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The Use of Petri Net Techniques to Characterize the Regulatory Mechanism of Mammalian Circadian Rhythm Genes

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ABSTRACT

Many cellular and physiological processes in most living organisms have been shown to display a rhythm of about 24 hours. This so-called circadian rhythm is based on a genetic regulatory network with interlocked negative and positive molecular feedback loops. Its functions are to allow organisms to adapt their physiology to the natural alternation of day and night, as well as to environmental stimuli. Disruption of the circadian rhythm has been linked to diabetes, obesity, and cancer. The aim of this study was to investigate the relationship between *mPer*, a gene involved in mammalian circadian rhythm, and cancer. Cancer cells can inhibit the expression of *mPer* to extend their own life cycle. We developed a model using Petri net techniques as an alternative to *in vivo* experimentation, whose use is made difficult by the uncontrollable expression of *mPer*. This model was then used to investigate the regulatory mechanisms of normal and abnormal circadian rhythms in mammals. We found that the cycle times of PER and PER/CRY were extended to almost 369 hours when *mPer* expression was completely inhibited. The results are significant since they show that normal cells could turn into cancer cells if the transcription of *mPer* is strongly inhibited.

INTRODUCTION

Many cellular and physiological processes in most living organisms have been shown to display a rhythm of about 24 hours. This so-called circadian rhythm [1] is based on a system of interlocked negative and positive molecular feedback loops. The suprachiasmatic nuclei (SCN) play the central role in the regulation of the mammalian circadian rhythm [2].

Some remarkable advances in understanding the molecular basis of circadian rhythms have been made in mutants of the fly *Drosophila* due to its ease of maintenance and reproduction [3]. A breakthrough for the mechanism of circadian rhythms in *Drosophila* was the finding that *mPer* mRNA is produced in a circadian manner. This periodic variation is accompanied by a circadian rhythm in the abundance of PER. The peak in *mPer* mRNA synthesis precedes the peak in PER production by 4 to 8 hours, suggesting that the *Drosophila* circadian rhythm results from negative feedback exerted by the PER protein on the synthesis of *mPer* mRNA. The first circadian rhythm gene in mammals was discovered by Takahashi [4], and was named *mClock*, with m designating that the gene is mammalian. Since then, the *mClock* gene has been successfully characterized, along with its protein product CLOCK, and the related BMAL (brain and muscle *Arnt*-like) protein. CLOCK and BMAL can form a complex which functions as the switch to turn the expression of *mPer* and *mCry* genes on or off [4, 5].

The Petri net is a mathematical model employed in many fields to describe discrete interactions in a system. Many extensions to the simple Petri net model [6] have been developed for various modeling and simulation purposes. The major categories of Petri net extensions are: (a) hierarchical Petri nets, which allow the previously defined net to present in a new net as an entity or process; (b) hybrid Petri nets, which allow the components to deal with continuous values instead of integer numbers of tokens; (c) timed Petri nets [7], which introduce the concept of deterministic time delays; (d) stochastic Petri nets, in which entities and processes may be assigned delays which are represented by a probability distribution; and (e) colored Petri nets [8], which allow more complex firing rules in the processes.

However, it is only recently that the Petri net has had an influence in biological applications, especially in areas such as metabolic pathways and other similar networks. A novel variation of Petri net called the hybrid functional Petri net (HFPN) and its enhanced version, the hybrid functional Petri net with extension (HFPNe), has been developed by Masao Nagasaki et al. [9, 10]. Both methods extend and combine various different kinds of Petri nets, making them suitable for solving biological

problems. As a result, this paper utilizes Cell Illustrator 3.0, a software tool developed by Nagasaki based on the HFPNe [10], to construct and simulate the genetic regulatory mechanism of circadian control. This mechanism consists of feedback loops of *clock* genes, which ultimately, under normal circumstances, result in endogenous near 24-hour rhythms in mammals [5]. Using this model, we can study the effect of abnormal gene expression on circadian behavior in mammals.

