Systems Biology of Mammalian Circadian Clocks

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Abstract

Systems biology is a natural extension of molecular biology; it can be defined as biology after identification of key gene(s). Systems-biological research is a multistage process beginning with (*a*) the comprehensive identification and (*b*) quantitative analysis of individual system components and their networked interactions, which lead to the ability to (*c*) control existing systems toward the desired state and (*d*) design new ones based on an understanding of the underlying structure and dynamical principles. In this review, we use the mammalian circadian clock as a model system and describe the application of systems-biological approaches to fundamental problems in this model. This application has allowed the identification of transcriptional/posttranscriptional circuits, the discovery of a temperature-insensitive period-determining process, and the discovery of desynchronization of individual clock cells underlying the singularity behavior of mammalian clocks.

INTRODUCTION: SYSTEMS BIOLOGY AS BIOLOGY AFTER IDENTIFICATION

Recent large-scale efforts in genome sequencing, expression profiling, and functional screening have produced an embarrassment of riches for life science researchers, and biological data can now be accessed in quantities that are orders of magnitude greater than were available even a few years ago. The growing need for interpretation of data sets, as well as the accelerating demand for their integration to a higher-level understanding of life, has set the stage for the advent of systems biology (1, 2), in which biological processes and phenomena are approached as complex and dynamic systems. Systems biology is a natural extension of molecular biology and can be defined as biology after identification of key gene(s).

We consider systems-biological research as a multistage process (**Figure 1**) that comprises four steps: (*a*) identification of the whole network structure through functional genomics and comparative genomics (system identification), (*b*) prediction and validation of the system's behavior to derive the design principle through the accurate measurement and (static) perturbation of network dynamics (system analysis), (*c*) control and repair of the network state toward the desired state through the precise and

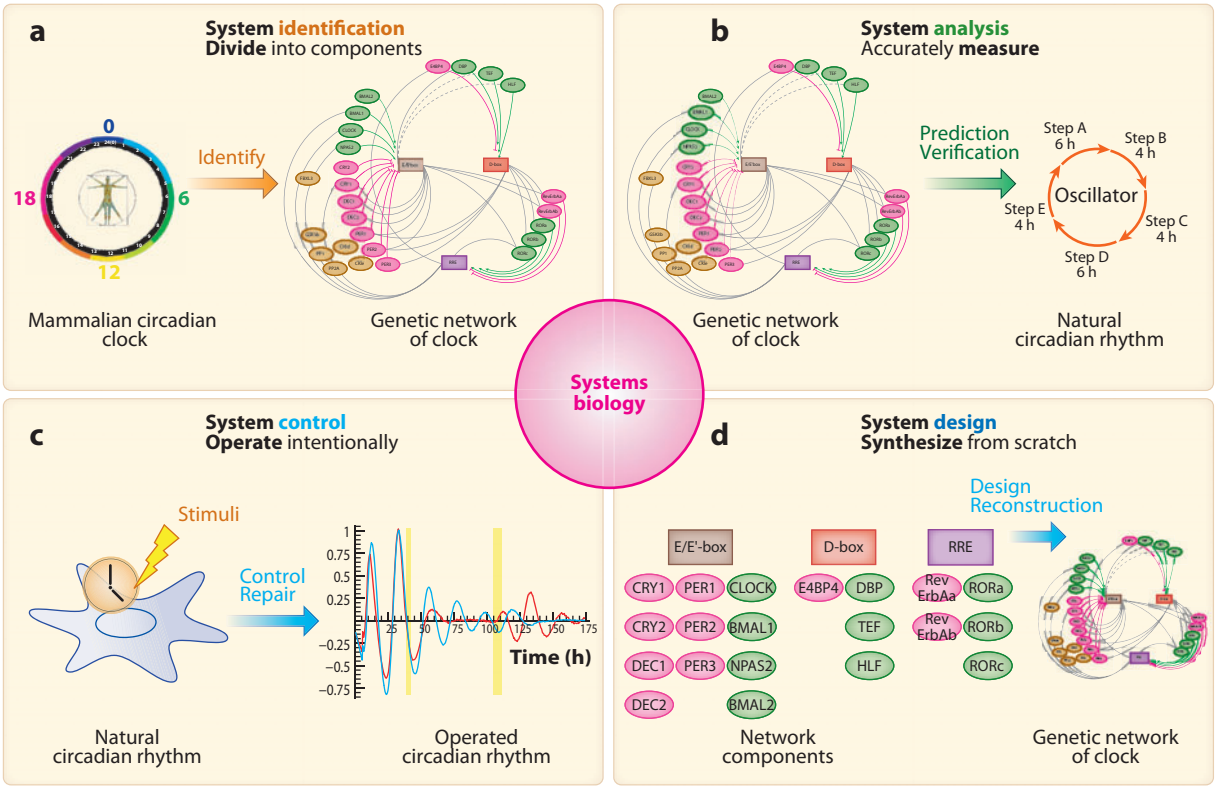

Figure 1

Systems biology. (*a*) Systems-biological research begins with comprehensive identification. In this step, individual system components and their networked interactions are comprehensively identified. (*b*) In the second step, to derive the design principle of a target system, the behavior of the system is predicted and validated through an accurate measurement with perturbations. (*c*) An understanding of the design principle of the system is needed to derive the method of controlling the system toward the desired state. (*d*) Finally, the level of understanding is confirmed by reconstruction of the system.

dynamic perturbation of its components (system control), and (*d*) reconstruction and design of systems based on the design principles derived from the identified structure and observed dynamics (system design). These processes are required to elucidate the design principles of complex and dynamic biological systems, such as the mammalian circadian clock.

MAMMALIAN CIRCADIAN CLOCK AS A MODEL SYSTEM

The mammalian circadian clock is an ideal model system for the study of complex and dynamic biological systems. The mammalian circadian clock consists of complexly integrated feedback and feed-forward loops (3) and exhibits well-defined dynamical properties (4), including (*a*) endogenous oscillations with an approximately 24-h period, (*b*) entrainment to external environmental changes (temperature and light cycle), (*c*) temperature compensation over a wide range of temperatures, and (*d*) synchronization of multiple cellular clocks despite the inevitable molecular noise. All of these dynamical properties would be difficult to elucidate without utilizing such system-level approaches. In addition to its advantages as a basic model system for systems-biological research, the circadian clock is intimately involved in the control of metabolic and physiological processes (3, 5), and its dysregulation is associated with the onset and development of numerous human diseases, including sleep disorders, depression, and dementia. An improved system-level understanding promises to provide biomedical and clinical investigators with a powerful new arsenal with which to attack these conditions.

Some systems-biological efforts to elucidate the design principles of complex and dynamic biological systems such as the mammalian circadian clock have been reported. In the following sections, we describe in detail the strategies and technologies developed for systems-biological research, in addition to their applicability to the specific case of the mammalian circadian clock.

IDENTIFICATION OF CLOCKS

Following the completion of genome projects for species such as mouse and human, genome-wide resources such as small interfering RNA (siRNA) and complementary DNA (cDNA) libraries have undergone considerable expansion. Development of high-throughput technologies also allows efficient use of these resources. These genome-wide resources and technologies, as well as genome-associated information, allow us to comprehensively identify complex systems such as the mammalian circadian clock (system identification).

Temperature compensation: phenomenon that causes the period of an oscillation to be stable despite temperature change

SCN: suprachiasmatic nucleus

Identification of the Transcriptional Circuit

The mammalian circadian master clock is primarily located in the suprachiasmatic nucleus (SCN) (3). Transcript analyses have indicated that circadian clocks are not restricted to SCN but are found in various tissues (6), including liver, and in cultured fibroblast cells such as Rat-1 (7, 8), NIH3T3 (9), and U2OS (10) cells. Increasing numbers of so-called clock genes were identified in mammalian clocks; these genes include three basic helix-loop-helix (bHLH)-PAS (PER-ARNT-SIM) transcription factors (*Clock*, *Npas2*, and *Arntl*; the latter is also known as *Bmal1* or *Mop3*) (11–14), two period genes (*Per1* and *Per2*) (15, 16), two cryptochrome genes (*Cry1* and *Cry2*) (17, 18), casein kinase I epsilon and delta (*Csnk1e* and *Csnk1d*) (19, 20), and two orphan nuclear hormone receptors (*Nr1d1* and *Rora*, also known as *RevErbα* and *Rorα*, respectively) (21, 22). A number of other transcriptional factors also thought to function in the circadian regulation of gene expression were gradually clarified; these include four bZip-family genes (*Dbp*, *Tef*, *Hlf*, and *Nfil3*; the latter is also termed *E4bp4*) (23, 24), three bHLH transcription factors (*Arntl2*, *Bhlhb2*, and *Bhlhb3*, also known as *Bmal2*, *Dec1* or *Stra13*, and *Dec2*, respectively) (25), one period-related gene (*Per3*) (26), and three genes related to *Nr1d1* and *Rora* (*Nr1d2*, *Rorb*, and *Rorc*, also known as *RevErbβ*, *Rorβ*, and *Rorγ*, respectively) (22, 27).

