Canaple et al. 2006; F. Gachon and U. Schibler, unpubl.). Free fatty acids are natural activators of PPARα, and we observed that the hepatic concentrations of both *Pparα* mRNA and several

Table 1. List of System-driven Diurnally Expressed Genes

Affy gene name	Full gene name	Putative gene function	Ratio –Dox/+Dox
Hspa4l	Heat shock 70-kD protein-4-like (HSP110 gene family)	chaperone, osmotolerance	1.5
Atf3	Activating transcription factor 3	stress-induced by cytokines, genotoxic agents, amino acid starvation	1.5
Afp	α-fetoprotein	transport of lipophilic compounds	1.5
Actg1	Actin, γ, cytoplasmic 1	cytoskeleton	1.5
Hspca	HSP1, α, HSP90α	chaperone, regulator of NRs and other TFs	1.4
Dnclc1	Dynein, cytoplasmic, light chain 1	dynein microtubule motor	1.4
Tuba4	Tubulin, α 4A	tubulin cytoskeleton, microtubules	1.4
Hspa4	Heat shock 70-kD protein 4	chaperone	1.3
Klhl24	Kelch-like 24	?	1.3
Tuba4	Tubulin, α 4A	tubulin cytoskeleton, microtubules	1.3
Afp	α-fetoprotein	transport of lipophilic compounds	1.3
Hspca	HSP1, α, HSP90α	chaperone, regulator of NRs and other TFs	1.3
Ccrn4l	CCR4 carbon catabolite repression 4-like, nocturnin	mRNA deadenylase?	1.3
1431214_at			1.3
Ldb1	LIM domain-binding 1	cofactor of TFs (e.g., Nkx5), development	1.2
1423672_at			1.2
Trpm4	Transient receptor potential cation channel, subfamily M, member 4	cellular sensors of Ca^{++}, temperature	1.2
Hddc3	HD-domain-containing 3		1.2
Tubb6	Tubulin, β6	tubulin cytoskeleton, microtubules	1.2
Heca	Headcase homolog (*Drosophila*)	basic protein, *Drosophila* imaginal disk cell proliferation	1.1
Cyp51	Sterol 14 α-demethylase (cholesterol synthesis)	cholesterol synthesis	1.1
Hspa1a	Heat shock 70-kD protein 1A	chaperone	1.1
Hspa8	Heat shock 70-kD protein 8	chaperone	1.1
Fus	Fusion involved in t(12;16) in malignant liposarcoma (TLS)	RNA/DNA-binding protein	1.1
Hspca	HSP1, α, HSP90α	chaperone, regulator of NRs and other TFs	1.1
Slc25a15	Slc25a15 solute carrier family 25	mitochondrial carrier ornithine transporter	1.1
Hspa1a	Heat shock 70-kD protein 1A	chaperone	1.1
Hsp110	HSP110	chaperone, apoptosis regulator	1.0
Fbxl20	F-box and leucine-rich repeat protein 20	ubiquitination, substrate receptor	1.0
Chordc1	Cysteine and histidine-rich domain (CHORD)-containing, zinc-binding protein 1	chromatin regulator? histone methyl transferase? associated with HSP90	1.0
Hsp110	HSP110	chaperone, apoptosis regulator	1.0
Fus	Fusion involved in t(12;16) in malignant liposarcoma (TLS)	RNA/DNA-binding protein	1.0
Ris2	Retroviral integration site 2 (Human homolog = CDT1)	DNA replication licensing factor, complex with geminine	1.0
Hspa8	Heat shock 70-kD protein 8	chaperone	1.0
Cyp2b10	Cytochrome p450, family 2, subfamily b, polypeptide 10	xenobiotic detoxification, CAR target gene	1.0
Cyp51	Sterol 14 α-demethylase (cholesterol synthesis)	cholesterol synthesis	1.0
Per2	Period 2	circadian core clock component	1.0
Rnf6	RNF6 ring finger protein	ubiquitin ligase regulates LIMK1→actin polymerase	1.0
Fgf21	FGF-21 fibroblast growth factor 21	MAPK activator	1.0
1453303_at			1.0
Hspa1a	Heat shock 70-kD protein 1A	chaperone	1.0
Per2	Period 2	circadian core clock component	1.0

(*Continued on following page.*)

Table 1. (*Continued*)

