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A NEW REGURATORY INTERACTION SUGGESTED BY SIMULATIONS FOR CIRCADIAN GENETIC CONTROL MECHANISM IN MAMMALS

Hiroshi Matsuno*, Shin-Ichi T. Inouye†, Yasuki Okitsu‡, Yasushi Fujii§

Faculty of Science, Yamaguchi University,
1677-1, Yoshida, Yamaguchi 753-8512, Japan
* *matsuno@sci.yamaguchi-u.ac.jp*
† *inouye@sci.yamaguchi-u.ac.jp*
‡ *okitsu@ib.sci.yamaguchi-u.ac.jp*
§ *a082dc@sty.cc.yamaguchi-u.ac.jp*

Satoru Miyano*

Human Genome Center, Institute of Medical Science, University of Tokyo,
4-6-1, Shirokanedai, Minatoku, Tokyo 108-8639, Japan
miyano@ims.u-tokyo.ac.jp

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Knowledge of molecular biological systems is increasing at an amazing pace. It is becoming harder to intuitively evaluate the significance of each interaction between the molecules of the complex biological systems. Hence, we need to develop an efficient computational method to explore the biological mechanisms. In this study, we employed a hybrid functional Petri net in order to analyze mammalian circadian genetic control mechanisms, which consists of feedback loops of clock genes and generates endogenous near 24 h rhythms in mammals. We constructed a computational model based on the available biological data, and by using Genomic Object Net, we performed computer simulations of the time courses of clock gene transcription and translation. Although the original model successfully reproduced most of the circadian genetic control mechanisms, two discrepancies remained despite a wide selection of the parameters. We found that addition of a hypothetical path into the original model result in successful simulation of time courses and phase relationships among clock genes. This also demonstrates the usefulness of the hybrid functional Petri net approach to biological systems.

Keywords: Hybrid Petri net; Genomic Object Net; circadian rhythms.

1. Introduction

Circardian rhythmicity, which consists of endogenously generated near 24 h rhythms, is observed in virtually all mammalian physiological functions and behavior. These rhythms are centrally regulated by the suprachiasmatic nucleus (SCN) of the hypothalamus. Most neurons in the SCN become active during the day and

are believed to comprise the biological clock.

Along with the recent discovery of the clock genes that are involved in circadian rhythms, basic mechanisms of the biological clock have been partially elucidated. Several mathematical models have provided insight into the mechanisms of oscillation in the negative feedback loop of the molecular circadian clock[1,2,3]. However, as biological research reveals increasingly complicated interactions, the significance of a particular interaction among the clock genes becomes harder to comprehend.

Matsuno *et al.*[4] have recently defined the notion of a hybrid functional Petri net (HFPN) that allows us to model biological mechanisms without utilizing any skills in mathematical descriptions and programming techniques. Since the HFPN is represented graphically, our intuitive understanding of a biological mechanism can be reflected in the HFPN, even if the biological mechanism constitutes a large network.

Genomic Object Net (GON) [5,6] is a biosimulation tool that employs the HFPN as the basic architecture. Since GON is equipped with a user-friendly graphical user interface (GUI) system, we can easily describe the HFPN of the biological system and manipulate the HFPN model by using different parameters to simulate the biological mechanisms under various conditions. Many biological systems including λ phage genetic switch control[7], Fas ligand induced apoptosis[4], *lac* operon genetic switch control[8], and fission yeast cell cycle[9] have been modeled using GON.

This paper demonstrates how a computational model is used to understand the molecular interaction mechanisms using the mammalian circadian clock as a concrete example. We constructed an HFPN model of a circadian genetic control system in mammals. Using GON simulations of the constructed HFPN model, we evaluated the mammalian circadian genetic control system, and found two inconsistencies in the oscillations of mRNAs in comparison with the known biological facts. In order to resolve these two inconsistencies, we compared the circadian genetic control systems of mammals and fruit flies. Subsequently, we found a molecular interaction path that exists in the circadian mechanism of fruit flies but not in that of mammals. When this path was introduced into the constructed HFPN model simulated by GON, mRNA concentration behaviors were found to be consistent with biological observations.

