Research News

Circadian clock and microarrays: mammalian genome gets rhythm

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Circadian (daily) rhythms are found in most living organisms from cyanobacteria to mammals. They are generated by an internal 'clock' that is reset by external time cues and that regulates a variety of physiological functions through downstream target genes. Analysis of the mammalian transcriptome using DNA microarrays is now identifying hundreds of tissue-specific clock-controlled genes, which regulate an impressive diversity of biological processes.

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Circadian clocks are time-keeping systems that allow most living organisms to adapt their physiology and behaviour in an anticipatory manner to the rhythmic changes in their environment. The genetic basis of circadian rhythms was established in *Drosophila* more than 30 years ago, and clock genes that generate and/or maintain circadian oscillations have been identified subsequently in all model systems [1,2]. How these clock genes interact to generate a ~24-h period molecular oscillator has been the focus of much effort in the past decade, and these studies have collectively provided a general model for the circadian pacemaker that is based on a self-sustained transcriptional–translational feedback loop [3]. In mammals, the 'master clock' controlling circadian rhythms resides in the suprachiasmatic nuclei (SCN) of the hypothalamus and is reset by light through the retinohypothalamic tract [4]. Unexpectedly, endogenous oscillators have also been identified recently in peripheral organs and isolated cells, and they appear to be entrained by humoral signals and feeding schedule [5–8]. Although the proximal pacemaker has been extensively defined in both the SCN and peripheral organs, the output pathways regulated by these pacemakers, which ultimately regulate rhythmic physiological functions, have remained a black box. A first step towards a comprehensive understanding of the output pathways in the mammalian circadian system is the identification of the genes that are downstream of the circadian pacemakers.

This important issue has now been addressed by several groups who have carried out circadian gene expression screens in the SCN, peripheral organs and cultured cells using DNA microarray technology [9–16]. Both cDNA and high-density oligonucleotide (HDO) microarrays have been used, and genes with a circadian expression profile were identified following various data analysis and filtering protocols. All the studies could identify some genes that were previously known to cycle, including clock genes, thus validating the microarray approach for circadian gene expression analysis.

Is there a process not regulated by the circadian clock?

The first surprise from these data is that a circadian pattern of gene expression is a significant phenomenon that involves hundreds of genes in the SCN and peripheral organs (Fig. 1). Because approximately a third of the mouse or rat genomes was analysed in these experiments, the total number of rhythmic transcripts in a given organ is likely to be higher. Most of the identified transcripts were not previously known to be regulated by the circadian clock and their functional