METHODS

Research flowchart . Figure 1 shows the research flowchart for this paper. The first step of the study was to collect literature related to circadian rhythms. Collection of the important kinetic reactions and various parameters from biological experiments enables the construction of a model of circadian rhythm in mammals using the newly developed state-of-the-art Petri net techniques described above. The HFPNe model allows us to further investigate the effect of many kinds of perturbation. For example, the relationship between changes in *mPer* expression levels and cancers can be investigated, along with the effect of such changes on the cell cycle [11, 12]. The model is then validated by available experimental biomedical data and should, for example, display circadian oscillations with a period of approximately 24 hours. If the model does not exhibit the correct periodical behavior, the kinetic parameters must be modified to fix the errors. The aim of this study was to characterize the important role of the *mPer* gene in circadian rhythm and the effect of *mPer* gene expression cycles in cancer cells [13]. This will be accomplished by adjusting the threshold values for the *mPer* gene.

FIG. 1. Research flowchart for this study.

The regulatory mechanism of circadian rhythms in mammals . Autonomous cellular circadian oscillators contain positive and negative elements that form feedback loops.

Usually, the positive elements of the loop activate the transcription of so-called 'clock genes' that encode the negative elements of the system. As a result, the concentrations of the negative elements rise, and they physically interact with the positive elements to inhibit their activity. This inhibition reduces transcription of the negative element genes, which then decreases the concentration of the negative elements and leads to reactivation of the positive elements, allowing the cycle to start again.

As an example, the regulatory mechanism of circadian rhythms in mammals is illustrated in Figure 2. A complex between two activators, the CLOCK and BMAL proteins, promotes the expression of the *mPer* and *mCry* genes, which then produce greater levels of both PER and CRY proteins during the daytime. Before the onset of nighttime, after production of sufficient levels of PER and CRY proteins, these two proteins form a complex that is secreted back into the nucleus. The complex exerts negative feedback on CLOCK/BMAL, inhibiting the expression of *mPer* and *mCry* and reducing the levels of the PER and CRY proteins. During the daytime, the cycle starts over, with CLOCK/BMAL regaining its activity and resuming promotion of *mPer* and *mCry* transcription [14].

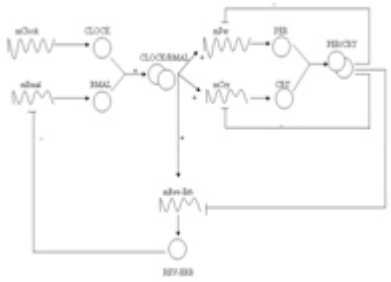

FIG. 2. The regulatory mechanism of circadian rhythms in mammals.

Modeling the mammalian circadian clock. This section illustrates the application of the Petri net to modern simulation work, especially in biological applications. This discussion will: (1) encompass the important research capabilities of the Petri net along with its inherent strengths and weaknesses, (2) introduce the basic elements of HFPN, (3) describe our modified mathematical model, and (4) discuss how to construct our model with Cell Illustrator 3.0, a useful tool developed by Masao Nagasaki et al. [10].

An important role for the Petri net in biological simulation. Several simulation tools, such as E-Cell and BioSpice, have been developed to model biological pathways or

metabolic pathways. Since many biological systems are modeled as a series of ordinary differential equations (ODEs), it would be rather complicated to use E-Cell or BioSpice to model them. It is likewise difficult for biologists to learn how to construct their own models from complicated mathematical equations. Therefore, the Petri net holds an advantage because biologists can model the system based solely on biological knowledge. Also, the inherent properties of the Petri net, such as extendibility, abstraction, structural reduction, boundedness, P-invariants, T-invariants, and liveness, are especially suitable for biological applications. In brief, Petri nets are more suitable than other simulation methods for the construction and simulation of biological models.