CCEs: clock-controlled elements

***dLuc*:** destabilized luciferase

In cellulo cycling assay: means of performing real-time monitoring of transcriptional dynamics of clock-controlled promoters in a cell via *dLuc*

Molecular interactions among these clock genes and clock-related genes have also been at least partly identified. For example, the transcription factors CLOCK and BMAL1 dimerize and directly and indirectly activate transcription of the *Per* and *Cry* genes through E-box elements (5′-CACGTG-3′) (28, 29). The PER and CRY proteins accumulate in the cytosol and are then translocated following phosphorylation into the nucleus, where they inhibit the activity of CLOCK and BMAL1 (3). The turnover of the inhibitory PER and CRY proteins leads to a new cycle of activation by CLOCK and BMAL1 via E-box elements. The transcriptional regulation network of these genes forms a circadian clock oscillator, which is known to control output genes and to affect physiological and metabolic processes (3, 5). This type of transcriptional feedback mechanism underlying circadian rhythms seems to be conserved across species (4). Despite reports of many transcriptional regulations of each gene, however, an overview of the circadian clock core network remains to be put forward.

Complicated networks cannot be elucidated without both a comprehensive identification of network circuits and an accurate measurement of system dynamics. In the first step toward complete identification of the circadian clock core network, several groups utilized DNA microarray to comprehensively and quantitatively measure genome-wide gene expression of mammalian circadian clocks (27, 30–54). For example, Ueda et al. (27) identified so-called clock-controlled genes and demonstrated circadian oscillation with characteristic expression patterns in mouse SCN and liver through use of a high-density oligonucleotide probe array (55) and biostatistics. In the second step, the authors comprehensively determined the transcription start sites via the oligo-capping method (56) to construct the genome-wide promoter/enhancer database. Using these data, the authors predicted a relationship between expression patterns of identified genes and DNA-regulatory elements on their promoter/enhancer regions. Three types of clock-controlled elements (CCEs)—E-boxes (5′-CACGTG-3′) (57) plus E′-boxes (5′-CACGTT-3′) (58, 59), D-boxes [5′-TTATG(C/T)AA-3′] (60), and RevErbA/ROR-binding elements (RREs) [5′-(A/T)A(A/T)NT(A/G)GGTCA-3′] (61)—are distributed throughout the oscillatory genes.

To determine the role of these elements in the circadian clock, Ueda et al. (27, 58) utilized a cell-culture system in which circadian rhythms in transcriptional dynamics are monitored via a destabilized luciferase (*dLuc*) reporter driven by clock-controlled promoters. In this cell-culture system—termed the in cellulo cycling assay—cultured Rat-1 cells were transiently transfected by reporter constructs and stimulated with dexamethasone, and their bioluminescences were measured. Dexamethasone was administrated to induce macroscopic circadian oscillations in the cultured cells. Through the genome-wide searching process described above, the authors found CCEs on 16 clock/clock-controlled gene promoters/enhancers. Via the cellulo cycling assay system, Ueda et al. revealed that functionally and evolutionarily conserved E/E′-boxes are located on the noncoding regions of nine genes (*Per1*, *Per2*, *Cry1*, *Dbp*, *Rorγ*, *RevErbα/Nr1d1*, *RevErbβ/Nr1d2*, *Dec1/Bhlhb2*, and *Dec2/Bhlhb3*); that D-boxes are located on those of seven genes (*Per1*, *Per2*, *Per3*, *RevErbα*, *RevErbβ*, *Rorα*, and *Rorβ*); and that RREs are located on those of six genes (*Bmal1/Arntl*, *Clock*, *Npas2*, *Cry1*, *E4bp4/Nfil3*, and *Rorγ*). On the basis of this functional and conserved transcriptional regulatory mechanism, investigators successfully drew transcriptional circuits underlying mammalian circadian rhythms (**Figure 2*a***) (58). We note that further characterization of these three CCEs was recently performed (62–65).

Ueda et al. (58) further suggested that regulation of E/E′-boxes is the topological vulnerability point in the mammalian circadian clock, and they functionally verified this concept using in cellulo cycling assay systems. Overexpression of repressors of E/E′-box regulation [CRY1 (29)], RRE regulation [REVERBα (22, 27)], or D-box regulation [E4BP4 (24)] affected

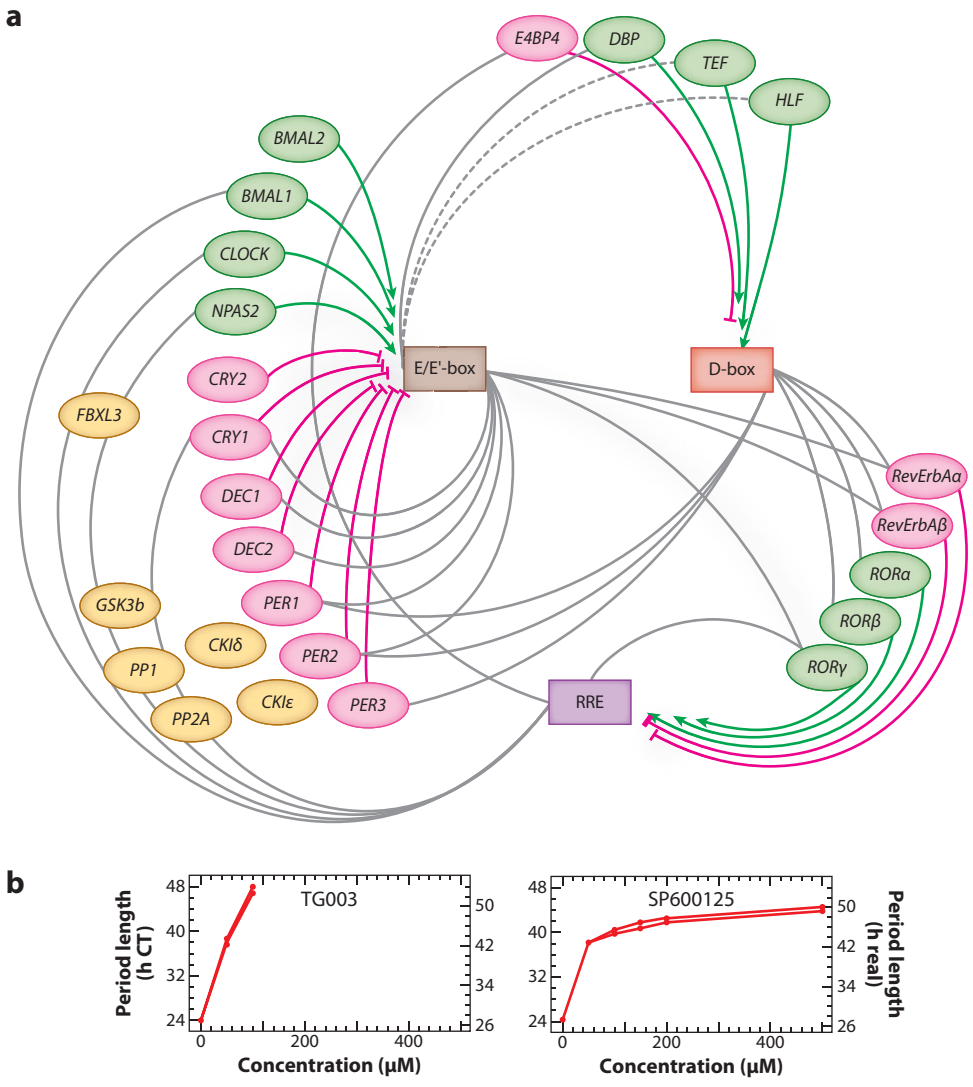

Figure 2

System identification of clocks. (*a*) Schematic representation of the transcriptional network of the mammalian circadian clock. Genes and clock-controlled elements are depicted as ovals and rectangles, respectively. Transcriptional/translational expression, activation, and repression are depicted as gray, green, and magenta lines, respectively. (*b*) Dose-dependent effects of SP600125 and TG003 on period length in U2OS-*hPer2-Luc* cells. The period length is indicated both in real time (*right axis*) and in circadian time (CT; *left axis*). For CT, the average period length in two independent control experiments was assigned as 24 h. The two lines in each graph correspond to two independent experiments (99). Each value represents the mean ± SEM (standard error of the mean). At the concentrations without data points, the cells behaved arrhythmically. High-dose pharmacological inhibition of the casein kinase I epsilon (CKIε) activity lengthened the circadian (~24-h) period to an almost circabidian (~48-h) period.

circadian rhythmicity in *Per2* or *Bmal1* promoter activity. The effects differed, however, among repressors, and the severest effect was observed when the E/E′-box was attacked. Such different modes of effect cannot be explained by mere quantitative differences in the strength of these three repressors, indicating that there is some qualitative difference among E/E′-box, D-box, and RRE regulation in circadian rhythmicity (58).