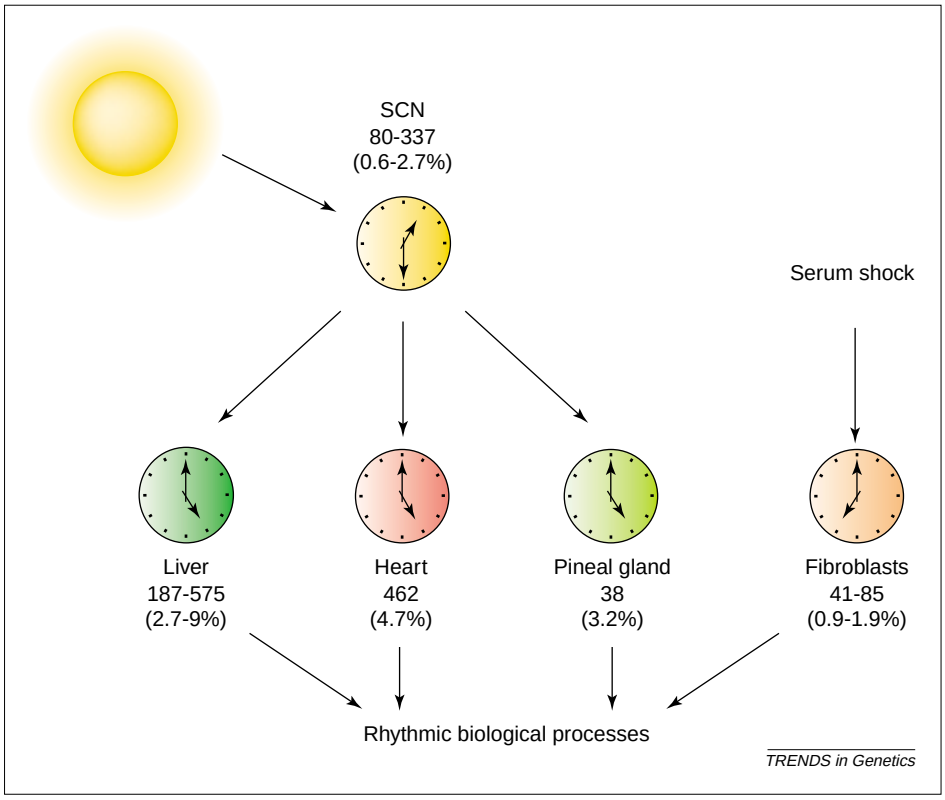

Fig. 1. Clock-controlled genes in the mammalian circadian system. In mammals, the 'master clock' resides in the suprachiasmatic nuclei (SCN) of the hypothatlamus and is believed to entrain peripheral clocks through neurohormonal signals. The SCN clock is entrained by the solar cycle and peripheral clocks control rhythmic functions of the organism. Clock-controlled gene expression has been analysed using microarrays with samples from the SCN [9,11], liver [9–13], heart [10] and pineal gland [14]. Circadian gene expression has also been analysed in serum-shocked fibroblasts [15,16]. The number of rhythmic transcripts that were identified in these studies as well as their proportion relative to the total number of genes analysed are indicated. It is known that the liver and heart clocks are delayed by 4–6 hours relative to the SCN clock (see times on the clock faces). However, it is not known whether this holds true for the pineal gland. Fibroblasts are experimentally reset, so in this case the time is not significant.

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annotation revealed that they control a variety of key pathways such as metabolism, transcription, translation, protein turnover, immune response, cell cycle, cell death, vesicle trafficking, ion transport and signal transduction. In addition, the well-documented analysis of these pathways by Panda *et al.* shows that clock-controlled genes regulate a given pathway cycle in phase; in other words, circadian regulation is coordinated [9]. The important message from these observations is that almost every biological process in a cell or an organism seems to be affected at some level by the circadian clock.

Tissue-specificity of circadian gene expression is the rule

Whether the SCN and peripheral pacemaker control a similar or a divergent set of genes was another crucial issue that was investigated in several of these studies. Although one could obviously predict that some tissue-specificity might occur, the comparison of the SCN and liver sets of clock-controlled genes demonstrates that the difference between the two tissues is dramatic. Indeed, Panda *et al.* [9] and Ueda *et al.* [11] found only 28 and 21 overlapping genes respectively, which means that less than 5% of the rhythmic transcripts are cycling in both organs (Fig. 2). Another study comparing the liver and heart transcriptomes ended up with a similar figure, yet the mapping of the genes to the hierarchical gene ontology of biological processes showed that both sets of genes were involved in similar functions [10]. The unexpected degree of tissue-specificity suggests that every gene in the genome could be under circadian regulation in one organ/cell type or another.

Among the few transcripts that are cycling in both the SCN and the liver (21–28 genes) or in both the heart and the liver (37 genes), *Rev-erbα* and *Rev-erbβ* appear as novel ubiquitously expressed clock-controlled genes. These two genes encode orphan nuclear receptors that behave as transcriptional repressors [17]. The Rev-erbα receptor was recently shown to be essential for the control of period length and phase shifting [18]. These two nuclear receptors, together with two other transcription factors, albumin D-box binding protein (DBP) and E4BP4, could constitute a small group of 'molecular outputs' used by the circadian pacemaker in the SCN and peripheral organs to control

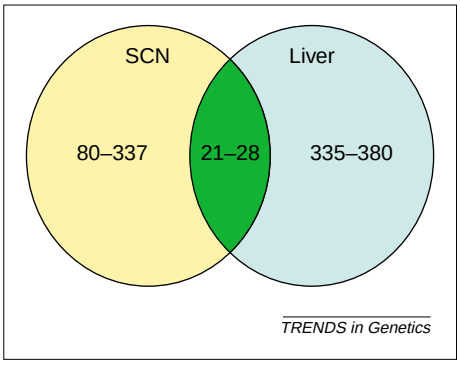

Fig. 2. Tissue specficity of circadian regulation in mammals. Although a significant number of genes are regulated by the circadian clock in the suprachiasmatic nuclei (SCN) and liver, very few rhythmic transcripts are identified in both tissues [9,11].