The main advantage of using HFPN techniques is the ability to integrate various biochemical reactions into a single graphical platform. Other advantages include: dynamic transfer and degradation of proteins or metabolites, the addition of co-substrates into a specific reaction, the addition of inhibitory or active parameters to the connectors, and control of substrate concentration flux into the cycle using a time-delay setting method.

Basic elements of the Hybrid Functional Petri Net. The HFPN contains several key components: entity (place), process (transition), connector (arc), and tokens (markings). An entity represents a protein, gene, metabolite, or any signal factor. Processes represent reactions, binding, separation, transcription, translation, or other biochemical reactions. Connectors link each component and make a network. Tokens, or markings, indicate the value held by each entity. Different kinds of components and reactions are shown in Figures 3 and 4 [15].

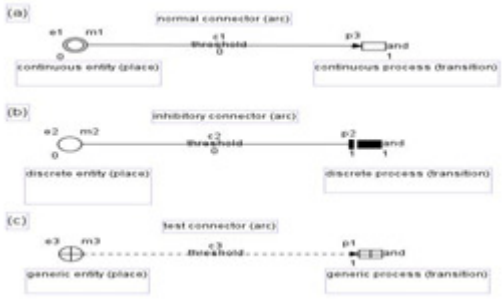

FIG. 3. The different kinds of entities, processes, and connectors in HFPN. (a) Continuous entity, normal connector, and continuous process. (b) Discrete entity, inhibitory connector, and discrete process. (c) Generic entity, test connector, generic process.

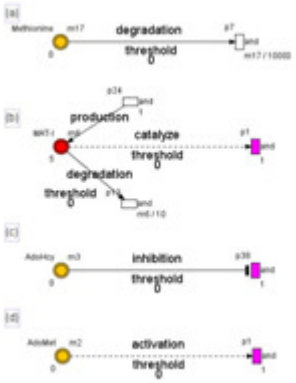

FIG. 4. Various reactions in the model established in this study. (a) Continuous entity, process, and normal connectors represent degradation in a metabolic cycle. (b) Test connectors stand for enzyme catalysis, and normal connectors stand for protein production. (c) Inhibitory connectors are used for inhibition. (d) Test connectors are used for activation.

The simple components above can interconnect to represent a complete biological pathway. The next section describes the methods used to construct the mammalian circadian clock model using a hybrid functional Petri net. These methods are: (1) build a circadian genetic control system based on biological knowledge, (2) introduce known rate equations into processes, (3) make assumptions regarding unknown reactions in accordance with biological knowledge, and (4) run simulation and adjust parameters in order to observe the behavior of the mammalian circadian clock.

Model construction. Figure 5 shows the constructed HFPN model of a circadian genetic control system in mammals. In this model, the first loop involves negative regulation of the *mPer* and *mCry* genes and their protein products. The second loop consists of the positive feedback between the *mClock* and *mBmal* genes and their protein products. Both loops are interconnected by the *mRev-Erb* gene and its protein products. The PER/CRY complex inhibits the amount of proteins produced from the *mPer*, *mCry*, and *mRev-Erb* genes. The REV-REBα protein inhibits the expression of *mBmal*. The BMAL/CLOCK complex, meanwhile, exerts positive feedback on the transcription of *mPer*, *mCry*, and *mRev-Erb* [16].

FIG. 5. HFPN model of the mammalian circadian gene regulatory system.