We also note that a novel strategy termed gene-dosage network analysis was recently developed by Baggs et al. (66). In this strategy, siRNA-induced dose-dependent changes in gene expression were used to build gene-association networks consistent with known biochemical constraints (66). Baggs et al. observed that several genes are upregulated following knockdown of their paralogs, which suggests that the clock network utilizes active compensatory mechanisms rather than simple redundancy to confer robustness and maintain function.

The Role of Negative Feedback

The identification of transcriptional circuits of mammalian circadian clock has revealed the topological importance of E-box, the so-called morning element. This is consistent with the prevailing transcriptional feedback model, which is believed to be mediated in the mammalian circadian clock by the CRY1 and -2 (17, 18, 29) and PER1 and -2 (16, 67, 68) proteins. More specifically, the CRY and PER proteins are hypothesized to autoregulate their own expression by repressing the heterodimeric complex of the bHLH-PAS-domain transcriptional activators CLOCK and BMAL1, which bind to E-box elements in the CRY (69) and PER (28, 58, 59) promoters. Similar autoregulatory loops, in which negative feedback regulation of transcriptional and/or translational processes are involved, seem to be evolutionarily conserved among a wide variety of species (3, 70, 71). Thus, transcriptional/translational feedback repressions are proposed to generate a 24-h periodicity. The question of universal necessity for transcriptional/translational feedback repression, however, arose primarily from recent studies of cyanobacterial circadian rhythms, in which the repression was shown to be not necessary (72, 73). Direct evidence for the requirement of CRY-mediated repression of CLOCK/BMAL1 transcriptional activity in the maintenance of circadian clock function thus remains to be presented.

To determine the requirement of feedback repression in circadian clock function, Sato et al. (78) changed the molecular parameter for feedback repression by functional genomics, then tested the cellular phenotype caused by this parameter change via the in cellulo cycling assay system. The authors sought to identify the CLOCK alleles that were insensitive to CRY1 repression but maintained normal transcriptional activity. They generated a library of ~6000 random point mutants of human alleles for both CLOCK and BMAL1, then screened clones individually in cell-based reporter assays with wild-type *Bmal1* cDNA and a *Per1* promoter–luciferase (*Per1*-Luc) construct (28) in the presence of cotransfected *Cry1*. Compared with wild-type alleles, of the CLOCK and BMAL1 clones screened, several reproducibly maintained threefold or greater reporter activity in the presence of CRY1. Notably, these clones demonstrated similar transcriptional activities as wild types in the absence of cotransfected *Cry1*, suggesting that these mutations do not cause overt alterations in the heterodimerization, nuclear localization, DNA-binding, and transactivation properties of the mutant CLOCK/BMAL1 complex.

The prevailing transcriptional feedback model predicts that impairment of CRY-mediated repression should have marked effects on circadian expression of the *Per* genes. This notion is supported by in vivo observations that expression of *Per1* and *Per2* is constitutively elevated in *Cry1/Cry2* double-knockout mice (18, 74). To determine whether these mutations in CLOCK and BMAL1 cause phenotypic changes in circadian gene expression, investigators performed in cellulo cycling assays

(27, 58). Mouse NIH3T3 cells were transfected with plasmids harboring *dLuc* driven by the *Per2* or SV40 basic promoters, along with the BMAL1 and CLOCK mutant alleles, and were monitored via the in cellulo cycling assay. Compared with empty vector transfection, cotransfection of wild-type CLOCK and BMAL1 did not substantially alter rhythmicity, and the transfected cells' period lengths were 21.4 ± 0.4 h. In contrast, transfection of either CLOCK or BMAL1 mutant alleles, when compared with wild-type CLOCK/BMAL1, resulted in substantial impairment of circadian rhythmicity after one or two cycles of oscillations. Notably, cotransfection of CRY-insensitive mutant CLOCK and BMAL1 together resulted in the loss of circadian *Per2* promoter activity. Sato et al. (78) therefore demonstrated that the transcriptional repression of CLOCK/BMAL1 by CRY was required for circadian E-box activity.

In addition to that of *Per* and *Cry*, the rhythmic expression of *Bmal1* mRNA is also subject to circadian clock regulation (75). The *Bmal1* promoter used in this study, however, does not have E-box sites and instead contains RRE (22, 27), whose activities are reciprocally controlled by the rhythmically expressed transcriptional repressor REVERBα (22) and the activator RORα (21). In an additional test for circadian clock function, the effects of mutant CLOCK and BMAL1 on rhythmic RRE activity were examined via in cellulo cycling assays with a *Bmal1-dLuc* reporter. Similar to the results obtained from the *Per2-dLuc* reporter, transfection of single CLOCK or BMAL1 mutants resulted in the decreased amplitude of cycling of *Bmal1-dLuc* activity compared with wild-type CLOCK/BMAL1 transfection. Moreover, this decrease in cycling amplitude was further exacerbated upon cotransfection of the double-mutant heterodimer. These results indicate that transcriptional repression of CLOCK/BMAL1 by CRY is also required for circadian BMAL1 expression through RRE, which in turn depends on transcriptional, translational, and posttranslational actions of endogenous cellular factors.

Arrhythmic *Per2* expression, observed in a population of cells expressing the double-mutant CLOCK/BMAL1 complex, may be due to the disruption of oscillator function or a lack of synchrony between individual rhythmic cells. To address these possibilities, quantitative imaging of *Per2-dLuc* reporter activity from individual NIH3T3 cells was measured by an approach similar to that used in analyzing *Bmal1* reporter rhythms from single cells (76). As in the whole-well assays, the median reporter activity for the population of imaged individual cells coexpressing wild-type CLOCK/BMAL1 oscillated rhythmically. In contrast, the population of individual *Clock/Bmal1* mutant cells was visibly arrhythmic. Individual reporter activities from single wild-type cells were rhythmic, as expected, whereas individual *Clock/Bmal1* double-mutant cells showed arrhythmic reporter activities. These differences in activity patterns were evaluated by two independent statistical methods that score the circadian rhythmicity of experimental time-course data. These data provide direct evidence that CRY-mediated feedback repression of the CLOCK/BMAL1 complex is required for mammalian clock function.

We also note that functional genomics technology utilized in Sato et al.'s study has successfully been applied to other clock proteins (CRY1 and CRY2) to further characterize their molecular properties (77). Thus, the application of cellular genetics technology will probably have as significant an impact on mammalian biology as similar approaches have had on prokaryotic and yeast biology.

The Identification of the Rate-Limiting Process

The E-box-mediated transcriptional/posttranscriptional loop is the critical core loop of the mammalian circadian clock (58, 78). Despite this finding, the specific processes in the core loop that determine the period length remain elusive. In this section, we describe the identification of the rate-limiting process of the mammalian circadian clock.

Circadian time (CT): standardized 24-h notation of the phase in a circadian cycle. CT0 indicates the beginning of a subjective day, and CT12 indicates the beginning of a subjective night

As described above, comprehensive gene-expression profiling is a basic approach to system identification (27, 30–54), and such gene-expression profiles are integrated with comparative genomics to characterize the clock-controlled DNA elements in the mammalian genomes (62, 64, 65). However, mammalian circadian clocks cannot be understood only through the identification of transcriptional circuits; posttranscriptional regulations need to be identified (79–82). This lack of information about posttranscriptional regulations will be able to be complemented by high-throughput measurement technologies such as proteomics (46, 83), metabolomics (84, 85), and other functional genomics resources such as RNA interference (RNAi) (10, 66, 86, 87) and chemical or peptide libraries (88, 89). Using these technologies and resources, Nakahata et al. (88) identified 15d-PGJ_2 and Hatcher et al. (84) identified little SAAS as entrainment factors for NIH3T3 cells and SCN, respectively. In a high-throughput RNAi-based genetic screening, Maier et al. (86) identified CK2 as a PER2-phosphorylating kinase and component of the mammalian circadian clock.