Affy gene name	Full gene name	Putative gene function	Ratio –Dox/+Dox
Alas1	Aminolevulinate, δ-, synthase 1	heme synthesis	0.9
Rnf125	Ring finger protein 125	ubiquitin ligase, inhibits RIG-1 signaling, dsRNA sensor	0.9
Erbb3	v-*erb*-b2 erythroblastic leukemia viral oncogene homolog 3	EGF receptor family, HGF receptor, PI3K induction	0.9
Rbl2	Retinoblastoma-like 2 (=p130)	cell cycle regulator, cancer	0.9
Tgoln1	(=Ttgn1) *trans*-Golgi network protein	protein trafficking, EGFR endocytosis?	0.9
Slc25a25	Slc25a15 solute carrier family 25	mitochondrial carrier ornithine transporter	0.9
1431213_a_at			0.9
Hmgcs1	3-hydroxy-3-methylglutaryl–coenzyme A synthase 1	cholesterol synthesis	0.9
Errfi1	ERBB receptor feedback inhibitor 1 (=Mig6 ortholog)	inhibitor of EGFR and MAPK signaling	0.9
Sqle	SQLE squalene epoxidase	cholesterol metabolism	0.9
Stip1	stress-induced-phosphoprotein 1	HSP70/HSP90-organizing protein	0.9
Efna1	Ephrin-A1	ephrin signaling, ligand of EphA2 receptor	0.9
Cirbp	Cold-inducible RNA-binding protein	cold stress regulator?	0.9
Fbxo21	F-box-only protein 21	ubiquitination, substrate receptor	0.9
Tcp11l2	Tcp11l2 t-complex 11 (mouse)-like 2	?	0.9
Errfi1	ERBB receptor feedback inhibitor 1 (=Mig6 ortholog)	inhibitor of EGFR and MAPK signaling	0.9
Ddx46	DEAD (Asp-Glu-Ala-Asp) box polypeptide 46	RNA helicase	0.9
Per2	Period 2	circadian core clock component	0.9
Calcoco1	Calcium binding and coiled-coil domain 1	transcriptional coactivator	0.9
March7	Membrane-associated ring finger (C3HC4) 7	membrane-bound ubiquitin ligase, endos., Endos. transport	0.9
Sc4mol	Sterol-C4-methyl oxidase-like	cholesterol synthesis	0.8
1455892_x_at			0.8
Ctgf	Connective tissue growth factor	extracellular-matrix-bound signaling factor, activated by SRF,	0.8
1452418_at		repressed by soluble actin	0.8
1435084_at			0.8
Hmgcs1	3-hydroxy-3-methylglutaryl–coenzyme A synthase 1	cholesterol synthesis	0.8
Ass1	Argininosuccinate synthetase 1	arginine synthesis, urea cycle, induced by amino acid starvation	0.8
Idi1	Isopentenyl-diphosphate δ isomerase	cholesterol synthesis	0.8
Fbxo21	F-box-only protein 21	ubiquitination, substrate receptor	0.8
Hmgcs1	3-hydroxy-3-methylglutaryl–coenzyme A synthase 1	cholesterol synthesis	0.8
Alas1	Aminolevulinate, δ-, synthase 1	heme synthesis	0.8
Fdps	Farnesyl diphosphate synthetase	isoprenoid metabol, FGF signaling?	0.8
1428531_at			0.7
Enpp3	Ectonucleotide pyrophosphatase/phosphodiesterase 3	hydrolysis of extracellular nucleotides	0.7
Idi1	Isopentenyl-diphosphate δ isomerase	cholesterol synthesis	0.7
Alas1	Aminolevulinate, δ-, synthase 1	heme synthesis	0.7
Lss	Lanosterol synthase (2,3-oxidosqualene-lanosterol cyclase)	cholesterol synthesis	0.7
Camk2b	Calcium/calmodulin-dependent protein kinase (CaM kinase) II β	activation of HSF1, inhibition of CREB	0.7

Liver transcripts whose accumulation continues to be diurnal in the absence of functional hepatocyte oscillators are listed according to the ratio of average hybridization signals obtained in the absence and presence of doxycycline. The putative functions of these genes are indicated in column 3.