2. Biological Facts on the Mammalian Circadian Genetic Control Mechanism

Molecular clocks reside within SCN cells. Each molecular circadian clock consists of a negative feedback loop of gene transcription and its translation into protein. The loop includes several genes and their protein products. In case of mammals, three Period genes (*Per1*, *Per2* and *Per3*) and two Cryptochrome genes (*Cry1* and *Cry2*) comprise the negative limb, while *Clock* and *Bmal1* (*Bmal*) genes constitute the positive limb of the feedback loop in the molecular circadian clock.

In the morning, transcription of the *Per* and *Cry* genes begins to increase with

the concomitant binding of CLOCK/BMAL dimers to the E-box enhancer regions of the genes. In the afternoon, when the number of *Per* or *Cry* mRNA increases, the protein products (PER and CRY) encoded by these genes are actively synthesized in the cytoplasm. Although the proteins are actively degraded by phosphorylation, they start to move into the nucleus when the amount of the protein synthesized exceeds the threshold required to form the dimers. When they enter the nucleus, presumably early at night, they block the association of CLOCK/BMAL heterodimer with, or reduce their binding on the E-box sequences by an unspecified mechanism, thus resulting in a decrease in the transcription of *Per* and *Cry*. Hence, during the late night period, transcription of the clock genes *Per* and *Cry* and levels of their protein products decrease. The decrease in the PER and CRY protein levels releases their inhibition onto CLOCK/BMAL dimers, and transcription of *Per* and *Cry* start to increase again the following day.

This negative feedback loop of transcription and translation of clock genes is further regulated by supplementary interactions. CLOCK/BMAL dimers also induce transcription of *Rev-Erbα* (*Rev-Erb*), while the E-box sequence in the enhancer region and protein products of *Rev-Erb*, in turn, suppress the transcription of *Bmal*.

3. Evaluation of the Present Circadian Gene Regulatory Model using Simulations

3.1. *Molecular interactions in a computational model*

Since biological research has found complicated interactions both within and outside the molecular circadian mechanism, it is harder to distinguish the interactions that are indispensable to the maintenance of oscillation from those that are accessory. Therefore, the objective of this paper is to evaluate the significance of each interaction involved in the circadian negative feedback loop. In order to simplify the model and gain an insight into each interaction path, we examine two group of genes *Per1*, *Per2*, and *Per3* genes and *Cry1* and *Cry2* genes collectively referred to as *Per* and *Cry*, respectively, form this point forward. This is justified by similar biological effects that were found in knockout mouse experiments.[10]

In the present model, *Per* and *Cry* genes and their protein products constitute the first major circadian feedback loop. The second loop is composed of the *Clock* and *Bmal* genes and their protein products. These two pathways are connected by interactions that include *Rev-Erb* and its product. Expression of *Rev-Erb* was accerelated by the PER/CRY dimmers and REV-ERB protein suppresses transcription of the *Bmal* gene, as detailed in Fig. 1.

3.2. *HFPN model of mammalian circadian gene regulatory mechanism*

Petri net[11] is a network consisting of *place*, *transition*, *arc*, and *token*. A place can hold tokens as its content. At a transition, arcs coming from places and those going

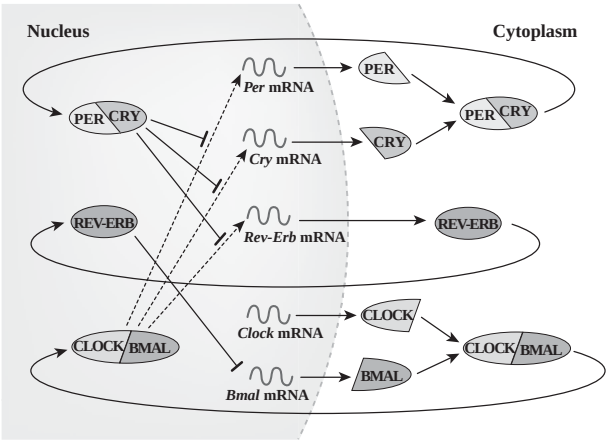


Fig. 1. Interaction map of the mammalian circadian gene regulatory system. *Per* and *Cry* genes are transcribed by the CLOCK/BMAL complex, and translated into proteins that form heterodimers before returning to the cytoplasm. Products of the *Clock* and *Bmal* genes heterodimerize to form the positive transcription factor for *Per*, *Cry*, and *Rev-Erb* genes; their effects are counteracted by the PER/CRY complex. The REV-ERB protein represses transcription of the *Bmal* gene.