In this model, temporal variation in the concentrations of mRNA and regulatory proteins is governed by the following 12 kinetic equations [16, 17]:

$$\frac{dmPer}{dt} = k_{12d} BMAL/CLOCK - k_{1t} mPer - k_{1d} mPer \quad (1)$$

$$\frac{dPER}{dt} = k_{1t} mPer - k_{2d} PER - k_{2t} PER \quad (2)$$

$$\frac{dmCry}{dt} = k_{12d} BMAL/CLOCK - k_{3t} mCry - k_{3d} mCry \quad (3)$$

$$\frac{dCRY}{dt} = k_{3d} mCry - k_{4d} CRY - k_{4t} CRY \quad (4)$$

$$\frac{dPER/CRY}{dt} = k_{2t} PER + k_{4t} CRY - k_{5d} PER/CRY \quad (5)$$

$$\frac{dmRev\text{-}Erb}{dt} = k_{12d} BMAL/CLOCK - k_{6t} mRev\text{-}Erb - k_{6d} mRev\text{-}Erb \quad (6)$$

$$\frac{dREV\text{-}ERB}{dt} = k_{6t} mRev\text{-}Erb - k_{7t} REV\text{-}ERB \quad (7)$$

$$\frac{dmBmal}{dt} = 1 - k_{8t} mBmal - k_{8d} mBmal \quad (8)$$

$$\frac{dBMAL}{dt} = k_{8t} mBmal - k_{9d} BMAL - k_{9t} BMAL \quad (9)$$

$$\frac{dmClock}{dt} = 1 - k_{10d} mClock - k_{10t} mClock \quad (10)$$

$$\frac{dCLOCK}{dt} = k_{10t} mClock - k_{11d} CLOCK - k_{11t} CLOCK \quad (11)$$

$$\frac{dBMAL/CLOCK}{dt} = k_{9t} BMAL + k_{11t} CLOCK - k_{12d} BMAL/CLOCK \quad (12)$$

Each ODE represents the change in concentration of reactants per unit time. The kinetic parameters used in this model are shown in Table 1 [16, 17]. Positive and negative signs represent input and output, respectively. In equations (8) and (10), "1" represents a constant input concentration.

Table 1. Parameter values used in the model

Parameter	Value	Function Description
k_{1t}	0.24hr^{-1}	Formation rate of PER
k_{1d}	0.24hr^{-1}	Degradation rate of mPer
k_{2d}	0.14hr^{-1}	Degradation rate of PER
k_{3t}	0.24hr^{-1}	Formation rate of CRY
k_{3d}	0.24hr^{-1}	Degradation rate of mCry
k_{4d}	0.12hr^{-1}	Degradation rate of CRY
k_{5t}	0.12hr^{-1}	Formation rate of PER/CRY
k_{5d}	0.87hr^{-1}	Degradation rate of PER/CRY
k_{6t}	0.12hr^{-1}	Formation rate of REV-ERB
k_{6d}	0.24hr^{-1}	Degradation rate of mRev-Erb
k_{7d}	0.15hr^{-1}	Degradation rate of REV-ERBα
k_{8t}	0.14hr^{-1}	Formation rate of BMAL
k_{8d}	0.24hr^{-1}	Degradation rate of mBmal
k_{9d}	0.83hr^{-1}	Degradation rate of BMAL
k_{10t}	0.24hr^{-1}	Formation rate of CLOCK
k_{10d}	0.24hr^{-1}	Degradation rate of mClock
k_{11d}	0.12hr^{-1}	Degradation rate of CLOCK
k_{12t}	0.12hr^{-1}	Formation rate of mBmal/Clock
k_{12d}	0.86hr^{-1}	Degradation rate of BMAL/CLOCK

TABLE 1. Parameter values used in the model.

RESULTS AND DISCUSSION

The regulatory mechanism of circadian rhythms in normal mammals . In this model, the *mPer* and *mCry* genes are first activated in the nucleus and transcribed into mRNA. The latter are transported into the cytosol, where they are translated into the PER and CRY proteins, which then form a complex (the PER/CRY complex). Figure 6 illustrates the simulation results for the cyclical behavior of *mPer, mCry*, PER, CRY, and the PER/CRY complex in normal mammals. The expression levels of the *mPer* and *mCry* genes follow the same curve, with a cycle time of 24 hours. The actual amount of proteins translated, however, is slightly different due to differences in the transport rate from the nucleus to the cytosol. In this case, the concentration of CRY is slightly higher than the concentration of PER. The peak in *mPer* and *mCry* mRNA precedes the peak in PER and CRY proteins. The PER/CRY complex also follows a 24-hour cycle. Notably, through its repression of transcription of *mPer* and *mCry*, the

PER/CRY complex starts to inhibit the production of PER and CRY proteins before it reaches its peak, resulting in a negative feedback function. Therefore, the PER/CRY complex declines to its nadir in response to the peak in *mPer* and *mCry* expression.