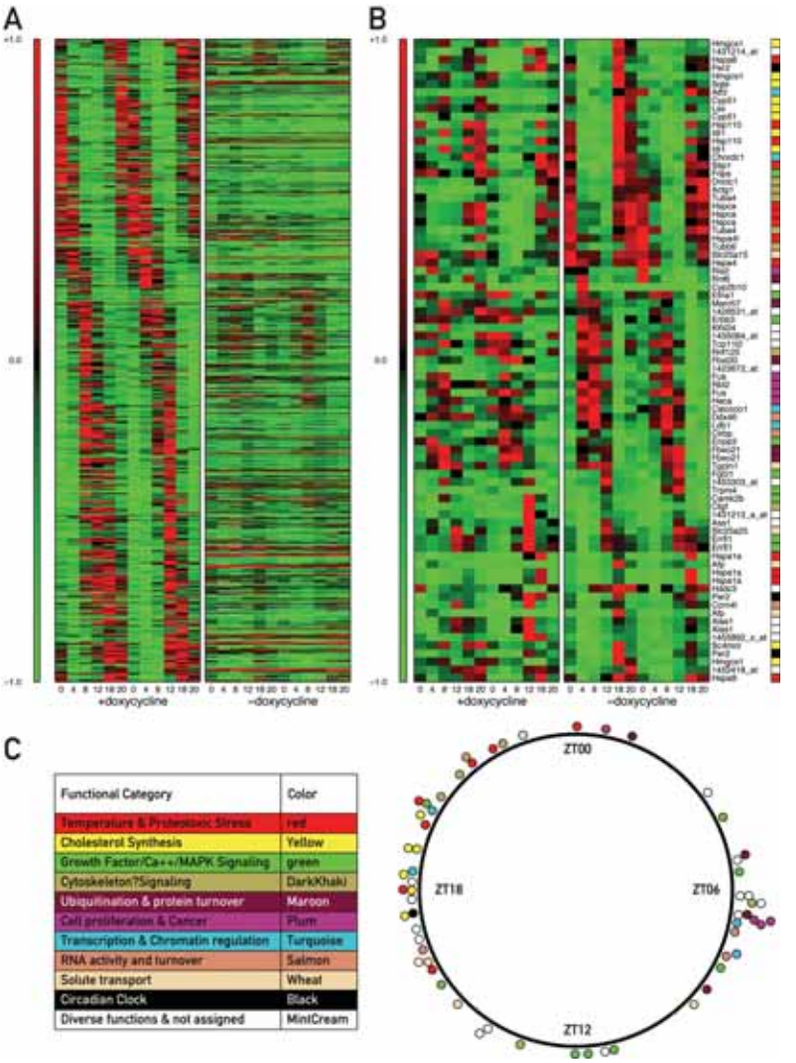

Figure 4. Phase maps of cyclically expressed transcripts in the presence and absence of Dox. Phase maps of genes selected from temporal Affymetrix data sets using the algorithms described by Kornmann et al. (2007). Briefly, the data sets composed of genome-wide hybridization signals obtained for liver RNA prepared from Tet-activator/rREV mice sacrificed at 4-hour intervals during 2 days were screened for circadian transcripts using a *p*-value of 0.05 for Fourier analysis and an amplitude of equal or greater than two. (*A*) Phase map of cyclic transcripts selected according to these criteria from the data sets obtained for Dox-treated animals. Note that most (but not all) of the transcripts selected in this way lose robust circadian accumulation in the absence of Dox. (*B*) Phase map of cyclic transcripts selected according to these criteria from the data sets obtained for untreated animals (–Dox). Note that most of the transcripts selected in this way also show a daily accumulation cycle in the presence of Dox, as expected. The color code bar on the right hand side of the panel indicates the putative functions of the proteins encoded by these systemically regulated genes. (*C*) Color code for functional gene categories for the transcripts displayed in *B* and circular phase map of these transcripts.

free fatty acids culminate toward the end of the postabsorptive phase (ZT08 to ZT12). Fibroblast growth factors signal through tyrosin receptor kinases that subsequently can activate various protein kinases, including mitogen-activated protein kinases (MAPKs) (Eswarakumar et al. 2005; Kurosu et al. 2007). MAPK phosphorylates serine and threonine residues in many protein substrates, including the cAMP-responsive element-binding protein (CREB). Both *mPer1* and *mPer2* are CREB target genes, and the activation of CREB has been shown to have an important role in the synchronization of circadian oscillators in SCN neurons (Morse and Sassone-Corsi 2002; Dziema et al. 2003; Hastings and Herzog 2004) and cultured fibroblasts (Balsalobre et al. 2000a; Yagita and Okamura 2000). In contrast to other fibroblast growth factors, FGF-21 has a weak heparin-binding domain and is thus not trapped in the extracellular matrix (Kurosu et al. 2007). Hence, it may act as a paracrine factor in the liver and as a hormone in distant tissues, such as muscle, fat, and kidney. As mentioned above, liver clocks get food-entrained more rapidly than clocks in other peripheral tissues, and the liver-specific expression of FGF-21 may provide a rational explanation for this finding.