A chemical-biological approach is also effective at elucidating the basic processes that underlie circadian clocks (90). Although performed on a relatively small scale, early studies revealed that several protein kinase inhibitors [such as IC261 (91, 92), CKI7 (93), lithium chloride (94, 95), SP600125 (92, 96), SB203580 (97), DRB, LY294002, BML-297, and SB202190 (98)], adenylate cyclase inhibitors [such as THFA (9-tetrahydro-2-furyl-denine), 2′5′-dideoxyadonosine, and 9-cyclopentyladenine (92)], and proteasome inhibitors [such as MG132 and lactacystin (91)] can lengthen the period of mammalian circadian clocks by 2% to ∼40%. High-throughput screening of a large chemical compound library was also performed (89). The results showed that the inhibition of glycogen synthase kinase 3 beta (GSK3β) shortens the period of mammalian clock cells, superseding a previous proposal about the function of GSK3β that was based on the period-lengthening effects of lithium (94, 95). This evidence supported the use of a chemical-biological approach in probing the fundamental processes of the mammalian circadian clock.

To systematically identify the fundamental processes involved in determining the period length of mammalian clocks, Isojima et al. (99) tested 1260 pharmacologically active compounds for their effect on period length in mouse and human clock cell lines, NIH3T3 and U2OS, and found 10 compounds that most markedly lengthened the period of both clock cell lines and affected both the central (SCN) and peripheral (mouse embryonic fibroblast) circadian clocks. Most compounds inhibited CKIε or CKIδ activity, and the siRNA knockdown of *Csnk1e* or *Csnk1d* exhibited great period-lengthening effects—more than 28 h in circadian time (CT). Moreover, the combinatorial knockdown of *Csnk1e* and *Csnk1d* additively lengthened the period of circadian oscillations to over 30 h in CT, and high-dose pharmacological inhibition of the CKIε/δ activity lengthened the circadian (∼24-h) period to an almost circabidian (∼48-h) period (**Figure 2*b***). Inhibition of CKIε/δ activity led to deceleration of the PER2 degradation rate, which is regulated by CKIε-dependent phosphorylation (100). Moreover, the period length of the circadian oscillation is strongly correlated with the PER2 stability under the administration of the CKI inhibitor. These data suggest that CKIε/δ-dependent phosphorylation on the PER2 protein is an important period-determining process in the mammalian circadian clock.

As these studies have shown, both the perturbation of components' quantity via cDNA, siRNA, and chemical libraries and the perturbation of components' quality (parameter change) via functional genomics are important for the identification of complex and dynamic biological systems, such as that of the mammalian circadian clock.

ANALYSIS OF CLOCKS

To derive the design principle of a system of interest, one must validate the behavior of a

predicted system through an accurate measurement with several types of perturbations (system analysis). In this section, we describe the quantitative analysis of identified period-determining biochemical reactions in the above section, which is probably important for determining the temperature compensation of the mammalian circadian clock.

A 24-h period that protects against environmental changes, such as in temperature and nutrition, is one of the most intriguing aspects of the circadian clock. The robustness of this protection against temperature change is known as temperature compensation. Typical biochemical reactions, such as enzymatic reactions, show temperature dependency, which is represented by a Q_{10} value of approximately two. In contrast, the period of circadian rhythm is independent of or compensated against temperature change (i.e., the Q_{10} value of the period is approximately one). The importance of temperature compensation in poikilotherms can be easily understood. This aspect of the mammalian circadian clock had been controversial, but in 2003 it was confirmed in cultured mammalian cells (101, 102). Temperature compensation has proved to be one of the general characteristics of circadian clocks, appearing in species ranging from cyanobacteria to humans. Despite our increasing knowledge of the molecular mechanism of circadian clocks, however, it is difficult to explain how circadian clocks sustain such a constant period against temperature change.

To explain this phenomenon, theoretical studies (103, 104) have proposed a balanced reaction model (**Figure 3*a***) in which (*a*) increasing kinetic parameters of some reactions in a negative feedback loop shorten the period and (*b*) other parameters prolong the period. If the period-accelerating and period-decelerating reactions were equally sensitive to temperature, the frequency of the circadian oscillation would increase with an increase in temperature. To reconcile this issue, the balance reaction model proposes that the period-accelerating reaction(s) are less temperature sensitive than the period-decelerating reaction(s) and, hence, that these two sets of enzymatic reactions are balanced to maintain a constant circadian oscillation. These theoretical explanations seem plausible, but the balance of effects on the period length between basic reactions can be easily broken by perturbations, such as inhibitors and point mutations on clock proteins. Furthermore, these theoretical models are inconsistent with the fact that many circadian clock mutants show diverse periods but sustain temperature compensation. How do circadian clocks acquire robustness against temperature change?

The circadian rhythm of KaiC (a circadian clock protein) phosphorylation can be reconstituted by only three Kai proteins, KaiA, KaiB, and KaiC, in a test tube, demonstrating that this rhythm is the central oscillator of the cyanobacterial circadian clock (72). In this test tube, the oscillation period of KaiC phosphorylation is unaffected by temperature change despite the occurrence of biochemical reactions. This observation indicates that the robustness of circadian oscillation against temperature difference in cyanobacterial cells depends on the biochemical properties of three clock proteins. Although the mechanism of the KaiC phosphorylation cycle remains unclear, these findings suggest a robust reaction model (**Figure 3*a***) for temperature compensation in circadian clocks, at least in cyanobacteria.

Isojima et al. (99) demonstrated that CKIε/δ activity on the PER2 protein is one of the most potent period-determining processes in the mammalian circadian oscillator. If potent period-determination processes were highly sensitive to temperature, it would be very difficult, if not impossible, to maintain temperature compensation over the entire circadian period. Thus, Isojima et al. (99) investigated the temperature dependency of this process in living clock cells and found that (*a*) the degradation rate of mPER2, which is regulated by CKIε-dependent phosphorylation (100), and (*b*) the period length were completely temperature insensitive in cellulo (**Figure 3*b*,*c***). These findings imply that the period-determination process is remarkably robust against temperature differences.

Q_{10}: the change in the rate of a process as a result of increasing the temperature by 10°C