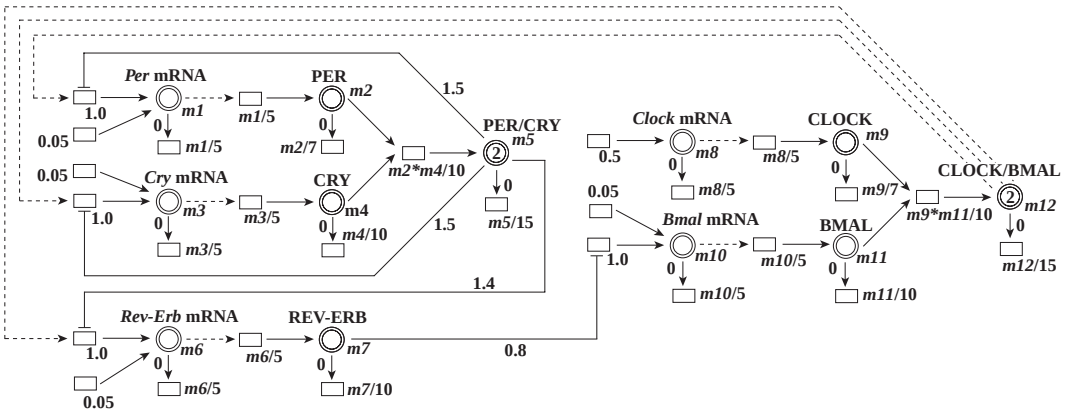


Fig. 2. The HFPN model of the mammalian circadian gene regulatory mechanism. Each continuous place holds the concentration of a gene product (mRNA or protein), and a formula for the speed of the corresponding biological reaction is assigned at the continuous transition. A continuous place and a continuous transition are connected by an arc that is chosen from a normal arc, test arc, or inhibitory arc depending on the biological relationship between two specified molecules.

out from the transition to some places can be connected. A transition with these arcs defines a firing rule with regard to the contents of the places to which the arcs are attached.

HFPN was defined by Matsuno *et al.*[4] as an extension of a hybrid Petri net.[12]

Fig. 2 is the HFPN model of the circadian gene regulatory mechanism in Fig. 1.

HFPN has two kinds of places, namely, discrete and continuous (doubled circles in Fig. 2) and two kinds of transitions, discrete and continuous (unfilled rectangles in Fig. 2). The concepts of discrete place and a discrete transition are the same as those in the traditional discrete Petri net.[a] A continuous place can hold a real number as its content. A continuous transition fires continuously at the speed of the parameter assigned to the continuous transition.

Three types of arcs are used with these places and transitions. A specific value is assigned to each arc as a weight. When a normal arc (a solid arc in Fig. 2 as the arc entering the continuous place CLOCK) with weight w is attached to a discrete/continuous transition, a certain number of tokens are transferred through the normal arc only if the content of the place at the source of the normal arc exceeds the weight w. An inhibitory arc (the line terminated with the small bar in Fig. 2 as the line emerging from the continuous place REV-ERB) with weight w enables the transition to fire only if the content of the place at the source of the arc is less than or equal to w. For example, an inhibitory arc can be used to represent repressive activity in gene regulation. The firing rule of a test arc (a dashed line in Fig. 2 as the arc leaving from the continuous place *Bmal* mRNA) is the same as that of a normal arc in terms of the weight, but the content of the place at the source of the test arc is not consumed by the firing. A test arc can be used to represent enzyme activity since the enzyme itself is not consumed.