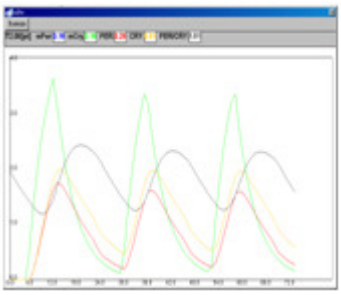

FIG. 6. Circadian oscillations of the PER/CRY feedback loop in normal mammals. The five curves correspond to the concentration (y-axis, nM) vs. time (x-axis, hr) of *mPer* (blue), *mCry* (green), PER (red), CRY (yellow), and PER/CRY complex (black).

The PER/CRY complex in the first loop inhibits the expression of the *mRev-Erb* gene (which functions as a molecular bridge between the first loop and the second loop). Figure 7 shows the circadian oscillations of the BMAL/CLOCK feedback loop in normal mammals. Levels of both *mRev-Erb* mRNA and REV-ERBα protein display a periodical behavior pattern with a 24-hour cycle. The production of REV-ERBα then inhibits the expression of the *mBmal* gene in the second loop. The production of BMAL protein continues to increase until it reaches a plateau after the first peak of *mRev-Erb* transcription. The production of CLOCK protein follows the same pattern as that of BMAL protein, and both reach a plateau after the first peak of *mRev-Erb*. Though the production of the BMAL/CLOCK complex continues to increase past the first few peaks of *mRev-Erb*, it will also reach a plateau at a later time.

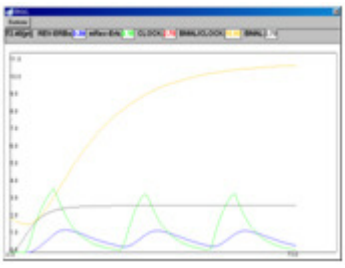

FIG. 7. Circadian oscillations in the BMAL/CLOCK feedback loop in normal mammals. The five curves correspond to the concentration (y-axis, nM) vs. time (x-axis, hr) of REV-ERBα (blue), *mRev-Erb* (green), CLOCK (red), BMAL/CLOCK (yellow), and BMAL (black).

REV-ERBα, the bridge between the first loop and the second loop, is regulated by PER/CRY in the first loop. The production of BMAL protein in the second loop, meanwhile, is regulated by REV-ERBα. BMAL and CLOCK combine to form the BMAL/CLOCK complex which then promotes the transcription of both the *mPer* and

mCry genes, leading to increased levels of the PER and CRY proteins. The levels of PER, CRY, PER/CRY, and REV-ERBα all display a normal 24-hour cycle. Note that, in contrast to the pattern of oscillation exhibited by the other proteins, the production of BMAL, CLOCK, and BMAL/CLOCK proteins all eventually reach a plateau.

The regulatory mechanism of circadian rhythms in abnormal mammalian systems. The previous section illustrated the successful building and validation of a model for normal circadian rhythms. The next step was to investigate what could occur if the *mPer* gene is not expressed correctly. By adjusting the threshold value for the PER/CRY complex from 1.5 (normal) to 0 (abnormal), the expression of the *mPer* gene was completely inhibited. As a result of this, neither PER nor PER/CRY was expressed at first. However, at around 369 hours, a slight amount of PER was produced. The second change occurred at around 738 hours. As shown in Figure 8 and 9, PER and PER/CRY followed a cycle of about 369 hours. This result suggests that transcription initiation of *mPer* is caused by positive feedback from the production of BMAL/CLOCK in the second loop, albeit at a much later time. The production of BMA is in turn inhibited by REV-ERBα. Therefore, the peak of BMAL appears just as the expression of REV-ERBα reaches its nadir. The other proteins (CRY, CLOCK, BMAL/CLOCK, and REV-ERBα) also display oscillation, albeit with much longer cycles (around 62 hours).