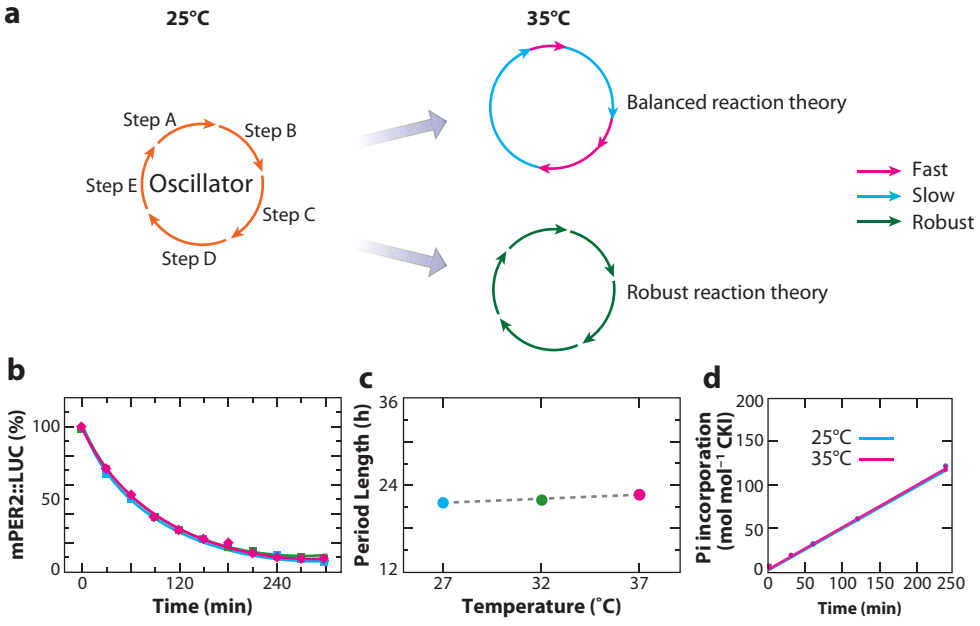

Figure 3

System analysis of clocks. (*a*) Two theoretical models of temperature compensation of the circadian clock. Steps A through E indicate the basic molecular reactions of circadian clock components. In the balanced reaction theory, increasing kinetic parameters of some basic reactions (*magenta*) shorten period, and those of other basic reactions (*blue*) lead to prolonging the period. These effects are offset so that the period is sustained. In the robust reaction model, such as the cyanobacterial circadian clock, temperature compensation of the circadian clock is caused by reactions of which kinetic parameters are independent (*green*). (*b*) The temperature dependency of decay for the mPER2::LUC bioluminescence in *mPer2*^{Luc} mouse embryonic fibroblasts (MEFs). The degradation of the mPER2::LUC protein was monitored after the addition of cycloheximide to MEFs. The time-course data of each sample were normalized to an approximate function in which time point zero was 100%. Each value represents the mean ± SEM (standard error of the mean) of the normalized data. The lines represent an approximated curve in which $y = 100$ at time zero and $y = 50$ at the averaged half-life time. The blue dots and line indicate the data at 27°C, green indicates 32°C, and magenta indicates 37°C ($N = 23$). (*c*) Temperature compensation in the period length of *mPer2*^{Luc} MEFs. The graph indicates the mean ± SEM. The gray broken line indicates the approximated line described by the equation $y = 19.02 + 0.097x$, and the Q_{10} value between 27°C and 37°C calculated from the equation is 0.957. (*d*) Temperature dependency of the ΔCKIε(wt) phosphorylation activity for the βTrCP-peptide substrate. Assays were performed at 25°C (*blue*) and 35°C (*magenta*). Abbreviations: ΔCKIε(wt), catalytic domain of wild-type (wt) casein kinase I epsilon (CKIε); Q_{10}, the change in the rate of a process caused by increasing the temperature by 10°C; βTrCP, β-transducin repeats–containing protein.

To examine the biochemical foundation underlying the observed temperature insensitivity, Isojima et al. (99) analyzed the phosphorylation activity of CKIε and CKIδ in vitro. They used synthetic peptide substrate derived from the putative β-transducin repeats–containing protein (βTrCP)-binding region of mouse PER2 and the catalytic domain of wild-type CKIε, lacking the C-terminal regulatory domain [ΔCKIε(wt)], to prevent the confusion that could result from the autophosphorylation of this regulatory domain and the subsequent repression of CKIε kinase activity. This use of the catalytic domain was also justified by evidence that CKIε is kept in a dephosphorylated, active state in vivo (105). Under this experimental condition, ΔCKIε(wt) phosphorylated the peptide substrate at similar rates both at 25°C

and at 35°C, indicating a strong temperature insensitivity (Q_{10} = 1.0) (**Figure 3*d***). Similar temperature insensitivity was also observed with ΔCKIδ(wt) at a Q_{10} of 1.2. However, the Q_{10} of ΔCKIε/δ(wt) activity for two clock-unrelated substrates—casein, a conventional protein substrate, and CK1tide, a commercially available peptide substrate for CKIε/δ, which is a peptide substrate already phosphorylated at two residues on the N terminus of the CKIε/δ phosphorylation site—was 1.6 for casein and 1.4 for CK1tide. This finding indicates that the temperature insensitivity depends substantially on the substrate. Earlier and Isojima et al.'s reports (99, 106) indicated that preincubating full-length CKIε with ATP repressed CKIε enzymatic activity ∼15-fold and 8-fold at 25°C and 35°C, respectively (99), probably as a result of the autophosphorylation of the C-terminal regulatory domain. Isojima et al. (99) investigated the effect of autophosphorylation of full-length CKIε on temperature insensitivity; their results indicated that autophosphorylation increases temperature sensitivity, suggesting that CKI-dependent phosphorylation can be temperature insensitive, depending both on the substrate and on the state of the enzyme. Moreover, the expression of exogenous full-length mPER2 protein and the catalytic domain of CKIε in NIH3T3 cells recapitulated the temperature insensitivity of degradation rate. On the basis of these experimental data, Isojima et al. (99) proposed that CKIε/δ-dependent phosphorylation is probably a temperature-insensitive period-determining process in the mammalian circadian clock.

The evidence for the existence of a temperature-insensitive reaction in vitro and in cellulo, with possible implications for temperature compensation in circadian clocks, suggests the surprising capability of CKIε/δ-dependent phosphorylation. Therefore, the remaining challenge is to obtain the atomic resolution model of this temperature-insensitive reaction.

In addition to the robustness of the circadian clock against temperature differences, mammalian circadian clocks also seem to have additional interesting dynamical characteristics. Recently, Dibner et al. (107) manipulated the general transcriptional rates by using a specific inhibitor of RNA polymerases II and III; they suggested that mammalian circadian oscillators are resilient to large fluctuations in general transcription rates. The authors also suggested that PER1 has an important function in transcription compensation (107). As these reports have shown, static perturbation of the system of interest and subsequent quantitative measurements of its dynamics yield important results that will aid our understanding of the system.

Singularity: the suppression of circadian rhythms by a critical perturbation

CONTROL OF CLOCKS

System control aims to regulate the target system toward the desired state through the precise perturbation of its components. To achieve this regulation, one must develop an assay system that can be controlled in a dynamic and quantitative manner.

The circadian clock is known to be entrainable by external cues such as light. Information obtained from light is transmitted to the circadian clock through sensing mechanisms containing photoreceptors, and as a result of light pulses the dynamics of the clock system change drastically. In this section, we describe the intentional control of the oscillating clock system in individual cultured cells via artificial light-sensing mechanisms (108, 109). We also explain how Ukai et al. (108) and Pulivarthy et al. (109) independently applied this photoperturbation system to a longstanding and unsolved biological phenomenon known as the singularity behavior of circadian clocks.

Circadian clocks exhibit various dynamic properties, making them difficult to elucidate without quantitative perturbation and precise measurement of their dynamics. One of the most fundamental but still unsolved dynamical properties of circadian clocks is singularity behavior, in which robust circadian rhythmicity can be abolished after a certain critical stimulus, such as a light or temperature pulse applied at

the appropriate timing and strength. Since the first report of singularity behavior in *Drosophila pseudoobscura* by Winfree (110), circadian clock singularities have been experimentally observed in various organisms, including unicells such as *Gonyaulax* (111), *Euglena* (112), and *Chlamydomonas* (113); fungi (114); insects (115); plants (116, 117); and mammals (118, 119). This evidence suggests that this behavior is a shared property of an extremely broad range of circadian clocks (4).

Although singularity behavior has been widely observed, little is known about its underlying mechanisms. Because such behaviors were experimentally observed at the multicell level (i.e., the collective behavior of unicells or the physiological or locomotor activity of multicellular organisms), two alternative single-cell-level mechanisms have been proposed to explain their collapse to singularity: (*a*) arrhythmicity of individual clocks (**Figure 4*a***) and (*b*) desynchronization of individual rhythmically oscillating clocks (**Figure 4*b***) (115, 120). In the former mechanism, individual clocks become arrhythmic; that is, the amplitude of the individual cells is substantially attenuated by the application of the critical light pulse. In contrast, in the latter mechanism of desynchronization, the phases of individual clocks are diversified by the critical light pulse. Although both mechanisms can explain substantial suppression of the multicell-level amplitude of circadian rhythm, the dynamical properties of the two fundamentally differ: The oscillations of individual cells are impaired in the arrhythmicity mechanism, whereas individual cells maintain their oscillations in the desynchronization mechanism. Importantly, although many researchers have observed multicell-level singularity behaviors in various organisms, it remains unknown whether arrhythmicity or desynchronization of individual clocks underlies the singularity behavior of circadian clocks.

Determination of the underlying mechanism for singularity behaviors of circadian clocks may require adjustable perturbation, given that the ability of a critical stimulus to drive circadian clocks into singularity depends on its timing and strength. Various stimuli, such as reagents and temperature, have been reported to directly reset mammalian cellular clocks (7–9, 101, 121–126). Unfortunately, it is difficult, although not impossible, for these factors to achieve the requisite flexibility in timing and strength. In contrast to perturbations achieved by the use of reagents or temperature change, photoperturbation provides an ideal range of adjustability in timing and strength. Whereas most mammalian cells cannot sense light, recent studies showed that mammalian cells (specifically Neuro-2a and HEK293 cells) become photoresponsive following the introduction of an exogenous $G_{\alpha q}$ protein–coupled photoreceptor, melanopsin (also known as *Opn4*) (127, 128). It was reported that photostimulation of melanopsin triggers a release of intracellular calcium mediated through the $G_{\alpha q}$ protein signaling pathway; importantly, there are several reports that mammalian cellular clocks can be reset by this pathway via a release of intracellular calcium (123, 124). These results suggest that melanopsin-dependent photoperturbation may enable the adjustable and quantitative perturbation of mammalian cellular clocks by changing intracellular calcium levels.