An HFPN model shown in Fig. 2 can be described according to the following simple rules. Each substance such as mRNA and protein corresponds to a continuous place. At each transition, a function of a form as $mX/10$ is assigned, and the speed of the corresponding reaction is defined. For example, the translation speed of PER protein is determined by the formula $m1/5$, where $m1$ is the concentration of *Per* mRNA. This reflects the biological observation that the reaction speed of transcription is determined based on the concentration of *Per* mRNA. Complex forming rate is generated using the formula of a form: $mX*mY/10$. For example, the formula $m2*m4/10$ for the complex forming rate of the proteins PER ($m2$) and CRY ($m4$) is assigned at the continuous transition. Continuous transitions without outgoing arcs are used to represent the natural degradation rate of mRNAs, proteins, and protein complexes. For example, as the degradation rates of the products of gene *Cry*, the formulas $m3/5$, $m4/10$, and $m5/15$ are assigned to the corresponding transitions, representing its mRNA degradation, protein degradation, and complex (with CRY protein) degradation, respectively. These differences in rates refect the two biological fact that proteins are more stable than mRNA and that protein PER becomes more stable by forming complex with protein CRY.[13]

After describing the HFPN of the biological mechanism, parameters of transition speed and initial values of places are selected based on biological knowledge

[a]A discrete place and a continuous place are represented by symbols of a single circle and a filled rectangle, respectively. These symbols are not used in the HFPN of Fig. 2.

and/or the facts described in biological literature. If a parameter can be determined from experimentally obtained data, that parameter is reflected in the model as the speed of the corresponding transition or the initial value of the corresponding place. Thereafter, the other parameters of reactions such as the transcription speeds of genes and degradation rates of proteins are going to be tuned such that the input/output concentrations of substances such as mRNA and protein correspond to biological facts that have been obtained experimentally or from previous literature. In general, many trial and error procedures will be required until the appropriate parameters for simulation are selected. Since GON provides the GUI specially designed for biological modeling, these selection procedures can be performed very easily and smoothly.

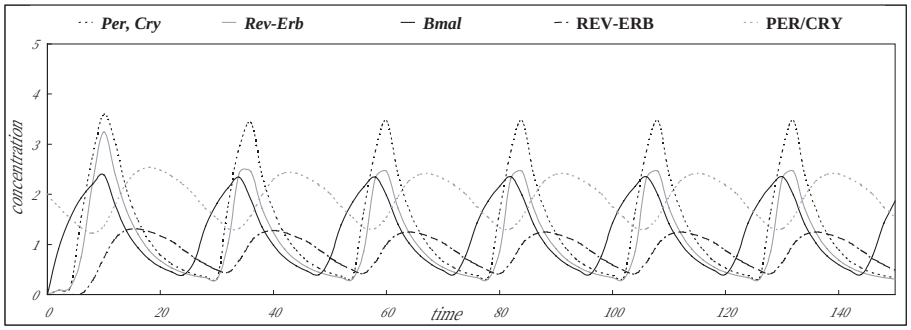
3.3. *Simulation results and their inconsistencies with the biological facts*

Simulations of the HFPN model of Fig. 3 were performed using Genomic Object Net.[4,6] This model produces periodic oscillations of mRNA and protein concentrations as shown in Fig. 3 (a). Modifications in the HFPN model were made to check for mutations such as *Per* gene disruption (removal of the normal arc going into the place PER) and preventing *Cry* gene from transcription (removal of the test arc entering the transition that is attached to the place *Cry* mRNA). The resulting behavior of these modifications corresponded well with the biological facts in the literature.[14,15] However, concurrently, we found the following two results that were inconsistent with the biological observations.