Figure 5 schematically illustrates the speculations proposed above on phase entrainment signaling pathways operative in peripheral tissues. Conceivably, PER1 and

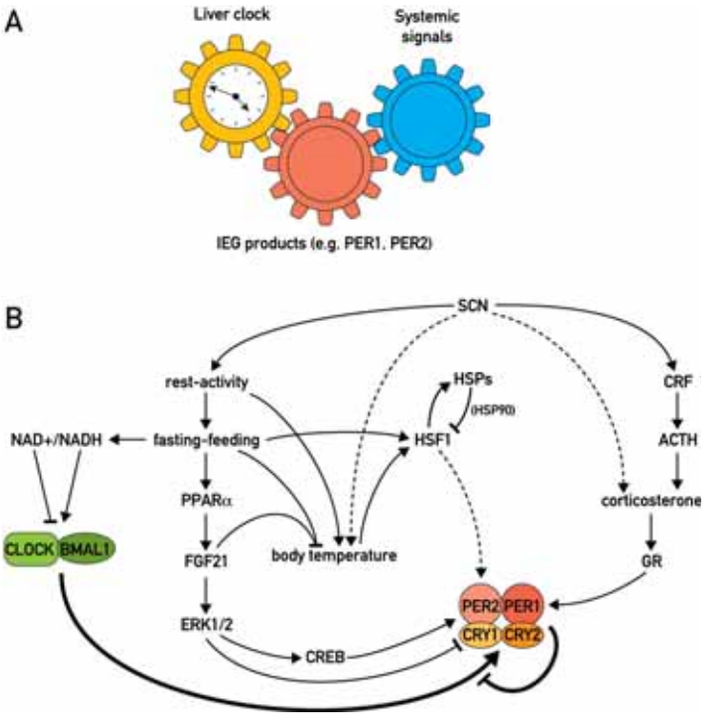

Figure 5. Interaction between system- and oscillator-driven genes. (*A*) Cogwheel diagram illustrating how systemically driven immediate-early genes, such as those encoding mPER1 and mPER2, could synchronize local circadian oscillators. Such components can serve as both core clock components and sensors of systemic zeitgeber cues controlled by the SCN. (*B*) Hypothetical scheme of molecular input pathways for peripheral circadian oscillators (see text for explanation).

PER2 serve as both cogwheels of the clockwork circuitry and immediate-early genes in its synchronization (Fig. 5A). Many molecular signaling pathways may contribute to this synchronization, and as hypothesized in Figure 5B, they are likely to be intertwined. For example, the feeding-dependent cyclic expression of FGF-21, in conjunction with fasting, may decrease body temperature and thereby down-regulate HSF1 activity during the second half of the postabsorptive phase. Furthermore, MAPK may not only increase PER1 and PER2 expression, but also inhibit the repressive activity of cryptochrome proteins on CLOCK-BMAL1-mediated transcription. In addition to these molecular signaling pathways depending on feeding behavior, glucocorticoids (and perhaps other hormones) are likely to serve as more direct messengers of the SCN in its endeavor to keep the phase of peripheral oscillators tuned (Balsalobre et al. 2000b; Le Minh et al. 2001).

CONCLUSIONS AND PERSPECTIVES

Overt daily cycles in behavior and physiology depend on the synchronization of countless cellular oscillators in the SCN and peripheral organs. The deciphering of molecular signaling pathways involved in the establishment of phase coherence in multicellular organisms such as mammals is thus a major challenge in circadian rhythm research. Although many signaling cascades can synchronize circadian clocks in cultured cells, the molecular routes by which the SCN phase entrains peripheral clocks remain subject to speculation. Their unambiguous identification requires a combination of state-of-the-art biochemical and genetic approaches. We have presented here our initial attempts toward the detection of immediate-early genes that participate in the interpretation of systemic signals in liver. In conjunction with studies recently published by us and other investigators (see above), these experiments hint toward a role of body temperature rhythms, FGF-21 signaling, and glucocorticoids in the daily phase resetting of peripheral clocks. Undoubtedly, additional pathways will soon join the list. This redundancy poses a major problem in the dissection of entrainment pathways, because the elimination of only one signaling pathway may only have no or only minor consequences on the steady-state phase. As illustrated in Figure 1A and as demonstrated by the ablation of glucocorticoid receptor signaling in liver, the kinetics of feeding-induced phase resetting is a much more sensitive readout in genetic loss-of-function studies. The recording of phase-resetting kinetics at a high temporal resolution requires novel experimental approaches, in which the phase of circadian gene expression can be monitored in real time and in live animals. Our laboratory is therefore investing a major effort toward the development of whole-animal imaging technologies that allow the measurement of circadian fluorescent protein expression in organs of freely moving mice.

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