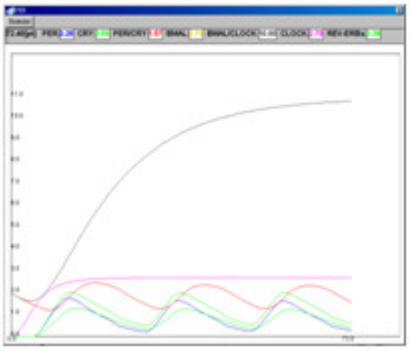

FIG. 8. Simulation results for PER, CRY, PER/CRY, BMAL, BMAL/CLOCK, CLOCK, and REV-ERBα proteins in normal mammals.

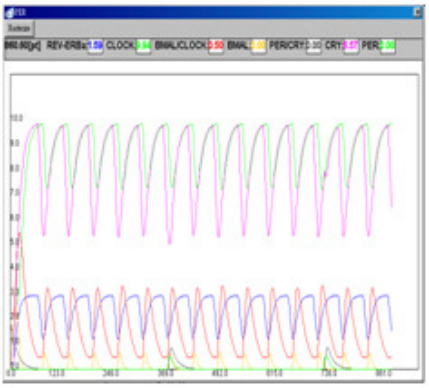

FIG. 9. Simulation results for PER, CRY, PER/CRY, BMAL, BMAL/CLOCK, CLOCK, and REV-ERBα proteins in abnormal mammalian systems.

Comparison of the circadian regulatory mechanism between normal and abnormal mammalian systems. By adjusting the threshold value for the PER/CRY complex from 1.5 (normal) to 0 (abnormal), it is possible to compare the differences between the circadian regulatory mechanisms of normal and abnormal mammals. Since the PER/CRY complex inhibits the transcription of *mPer*, the cycle times for PER and PER/CRY become extended to almost 369 hours in comparison with the normal cycle time of 24 hours. At the same time, none of the other proteins (CRY, BMAL, BMAL/CLOCK, CLOCK, and REV-ERBα) adhere to their normal 24-hour cycles either. Among these proteins, BMAL, CLOCK, and BMAL/CLOCK exhibit a somewhat unstable status.

CONCLUSION

Many cellular and physiological processes in most living organisms have been shown to display a rhythm of about 24 hours. This so-called circadian rhythm is based on a genetic regulatory network with interlocked negative and positive molecular feedback loops. Its function is to allow organisms to adapt their physiology to the natural alternation of day and night as well as to environmental stimuli.

In this study, we constructed an HFPN model of a circadian genetic control system in mammals. The model comprised two loops, where the first loop involves negative regulation of the *mPer* and *mCry* genes and their protein products, and the second loop involves positive regulation of the *mClock* and *mBmal* genes and their protein products. The two loops are interconnected by the *mRev-Erb* gene and its protein products. The PER/CRY complex inhibits expression of *mPer, mCry* and *mRev-Erb*. The

REV-ERBα protein inhibits *mBmal* expression. The BMAL/CLOCK complex, meanwhile, exerts positive feedback on *mPer, mCry*, and *mRev-Erb*.

Cancer cells can inhibit the expression of *mPer* in order to extend their life cycles. In our model, we found the cycles of PER and PER/CRY were extended to almost 369 hours when the expression of the *mPer* gene was completely inhibited. The results are significant since they show that normal cells could turn into cancer cells if the transcription of the *mPer* gene is strongly inhibited.

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FOOTNOTES

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