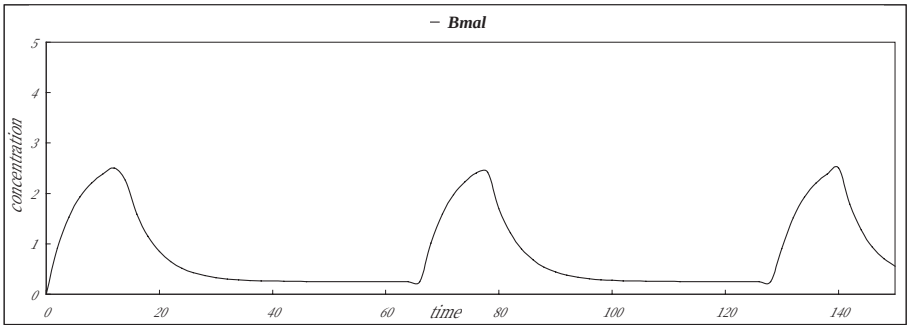
(a) In Fig. 3 (a), the *Bmal* mRNA peaks at almost the same time as the *Cry* and *Per* mRNAs. However, as per the biological observations[16], the peak of *Bmal* mRNA has to be located approximately at the mid point of two successive *Per* or *Cry* mRNA peaks. .
(b) Fig. 3 (b) shows the periodic oscillation of *Bmal* mRNA in *Cry* knockout mice. However, this contradicts the biological fact that *Bmal* gene stops oscillating in Cry knockout mice.[10]

In order to explain the results (a), we present the following two facts.

Fact 1 *Rev-Erb* mRNA exhibits a similar behavior in phase to *Cry* mRNA (*Per* mRNA).[17] In fact, the simulation results shown in Fig. 3 (a) indicates similar concentration behavior of *Rev-Erb* mRNA and *Cry* mRNA (*Per* mRNA) with the exception of the difference in their mRNA peaks.
Fact 2 Generally, the mRNA concentration peak of a gene occurs at the point when transcription of the gene stops. Since only the repression by the REV-ERB protein controls *Bmal* transcription depicted in Fig. 1, *Bmal* transcription should ideally be switched off when the concentration of the REV-ERB protein increases. Hence, the peak of *Bmal* mRNA is noted when the concentration of REV-ERB protein



(a) Expressions of *Bmal*, *Cry*, *Per*, *Rev-Erb*, and *Clock*



(b) Expression of *Bmal* gene when *Cry* gene is disrupted

Fig. 3. Simulation results of the HFPN model in which all known biological facts are reflected. Dark solid line: *Bmal* mRNA; dark broken line: *Per, Cry* mRNA; pale solid line: *Rev-Erb* mRNA; pale broken line: PER/CRY complex; and doted dash line: REV-ERB protein. (a) *Bmal* mRNA behaves similar to *Per* mRNA and *Cry* mRNA, wherein the *Bmal* mRNA peak is located approximately at the mid point of two successive *Per* or *Cry* mRNA peaks. (b) *Bmal* mRNA is expressed even when the *Cry* gene is disrupted.

increases.

From Fact 2, in order for the *Bmal* mRNA concentration peak to locate at the mid point of two successive *Per* or *Cry* mRNA concentration peaks, the period of REV-ERB protein concentration growing has to include that mid point. Hence, this observation and Fact 2 demonstrate that the difference in time between the peaks of *Rev-erb* mRNA and REV-ERB protein is over 12 h.[b] However, it is unrealistic to spend over 12 h for translation. In fact, the translation time for REV-ERB protein in mouse liver is known to be approximately 1 or 2 h.[18] Thus, with the model of circadian gene regulation of Fig.1 where *Bmal* gene expression is controlled only by REV-ERB protein, we can not locate the peak of *Bmal* mRNA concentration at the mid point of the two successive *Cry* (*Per*) peaks.

[b]Note that the period of circadian rhythm is approximately 24 hours.