To experimentally reveal the underlying mechanism of the singularity behavior of mammalian cells, Ukai et al. (108) synthetically implemented photoresponsive mammalian cells by exogenously introducing melanopsin (**Figure 4*c***). To continuously and quantitatively monitor the effect of photoperturbation on the state of cellular clocks, the authors devised a high-throughput monitoring system with a light-exposure unit. Using this system, Ukai et al. revealed that a critical light pulse drives cellular clocks into a singularity behavior in which robust circadian rhythmicity can be abolished after a certain stimulus. Theoretical analysis and subsequent single-cell-level observation consistently predicted and directly observed that desynchronization of

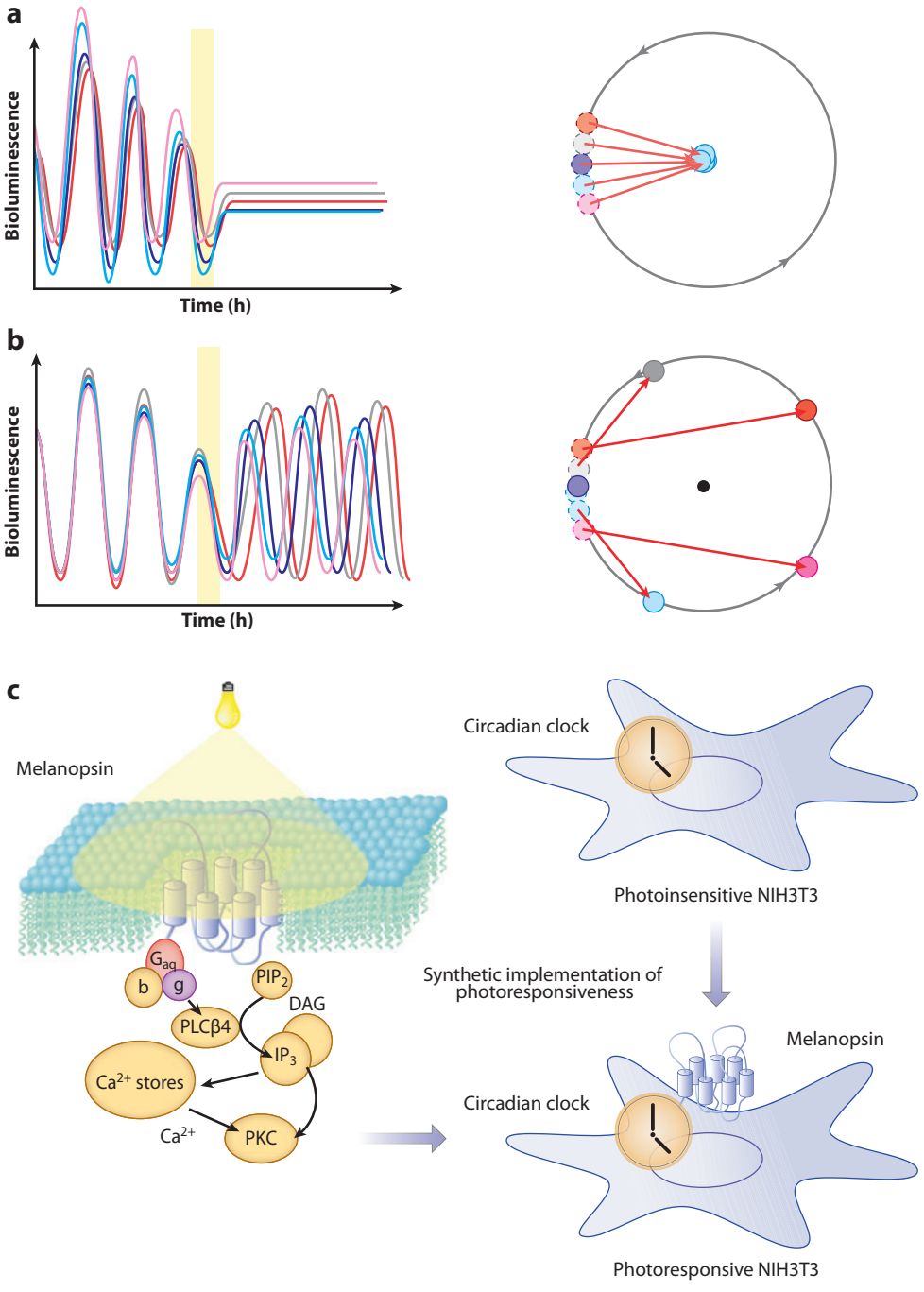

Figure 4

System control of clocks. (*a*,*b*) Diagrams of two alternative single-cell-level mechanisms for multicell-level singularity behavior. (*a*) Arrhythmicity and (*b*) desynchronization of individual cellular clocks. (*c*) Synthetic implementation of photoresponsiveness within mammalian clock cells. Schematic representation of melanopsin-dependent photoresponsive NIH3T3 cells and the known $G_{\alpha q}$ signaling pathway. Abbreviations: DAG, diacylglycerol; IP_3, inositol triphosphate; PIP_2, phosphatidylinositol 4,5-bisphosphate; PLCβ4, phospholipase C beta 4; PKC, protein kinase C.

individual cellular clocks underlies this singularity behavior. Ukai et al. (108) also constructed a theoretical framework to explain why singularity behaviors have been experimentally observed in various organisms, and they proposed desynchronization as a plausible mechanism for the observable singularity of circadian clocks.

In an independent study, Pulivarthy et al. (109) implemented the same photoreceptor (*Opn4*) into the immortalized fibroblast derived from *Per2*^{LUC} knockin mice and created *Per2*^{LUC};*Opn4* cell lines. Using this cell line, Pulivarthy et al. observed a 40% amplitude reduction of PER2::LUC bioluminescence levels of individual cells under the experimental condition inducing singularity-like behavior. This reduction had not been observed in Ukai et al.'s study using *Per2*-Luc and *Bmal1*-Luc. However, Pulivarthy et al. and Ukai et al. simultaneously observed the remarkable desynchronization of individual cellular oscillations. Although there are some differences between the two studies, which could be due to different means of readouts, each observed desynchronization of individual cellular oscillations, which thus seems to underlie the singularity behavior of mammalian circadian clocks. Importantly, these in cellulo and in silico findings are further supported by in vivo observations by Ukai et al. (108) that desynchronization actually underlies the multicell-level amplitude decrease in rat SCN that is induced by the critical light pulses, which in turn can predispose organisms' locomotor activity to transient amplitude decrease.

To elucidate the underlying mechanism of singularity behaviors, Winfree (115, 120) conducted a two-pulse experiment that revealed the unclocklike behavior of the circadian clock. In this experiment, the critical light pulse inducing the singularity behavior seemed to decrease the amplitude of the circadian clock without affecting its frequency. This finding seemed to contradict both the tenets of the simple limit-cycle model and its prediction that arrhythmicity underlies the singularity behavior. To explain this unclocklike behavior, Winfree proposed the so-called clockshop hypothesis, in which an organism-level circadian clock consists of multiple circadian oscillators with substantial fluctuations, and predicted that the desynchronization of individual circadian oscillators underlie the singularity behavior (multicell-level amplitude decrease). He was unable to test this prediction, however, as there was no way to observe single-cell-level circadian rhythmicity at that time (1975). The studies by Ukai et al. (108) and Pulivarthy et al. (109) proved Winfree's prediction on the desynchronization, at least in the mammalian circadian system, more than 30 years after it was originally proposed (120).

DESIGN OF CLOCKS

The final step in the systems-biological process is system design, the reconstruction and design of a dynamical system based on the design principles revealed through the efforts of system identification, system analysis, and system control. In *Escherichia coli*, various dynamical systems, including the toggle switch (129), the repressilator (130), the metabolic oscillator (131), and the tunable oscillator (132), have been designed and implemented. Tigges et al. (133) recently designed and implemented tunable oscillators in mammalian cells. Such a synthetic approach can be applied to a complex and dynamic system such as a mammalian circadian clock and will enhance its understanding. A synthetic approach is especially useful in validating the hypothesis derived from the identified structure and observed dynamics.

A possible synthetic approach is to extend the in cellulo cycling assay system described above (27) and utilize it as a physical simulator, which allows implementation of artificial transcriptional circuits to mimic transcriptional circuits of mammalian circadian clocks and, hence, to test the design principles of natural systems. To this end, Ukai-Tadenuma et al. (134) have developed an in cellulo system with which to validate the sufficiency of the components of a natural circadian phase-controlling mechanism. Alternatively, a more radical synthetic approach, such as an in vitro reconstruction of a mammalian circadian clock, could be undertaken. We describe these two synthetic approaches in the following sections.

