Contributions of Systems Biology to the Understanding of Circadian Rhythms in *Drosophila*

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***Abstract*— Circadian rhythms govern many aspects of an organism's behaviour and physiology. Until the advent of system biology, models of circadian phenomena had been based on studies of single genes. In the last several years, microarray technology has been applied to this area of research, and has resulted in significant advances in our understanding of circadian rhythms. Two traditional models of circadian rhythms are presented in this paper, and the results of three of the microarray studies are outlined as well. Some problems posed by microarray technology are also discussed.**

I. Introduction

Traditional molecular genetics and biology have been relatively successful in identifying the fundamental elements and mechanisms that enable cells to function. The major thrust of this work has been to identify the genes and proteins that are responsible for a specific phenotype or biological process. Among many other applications, standard techniques such as forward and reverse genetics and biochemistry have been used to investigate the fundamental nature of the biological circadian clock. This research has revealed that the core circadian oscillator consists of a transcriptional/translational feedback loop that drives circadian behaviour and physiological rhythms. However, little has been determined about the identities or functions of genes and proteins that are downstream from the core oscillator in the circadian control scheme. Until the advent of systems biology, there was no comprehensive method of identifying all of the rhythmically expressed genes in a particular organism, except by the time- and resource-consuming brute force method.

Within the past two years, several groups have used microarrays to study the biological circadian clock [1]–[4]. This new technique has allowed for a much more comprehensive identification of DNA motifs that mediate cyclical gene expression than was possible with traditional techniques. The results of this work have provided significant insights into the connections between the core oscillators and the physiological and behavioural outcomes of circadian gene expression.

A. Circadian Rhythms

Circadian rhythms are endogenous, self-sustaining oscillations that have a period of approximately 24 hours. These rhythms are expressed in organisms in the form of locomotor activity, feeding behaviour, sleeping or waking patterns, and certain physiological and metabolic pathways such as gene expression. Since environmental changes in light and temperature resulting from the Earth's rotation follow a predictable rhythm, it is evolutionarily advantageous for organisms to take advantage of this periodicity. Therefore, circadian rhythms play a key role in the adaptation of organisms to their environments.

In *Drosophila melanogaster*, several aspects of physiology and behaviour are governed by the circadian clock. For example, adult flies restrict flying, foraging for food, and mating activities to daytime, and exhibit a period of reduced activity ("sleep") at night. This behaviour persists when lighting conditions are changed. For example, adult *Drosophila* emerge from their pupal cases during the early morning. Panda et al. reported that pupae kept on a regular schedule of 12 hours of light followed by 12 hours of darkness, and then subjected to constant darkness, will emerge at the time of day when they expect dawn [4]. The circadian rhythm is very sensitive to the timing of dusk and dawn, and will adapt itself to seasonally changing

day lengths. However, it is relatively insensitive to short exposures to light, such as lightning [4].

Several compatible models of circadian rhythms exist, including a negative transcriptional feedback loop model and a limit cycle oscillator model, both of which are outlined below. However, both of these models have limitations that can be addressed by using a systems biology approach.

B. Negative Transcriptional Feedback Model

Circadian rhythms are regulated by a central pacemaker that receives external inputs, such as ambient light level, and keeps circadian time. The working principles of the circadian clock in *Drosophila* were determined some years ago using traditional genetics. An autoregulatory negative transcriptional feedback loop plays a central role in the mechanism of circadian rhythms [3], [5]. In *Drosophila*, the feedback loop is driven by a heterodimer of the transcription factors CLOCK (CLK) and CYCLE (CYC). This CLK:CYC heterodimer binds to the E boxes and activates the transcription of two genes, *period* (*per*) and *timeless* (*tim*). The protein products of these genes (PER and TIM, respectively) are believed to inhibit the function of the CLK:CYC heterodimer [3]. Since the CLK:CYC complex promotes the synthesis of PER and TIM, the inhibition of its action by these proteins forms a negative transcriptional feedback loop. The rate at which the TIM protein breaks down is governed by the ambient light level. Since light levels cycle predictably throughout a 24 hour period, the PER
TIM negative transcriptional feedback loop is responsible for circadian cycling.

The role of PER and TIM in circadian rhythms was deduced using genetic screens to identify periodically expressed genes. However, this model is not complete, as this approach does not provide an accurate perspective of the whole genome of *Drosphila*. Using sheer intuition to predict the dynamics of a complex regulatory system such as circadian rhythms quickly meets with limitations

C. Limit Cycle Oscillator Model

It has long been suggested that circadian rhythms can be modelled as limit cycle oscillators [5]. The time course of a circadian system can be plotted as a function of the concentrations of biochemical species. When two biochemical species are considered, sustained oscillations take the form of a close curve in $\mathbb{R}^2$ (where $\mathbb{R}$ denotes the set of real numbers). In the case of limit cycle oscillators, the same closed curve will be obtained for all initial conditions. Therefore, limit cycle oscillators represent a particularly stable class of