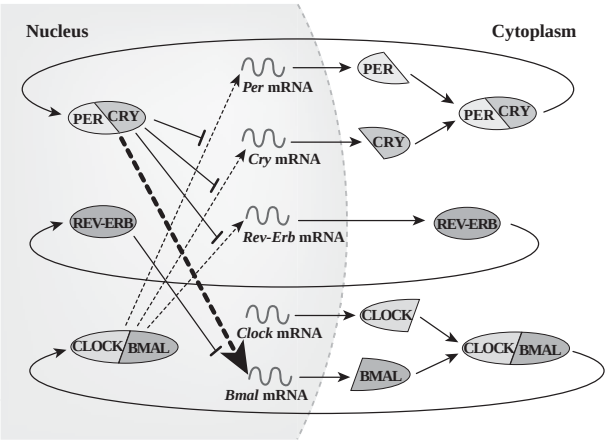


Fig. 4. New mammalian circadian gene regulatory mechanism. The bold dotted arc is the hypothetical interaction "PER/CRY activates *Bmal*." Inconsistencies found in the original model (Fig. 1) were resolved if the path illustrated by the bold dotted arrow was introduced.

In contrast, the reason for this the result (b) is simple. From the gene regulatory mechanism in Fig. 1, it is easily observed that the *Cry* gene disruption can not contribute toward blocking the auto-feedback system of *Bmal* transcription. CLOCK/BMAL complex composition, *Rev-Erb* gene activation, and repression of *Bmal* gene transcription. Hence, oscillation of the *Bmal* does not stop.

4. A New Hypothesis: PER/CRY Complex Activates the *Bmal* Gene

Circadian clock mechanisms have been examined in many living organisms such as cyanobacteria, fruit flies, and mice.[15,20] Many investigations have been conducted on the fruit fly (*Drosophila melanogaster*), and it is known that it has a similar circadian gene regulatory mechanism to that of mice. Thus, in order to resolve the inconsistencies pointed out in the previous section, we compared these two circadian mechanisms. Consequently, we noticed the following path observed in the *Drosophila* circadian mechanism has not been identified in mice.

- PER/TIM complex activates the gene *dClock*.[19]

Where TIM (timeless) is a protein in *Drosophila* that replaces CRY, and *dClock* is a gene in *Drosophila* that corresponds to *Bmal* in mice.

Fig. 4 is the new mammalian circadian gene regulatory mechanism incorporating the hypothetical interaction "PER/CRY complex activates the gene *Bmal*." Fig. 5 is the modified HFPN model in which the hypothetical path specified above was incorporated in the form of the bold dotted arc emerging from the place PER/CRY. Fig. 6 shows simulation results obtained using this modified HFPN model. This

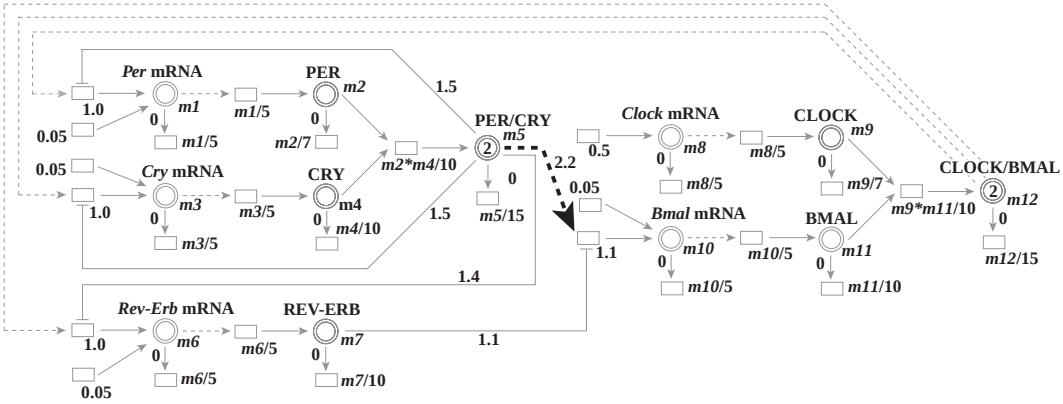


Fig. 5. Improved HFPN model in which the hypothetical path (bold dotted arrow) "PER/TIM activates the gene *Bmal*" is included.