Design of Transcriptional Circuits

The network structure comprising clock genes and CCEs has been comprehensively described.

However, the dynamic principles governing this transcriptional circuit remain elusive. A key issue concerning the logic of the mammalian circadian clock is how the expression peaks (phases) of circadian oscillating genes are determined. To understand the molecular logic of the phase-controlling system(s), Ukai-Tadenuma et al. (134) focused on the three main CCEs: the E/E′-box, the D-box, and the RRE. The transcriptional activator DBP activates gene expression via the D-box, whereas the transcriptional repressor E4BP4 represses gene expression (**Figure 2*a***). *Dbp* is regulated by the E-box, the morning control element; *E4bp4*, however, is regulated by the RRE, the nighttime control element. On the basis of this transcriptional circuit information, the investigators hypothesized that a morning activator and a nighttime repressor determine the daytime transcriptional output mediated through the D-box. Similarly, RRE activators (e.g., *Rorα*) are expressed during the daytime under the control of the D-box, and the RRE repressors (e.g., *RevErbα*) seem to be strongly influenced by a morning element (the E′-box; **Figure 2*a***). These observations led to the hypothesis that a daytime activator and a morning repressor can specify the nighttime transcriptional output mediated through the RRE. To test this hypothesis, Ukai-Tadenuma et al. (134) adopted a synthetic approach to physically simulate an identified structure, and they observed the resulting dynamics through use of artificial transcriptional circuits.

To design and implement artificial transcriptional circuits in mouse NIH3T3 cells, which have a self-oscillating circadian clock, Ukai-Tadenuma et al. (134) developed an in vitro cycling assay system composed of three components: (*a*) an artificial activator (a destabilized GAL4-VP16 fusion protein, dGAL4-VP16), (*b*) an artificial repressor (a destabilized GAL4 protein, dGAL4), and (*c*) an output reporter gene (*dLuc*) driven by a minimal TATA box fused with four tandem repeats of the upstream activator sequence (UAS; the GAL4-binding sequence) (**Figure 5*a***). Because the artificial activator and repressor are driven by the SV40 basic promoter fused with three tandem repeats of CCEs, the expression timing of these artificial regulators is controlled via the morning (E′-box), daytime (D-box), and nighttime (RRE) elements. The authors reasoned that if and only if the phases of the transcriptional activator(s) and repressor(s) are acceptable determinants of the phase of the downstream transcriptional output, then it should be possible to generate the natural phases using synthetic transcriptional regulators and promoters. If this proved to be true, it would be very significant, as transcription factors are regulated by various posttranscriptional mechanisms—including translation, phosphorylation, ubiquitination, sumoylation, and nuclear transportation—that are thought to contribute at least in part to the control of phases of the downstream transcriptional outputs.

UAS: upstream activator sequence

Using the in vitro cycling assay system as a physical simulator, Ukai-Tadenuma et al. (134) tested the hypothesis for daytime output by examining the dynamic behavior of the transcriptional output generated from the competition between an artificial morning activator controlled via E′-box and a nighttime repressor controlled via RRE. The phases of the activator and repressor in this experiment were detected at CT4.0 $\pm$ 0.29 ($n = 2$) and CT17.1 $\pm$ 0.37 ($n = 2$). The transcriptional output driven by these regulators exhibited circadian oscillation with a phase at CT7.7 $\pm$ 0.85 ($n = 2$), which is very close ($\leq$1.0 h) to the corresponding natural daytime (D-box) phase, CT8.7 $\pm$ 1.13 (**Figure 5*b***). These results suggest that morning activation and nighttime repression are sufficient to determine the daytime transcriptional output. Importantly, high-amplitude circadian oscillation was not observed when only the morning activator or only the nighttime repressor was expressed. The authors concluded that both morning activation and nighttime repression are necessary for daytime output.

Moreover, the hypothesis for nighttime output was tested through examination of another artificial circuit consisting of an artificial daytime activator under D-box control and a

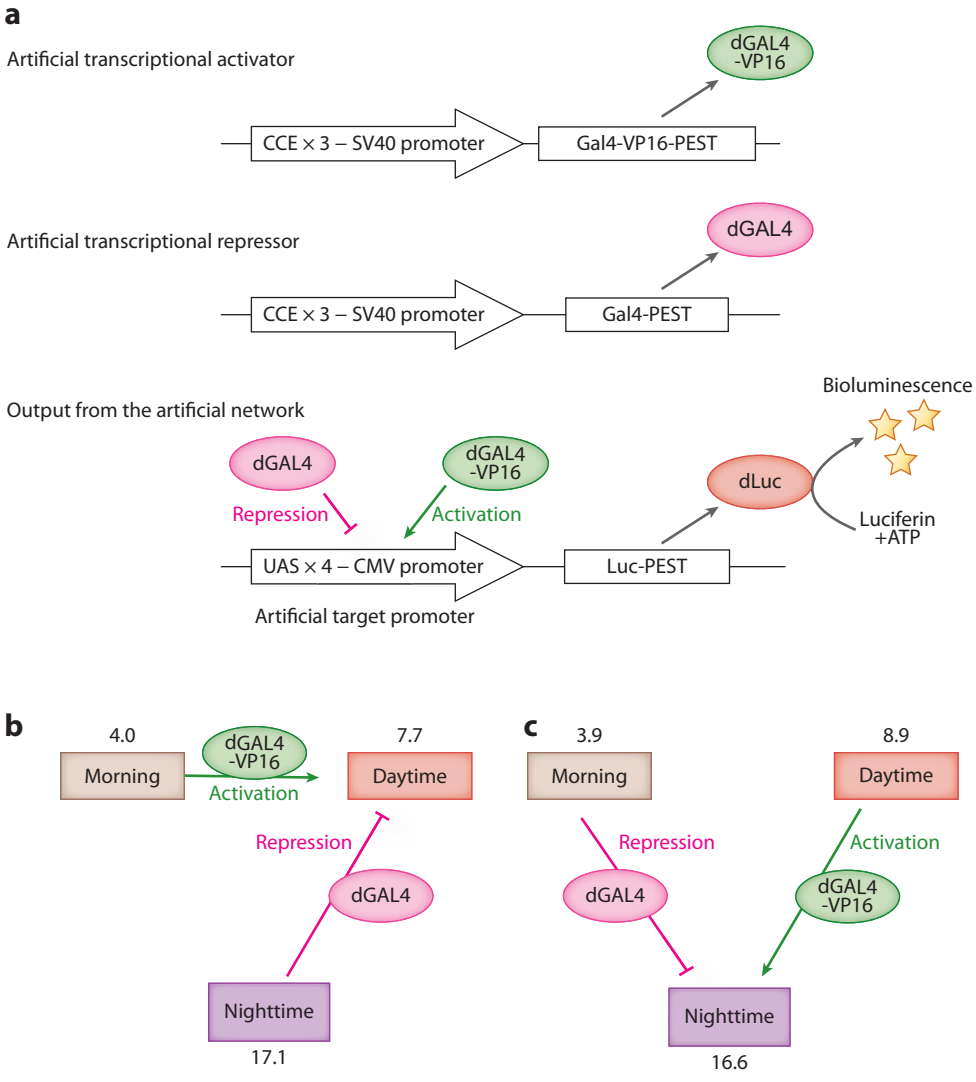

Figure 5

System design of clocks. (*a*) The artificial transcriptional system. dGAL4-VP16 and dGAL4 were used as the activator and the repressor, respectively. These transcriptional factors were expressed under the control of three tandem repeats of a clock-controlled element (CCE): the E′-box of *Per2*, the D-box of *Per3*, and the RRE (RevErbA/ROR-binding element) of *Bmal1*. The artificial activator and repressor competitively bind the four tandem repeats of the upstream activator sequences (UAS) in the artificial promoter to regulate the output reporter gene *dLuc*. NIH3T3 cells were transiently transfected with plasmids harboring the activator, repressor, or reporter, and the reporter activity was monitored under a real-time bioluminescence-measuring system, which allowed observation of this artificial circuit's dynamic behavior. (*b* and *c*) Proof by synthesis of daytime and nighttime transcriptional regulations. Synthesis of (*b*) daytime and (*c*) nighttime expressions from two different artificial transcriptional circuits: (*b*) the morning activator under E′-box control and the nighttime repressor under RRE control and (*c*) the daytime activator under D-box control and the morning repressor under E′-box control. The schemes summarize the timing of the peaks (i.e., phases) of promoter activity. The activator (*green oval and arrow*), repressor (*magenta oval and arrow*), and output reporter are indicated with their phases in circadian time (*numbers*).

morning repressor under E′-box control. The phases of the transcriptional activator and repressor in this experiment were at CT8.9 ± 0.28 ($n = 2$) and CT3.9 ± 0.13 ($n = 2$). The transcriptional output driven by the regulators exhibited circadian oscillation with a phase at CT16.6 ± 1.04 ($n = 2$), which is very close (≤1.0 h) to the corresponding natural nighttime phase (CT17.0 ± 0.81) (**Figure 5*c***). These results also suggest that daytime activation and morning repression are necessary and sufficient to generate the circadian nighttime output, which implies that the input phases of transcriptional regulators can determine the phases of transcriptional output. These findings led to the idea that various combinations of transcriptional regulators with CCEs for the three basic circadian phases (morning, daytime, and nighttime) may generate not only the basic phases but also other phases. Indeed, Ukai-Tadenuma et al. (134) succeeded in generating various phases though simple combinations of the transcriptional regulators of the three basic circadian phases.