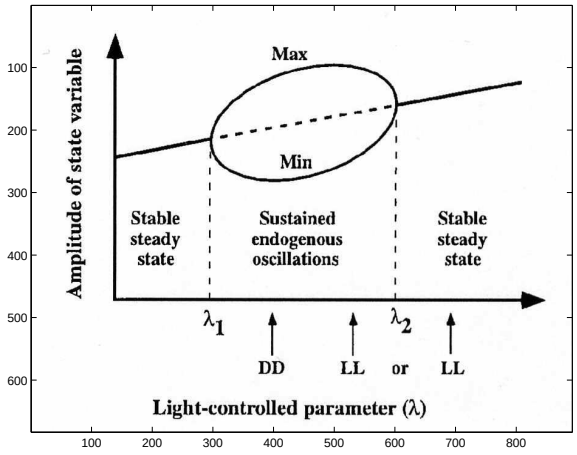

Fig. 1. Circadian Limit Cycle Oscillator [5]

periodic behaviour. Biological circadian rhythms are an example of limit cycle oscillation, as they maintain the same amplitude and period of oscillation in a changing environment.

However, sustained limit cycle oscillations only take place in a region of operation bounded by the critical values of some system parameters. In the case of circadian oscillations in *Drosophila*, the controlling parameter is modelled as the concentration of TIM protein [5]. Figure 1 illustrates the circadian limit cycle oscillator in *Drosophila*, where the concentration of TIM protein is denoted by λ. At low values of λ, the system reaches a stable steady state corresponding to some constant concentration of state variables. As λ increases, bifurcation occurs when λ reaches some critical value λ_1. The steady stable becomes unstable, and sustained oscillations occur. As λ increases further, the amplitude of the oscillations increases and then decreases again, until λ reaches a second critical value, λ_2. At this concentration of TIM protein, the sustained oscillations disappear and the system returns to a stable steady state.

The sustained oscillations in constant darkness that have been observed in *Drosophila* behaviour correspond to a value of TIM concentration λ_{DD} located in the domain of sustained oscillations (between λ_1 and λ_2) [5]. Similarly, oscillations that are damped in constant light correspond to values of λ_{LL} that are above the critical value λ_2. Thus,

$$\lambda_1 < \lambda_{DD} < \lambda_2 < \lambda_{LL}$$

The concept of circadian rhythms as limit cycle oscillators was first investigated using abstract models. This idea was then verified experimentally using traditional genetic analysis tools, resulting in the model outlined above. However, in such a simple model a very high

degree of cooperativity of repression would be needed to produce the limit cycle oscillations observed in circadian cycling. A more realistic model would require a smaller degree of cooperativity. Such a model would have to account for the saturable kinetics of mRNA and protein degradation, the phosphorylation of the PER and TIM proteins, and so on. This type of information can be gained relatively quickly and easily using microarray technology to interrogate a large number of DNA/protein strands simultaneously, but would be prohibitively time-consuming using traditional techniques.

II. Microarray Technology

The term microarray refers to an array of individual pieces of DNA or oligonucleotide that are bound to a substrate such as a microscope slide. The purpose of a microarray experiment is to determine the expression of mRNA in a particular sample. The target mRNA is extracted from the cells or tissues of interest, converted to DNA, and labelled. It is then hybrized to all the DNA or oligonucleotide spots on the microarray. Favourable matches are identified using phosphorescent or fluorescent imaging. The data resulting from a microarray experiment are just a list of measurements of spot intensities. Extracting useful information from this data requires identifying which results are meaningful and which are not.

Information is derived from microarray studies by comparing the gene expression patterns of a control organism to the expression profile of an organism that has been subjected to some process or treatment that induced biological changes in the organism. The use of DNA microarrays allows researchers to compare the expression of thousands of genes in parallel, which allows for great increases in experimental throughput.

In particular, microarrays can be used to identify the potential targets of key circadian clock components. This has been done by profiling the expression profiles of strains of the organism in question that have an inactivating mutation in a known core clock component. In *Drosophila*, microarray technology has been used to identify gene clusters that correspond to specific developmental stages in the fly life cycle. This work revealed that a relatively large portion of the fruit fly genome is dedicated to life stage-specific gene transcription [6]. Similar work has been conducted in *Arabidopsis*, where it has been shown that 2% to 6% of the genome is circadianly expressed under free-running conditions [6]. Specifically, the clusters of cycling genes whose expression peaked during the daylight hours corresponded to light-related pathways such as photosynthesis. Also, several of the clusters that peaked expression at dusk corresponded to genes that play a role in cold resistance.

III. Applications of Microarray Technology to Circadian Research

The past few years have been a time of great activity in circadian research. Many research groups have been applying microarray technology to their research. The results of three of these groups are discussed below.