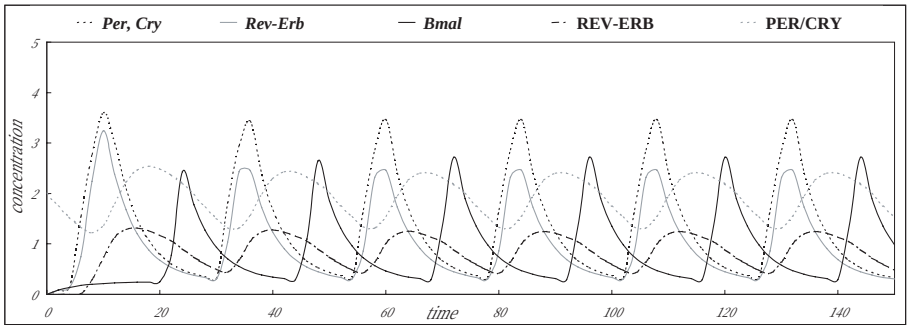
figure shows that the two biologically inconsistent points (a) and (b) presented in the subsection 3.3 were resolved by introducing this new path.

(a) Fig. 6 (a) demonstrates the effect of the hypothetical path on the concentration behavior of *Bmal* mRNA. Recall that, in the original model, the transcription switch of gene *Bmal* was controlled only by the inhibition by the REV-ERB protein. In contrast, in the new model, this transcription is controlled not only by the inhibition from the REV-ERB but also by the activation from PER/CRY protein complex. This activation from the PER/CRY complex allows the *Bmal* transcription to stop at the some point during the decrease in the PER/CRY complex concentration. In summary, two operations for *Bmal* expression of "inhibition from REV-ERB" and "activation from PER/CRY" enables the *Bmal* mRNA peak to locate at the mid point of two successive *Cry* (*Per*) mRNA peaks.

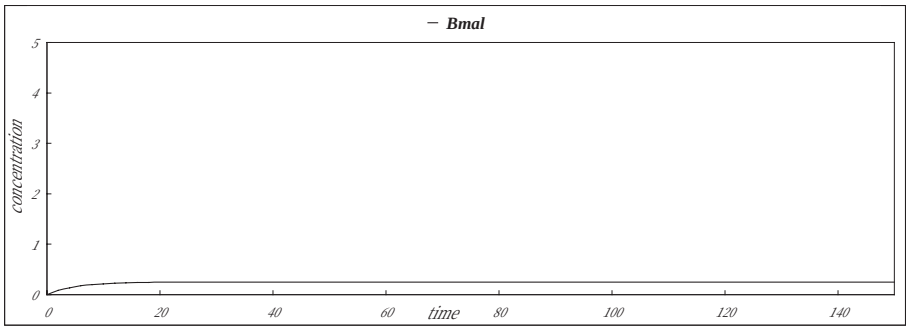
(b) In order to activate *Bmal* in the new model, both these conditions, namely, "REV-ERB represses *Bmal*" and "PER/CRY activates *Bmal*" have to be fulfilled. Hence, no oscillation of *Bmal* mRNA occurs when *Cry* is disrupted, as shown in Figure 6 (b).

5. Discussions

Five types of genes *Per*, *Cry*, *Clock*, *Bmal*, and *Rev-Erb* have been recognized as the essential genes of mammalian circadian clocks that consist of interlocking feedback loops as shown in Fig. 1. We simulated concentration behaviors in the oscillations of the original circadian clock model in an HFPN model shown in Fig. 2. However, this original model could not explain two established biological observations. The first was a discrepancy found in the original model wherein *Bmal* remained oscillating



(a) Expressions of *Bmal*, *Cry*, *Per*, *Rev-Erb*, and *Clock*



(b) Expression of *Bmal* gene when *Cry* gene is disrupted

Fig. 6. Simulation results of the HFPN model after adding the new hypothetical reaction: "PER/CRY activates the gene *Bmal*." Dark solid line: *Bmal* mRNA; dark broken line: *Per, Cry* mRNA; pale solid line: *Rev-Erb* mRNA; pale broken line: PER/CRY complex; and dotted dash line: REV-ERB protein. (a) The peak of *Bmal* mRNA is located almost at the mid point of two successive peaks of *Per* or *Cry* mRNA. (b) *Bmal* mRNA is not expressed when the *Cry* gene is disrupted.

even in the Cry knockout mouse, and the second observation was that *Bmal* mRNA concentration peak could not be made at the mid point between two successive peaks of *Per* or *Cry* mRNA concentration. In order to resolve these discrepancies, a new path was introduced into the original model. Introduction of the path in which PER/CRY enhance transcription of *Bmal* resolved both these inconsistencies.