These experiments showed that the transcriptional regulation of upstream transcription factors can determine the phase of the downstream output. This study presents a synthetic approach to the proof by synthesis of transcriptional logic, which provides us with a new strategy with which to (*a*) investigate the requirements for identified components and/or their interactions and (*b*) reveal as-yet-unidentified components or interactions. For example, although the authors successfully (re)generated two basic phases, daytime and nighttime—as well as additional phases near the subjective noon, dawn, dusk, and late night—from the three basic circadian phases, we have not yet been able to regenerate the basic morning phase, which is expected to be regulated directly or indirectly by the three basic phases to maintain circadian oscillations. Thus, morning transcriptional regulation remains a missing link in the mammalian clock system. Given that a strong repressor at evening phase seems indispensable to the reconstruction of the morning phase, a candidate transcription factor could be CRY1, a transcriptional repressor expressed during the evening. However, the expression-regulation mechanism of the *Cry1* gene remains unknown at present because more than one DNA element seems to be involved in this expression. Hence, the challenge of synthesizing evening and morning expression, as we work toward the complete reconstruction of the transcriptional circuits underlying mammalian circadian clocks, remains to be solved.

Reconstitution of Posttranscriptional Circuits

In the preceding section, we described the design of the transcriptional circuits of the mammalian circadian clock. The proof by synthesis of transcriptional circuits is useful because transcriptional feedback repression is important for circadian clock function, as we discussed in the section entitled The Role of Negative Feedback. However, the period length of circadian clock is largely determined by posttranscriptional circuits, as we discussed in the section entitled The Identification of the Rate-Limiting Process. Therefore, synthetic approaches based on biochemistry toward posttranscriptional circuits will be necessary for attaining a complete understanding of the mammalian circadian clock. Among such synthetic approaches, one of the most radical and fundamental is to perform an in vitro reconstruction, from scratch, of the mammalian circadian clock.

Indeed, biochemical studies on the circadian clock of cyanobacteria, the simplest organisms to possess such a clock, revealed the sufficiency of posttranscriptional circuits in the circadian clock (72). Interestingly, although ubiquitous molecular behaviors concerning the circadian clock, such as negative feedback regulation of clock genes, circadian oscillation of accumulation of RNA and clock proteins, and phosphorylation of clock proteins, are observed in cyanobacteria, the robust circadian oscillation of the phosphorylation state of KaiC was

reconstituted via a mixture of only three cyanobacterial clock proteins and ATP in a test tube (72). The circadian oscillation of KaiC phosphorylation was therefore demonstrated to be the central oscillator of the cyanobacterial circadian clock. This elegant study on the cyanobacterial circadian clock evoked the importance of using a biochemical approach to the circadian clock to answer fundamental questions, such as those of autonomous oscillation and temperature compensation.

As described in the section entitled Identification of Clocks, the clock-related genes have been comprehensively identified in mammals, as well as in other model organisms such as *Drosophila* and *Neurosphora* (3, 4, 70, 71). Furthermore, Isojima et al. (99) have discovered that CKIε/δ-dependent phosphorylation exhibits temperature insensitivity in vitro, as do cyanobacteria (72). Although it is not known whether a temperature-insensitive reverse reaction (i.e., dephosphorylation) exists in the mammalian circadian clock, its discovery would be a great step toward the complete reconstruction of the autonomous oscillator in the mammalian circadian clock.

Synthetic approaches based on cell biology and biochemistry can validate the sufficiency of transcriptional and posttranscriptional circuits for the dynamical property of interest. Following these approaches will lead to a deeper understanding of the design principles of the mammalian circadian clock.

PERSPECTIVES

In this review, we focused primarily on the intracellular mechanisms of the mammalian circadian clock. However, the mammalian circadian system is organized in a hierarchy of cellular oscillators and exhibits various intercellular dynamical properties derived from populations of clock cells. One example of such a property is singularity behavior (desynchronization of multiple circadian clock cells), discussed in the section entitled Analysis of Clocks. Other examples, which are not discussed herein, include the synchronization of multiple clock cells within the SCN (135–137) as well as the intertissue hierarchical coupling between master clock (SCN) and slave (peripheral) clocks (3, 138).

Synchronization of multiple clock cells within the SCN is one of the most important characteristics that distinguish the SCN from peripheral clocks. Coherent circadian rhythms, probably due to intercellular couplings of individual clock cells in the SCN, are generated in the SCN (135–137). Interestingly, not only are intercellular interactions among individual clock cells in the SCN important for the coupling of multiple circadian oscillators, they are also necessary for maintaining self-sustained circadian oscillations of individual clock cells (135). Indeed, self-sustained oscillations of individual cells in the SCN are highly sensitive to intercellular neural communications (135) and cAMP signaling (92). Furthermore, these intercellular coupling–dependent self-sustained oscillations within the SCN seem able to rescue the molecular defects of individual clock cells. For example, Liu et al. (139) recently discovered that *Per1* and *Cry1* are required for self-sustainable oscillations in peripheral clock cells and in neurons dissociated from the SCN. The authors also found that intercellular couplings in the SCN can compensate for those molecular deficiency.

Intercellular communications in the mammalian clock system are not limited to the synchronization of clock cells in the SCN. Intertissue hierarchical coupling between master clock (SCN) and slave clocks plays an important role in generation orchestrated circadian oscillations throughout the body. In fact, Kornmann et al. (140) discovered—under the condition that transcription of the essential core clock gene *Bmal1* is repressed in liver—that 31 genes, including core clock gene *Per2*, exhibited robust circadian oscillations irrespective of whether the liver clock was running. In contrast, in liver explants cultured in vitro, circadian cycles of *Per2* were only observed when hepatocyte oscillators were operational. These findings indicate that circadian oscillations of gene expression observed in the liver

of intact animals without functional hepatocyte oscillations are driven by systemic cues.

These intercellular dynamical properties would be difficult to understand without systems-biological approaches and techniques such as tissue-specific, conditional, and quantitative perturbation and subsequent quantitative measurement. Kornmann et al. adopted such conditional perturbation technique, in which REVERB*a* is conditionally overexpressed and represses the transcription of *Bmal1* in a doxycycline-dependent manner. Integrative research that makes use of quantitative perturbations with high spatial and temporal resolutions, quantitative measurements with high accuracy, and appropriate theoretical analysis will enable us to elucidate unsolved systems-level questions about complex and dynamic phenomena.

CONCLUSION

Following identification of key clock genes, demand for a higher-order understanding of the design principles of the mammalian circadian clock has increased. In this review we have described several steps, beginning with comprehensive identification of clock components and their networked interactions (system identification) and quantitative analysis (system analysis) of temperature-insensitive reactions and leading to the ability to control existing systems toward the desired state (system control) and to design new systems according to an understanding of structure and underlying dynamical principles (system design). We strongly believe that it is time to fully integrate these systems-biological approaches to solve system-level questions.

SUMMARY POINTS

1. Systems biology can be defined as biology after identification of key gene(s) and contains four steps: system identification, system analysis, system control, and system design.
2. System identification refers to identification of the whole network structure through functional genomics and comparative genomics.
3. System analysis involves prediction and validation to derive the design principle through the accurate measurement and (static) perturbation of network dynamics.
4. System control involves repair and control of the network state so as to achieve the desired state through the precise perturbation of its components.
5. System design refers to the reconstruction and design of systems according to the design principles derived from the identified structure and observed dynamics.

DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

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