Ceriani et al. used high-density oligonucleotide arrays to examine circadian transcriptional regulation in *Drosophila* head tissue [2]. They reported 712 genes cycling in flies exposed to a 12 hours light-dark regime, including 115 genes that also cycled in constant darkness. Also, 341 genes were found that cycled only in constant darkness. They also probed the high-density arrays with RNA isolated from body tissue, and found that a similar number of genes cycle in both the head and body tissues. However, only a small number of genes cycle in both tissues. They concluded from this data that the circadian clock controls different aspects of physiology in different tissues.

Ceriani et al. also studied circadian control of locomotion behaviour by the slowpoke binding protein gene *slob*. A mutation in this gene causes behavioural defects in *Drosophila*. The group found that *slob* mRNA cycled robustly in several fly tissues. They then examined locomotor activity in *slo* mutants. The wild-type flies showed increased locomotor activity near dawn and dusk and remained quiet the rest of the day. The *slo* mutant flies showed activity throughout the day, irregardless of light levels. From this, Ceriani et al. concluded that circadian expression of at least one gene that affects temporal regulation of locomotor activity is absent in *slo* mutant flies.

McDonald and Rosbash used cDNA arrays to identify circadianly cycling genes in wild-type *Drosophila* [3]. They entrained wild flies for 3 days in a regime of 12:12 hours of light and darkness (LD regime). The fly population was then sampled every four hours during the first full day in constant darkness (DD regime). They found that 134 genes exhibited circadian cycling in constant darkness. McDonald and Rosbash also noted that many of the cycling genes with similar functions were clustered in the same region of the chromosome, and appeared to be subject to transcriptional coregulation.

This group also used *clk* mutant flies to examine cycling mRNA in flies that exhibit arrhythmic behaviour. The mutant flies were entrained and sampled in the same way as the wild-type flies. The researchers found that none of the genes that cycled in the wild flies were cycling in the *clk* mutant flies. This indicated that there are no *clk*-independent oscillators in *Drosophila*, and hence the *clk* gene sits at the top of the circadian regulatory network.

Ueda et al. examined temporal patterns of gene ex-

pression in *Drosophila* in LD and DD regimes using high-density oligonucleotide arrays [2]. They used Affymetrix GeneChips, which are capable of representing the entire *Drosophila* genome on one chip. The fly population was sampled every four hours over two days in both LD and DD. They found that 683 genes were cycling in the fly head tissue in an LD regime. Of these, 103 genes were cycling under both LD and DD conditions. Also, 407 genes cycled under DD, but not under LD.

Several interesting cycling genes were identified by this group. For example, some of the enzymes involved in the metabolism of glutamate and GABA were found to be periodically regulated. In mammals, glutamate and GABA are neurotransmitters associated with clock function. This suggests that glutamate and GABA may have circadian aspects in fruit flies.

IV. Conclusions

The last several years have seen significant advancements in the state of knowledge of circadian rhythms. This progress has included the observation that a number of aspects of *Drosophila* physiology are under circadian control, including locomotion and neurotransmission. It was also discovered that circadianly regulated genes are grouped together on chromosomes, raising the possibility of the existence of *clk*-regulated transcription enhancers. In addition, it was shown that the *clk* gene sits at the top of the circadian control hierarchy. All of these advancements are due to the application of systems biology techniques to the study of circadian rhythms.

A. Limitations of Microarray Techniques

Microarray techniques have yielded many advances in the field of circadian rhythms in the past few years. However, they do present several problems that are discussed below.

Circadian expression patterns repeat every 24 hours. However, the technical and cost limitations experienced by most labs mean that organisms are sampled only every 3 or 4 hours. This results in poor temporal resolution of gene expression. Also, only high-density oligonucleotide arrays are capable of interrogating the entire genome of even a simple organism in a single experiment [6]. These systesm are expensive, and threrefore are not available to all research groups.

There can also be a high degree of variability in the results of microarray studies, especially for genes that have relatively low expression levels [7]. Table IV-A shows the number of genes that were found to cycle under different conditions by three research groups. There are significant discrepancies between the number of cycling genes reported by the three groups. In particular, only 14 genes are listed as cycling by all three groups.

	Ceriani	Ueda	McDonald
Technique	HDO	HDO	cDNA
LD cyc. genes	712	682	
LD/DD cyc. genes	115	103	
DD cyc. genes	341	407	134

TABLE I
NUMBERS OF CYCLING GENES FOUND BY DIFFERENT GROUPS

Ceriani et al. suggested that this discrepancy could be the result of differing experimental approaches or analysis techniques used [1]. When they compared their results to those of Ueda et al. [2], who used a similar experimental technique, Ceriani et al. found relatively good agreement in the lists of genes reported by the two groups as cycling in a light-dark regime. However, there was poor agreement as to which genes cycled in constant darkness. Ceriani et al. suggested that this discrepancy could be due to the damping of the output signal which characteristically occurs over time when experiments are conducted in constant darkness [1].

Another major problem with microarray technology is the sheer quantity of data that is generated. When faced with such a large amount of information, researchers may be tempted to look for genes that correspond to existing system models. This tendency may obscure the more novel results suggested by the microarray data.

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