Very recently, researchers[21,22] have identified that the *Rorα* gene is an essential gene that activates *Bmal* transcription.[c] Since the E-box enhancer is located upstream of *Rorα*, the transcription of the gene is inhibited by the PER/CRY complex and activated by CLOCK/BMAL complex. Fig. 7 is the genetic interaction map updated by the incorporation of these two interactions into Fig. 1.

Recall that the mechanism of *Bmal* expression is the difference between the interaction maps of Fig. 1 and Fig. 4. In other words, as pointed out in section 4,

[c]These papers have been published after we constructed the HFPN model of Fig. 2.

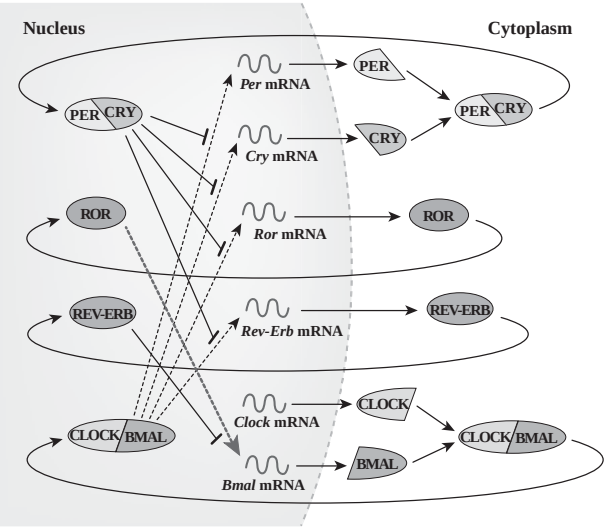


Fig. 7. Interactions of circadian genes after incorporating *Ror* gene and its product into Fig. 4. Simulations of the HFPN model constructed from this figure have produced the same behavior as Fig. 6 (a) in terms of the location of the *Bmal* concentration peak.

two operations for *Bmal* expression of "inhibition by REV-ERB" and "activation by PER/CRY" essentially function such that the *Bmal* mRNA peak at the mid point of two successive *Cry* (*Per*) mRNA peaks. Although the biologically identified interaction "ROR activates *Bmal*" is not the same as the suggested interaction in Fig. 4, these two operations of activation and inhibition is involved in Fig. 7.

We have conducted further simulations of an HFPN model constructed from Fig. 7 that displays the same behavior of *Bmal* expression as that in Fig. 6 with regard to peak locations. This implies that although it is partial, these recent researches[21,22] support our suggestion with respect to *Bmal* expression mechanism. Details will be reported elsewhere.

Gaining insights into the dynamic behavior of molecular interactions is difficult without the aid of simulations. In fact, the authors of the recent papers[21,22] did not mention the significance of the interaction of *Ror* gene in the phase relations between *Per* (*Cry*) and *Bmal* expressions.

6. Conclusions

In the biological literature, images such as Figures 1, 4, and 7 are commonly used to express molecular interactions, existing interactions and newly discovered interactions. Using the HFPN models constructed from these images, this paper demonstrates the discriminating feature of computer simulations by demonstrating an example that reveals inconsistencies in the molecular interactions.

Circadian rhythms are well-studied models in computer simulation research of

biological systems. However, many observations of molecular interactions whose underlying principles have not been systematically uncovered may still remain. One of these examples is the phase response that is induced by a light pulse in circadian rhythms. We have attempt dynamical analyses of this light-induced phase response using an HFPN model that provides a novel suggestion that implies the importance of interlocking loops to the phase response in mammals. These results will be reported in the near future.

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