downstream targets [4]. Interestingly, most clock-controlled genes lack the E-box response elements that are required for a direct regulation by the circadian pacemaker, suggesting that a major part of the circadian control is indirect and that circadian output pathways involve gene expression cascades in which one can find transcription factors such as Rev-erbα and Rev-erbβ or DBP as immediate circadian pacemaker targets [9]. A survey of the sets of genes identified in the different studies indicates that at least 50 different transcriptional regulators are under circadian regulation. A bioinformatic search has recently identified response elements for some of these factors (CREB, DBP, REVERB/ROR) in a number of clock controlled genes [11].

Persisting problems with microarray experiments

Statistical variation (noise) is a common problem in studies dealing with large datasets such as those generated by microarrays experiments. Although the authors of the above-mentioned studies were aware that circadian profiles could be detected by chance in large number of time series, the fact that several studies analysing the liver (or the SCN) identified very different sets of genes using very similar or nearly identical protocols suggests that further optimization of this type of experiment is needed. For instance, ~90 genes were identified in the liver by both Panda *et al.* [9] and Storch *et al.* [10]. In the SCN, Panda *et al.* [9] and Ueda *et al.* [11] identified 337 and 80 cycling transcripts, respectively. Of course, slightly different experimental settings, the use of different version of the HDO microarrays, data filtering and analysis procedures can contribute to such differences, but this will hardly explain why the vast majority of the transcripts identified by one group were not identified by another. In fact this clearly suggests that expression of a significant number of these genes is affected by factors other than time. Certainly, very few clock-controlled genes are strictly regulated by the circadian clock; the vast majority are probably under the control of multiple pathways. Each output gene might thus integrate circadian regulation into the particular physiological and/or metabolic context of the cell in which it is expressed. Then because many genes that are under circadian regulation cycle with a low amplitude (peak/through ratio <2) [10,12], their identification is likely to be uncertain and influenced by variations in factors such as feeding, animal housing and stress. For instance, Kita *et al.* found that liver circadian expression of only 191 out of 597 genes was not affected by the feeding status [13]. In addition, one cannot exclude that part of the noise in these experiments is caused by factors related to the processing of the samples.

The design of most published studies using microarrays only allows the analysis of the effect of one factor (e.g. time in circadian expression experiments). Many authors using HDO microarray replicate the hybridization step, yet the good reproducibility of this type of array make this caution less critical than, for instance, replicating the cDNA synthesis because the efficiency of the reverse transcription step can vary significantly with some mRNA. Ideally, more-complex experimental designs should be used to estimate the contribution of factors other than to time in the total variation of the whole dataset, using classical statistical techniques such as analysis of variance (ANOVA). In addition, a more reliable identification of low amplitude transcripts probably would require improved data analysis protocols.

DNA microarrays have revealed an unexpectedly large and diverse collection of tissue-specific clock-controlled genes. A comprehensive understanding of the role of these hundreds of genes in the mammalian circadian system will certainly challenge chronobiologists for some time.

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Reinforcing the idea of signalling in the stomatal pathway

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Stomata are plant epidermal structures that regulate gas exchange with the atmosphere. In *Arabidopsis*, mutations in *TOO MANY MOUTHS* (*TMM*) disrupt a range of processes related to stomatal development and patterning. Recently, the sequence and the expression pattern of *TMM* were reported. *TMM* encodes a leucine-rich-repeat-containing, receptor-like protein that lacks a cytoplasmic kinase domain and that is expressed in postprotodermal cells. Several lines of evidence suggest that TMM and STOMATAL DENSITY AND DISTRIBUTION1, a putative subtilisin-like serine protease, might act in the same signalling pathway.

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Stomata are plant epidermal structures that control essential functions such as the uptake of CO_2 for photosynthesis and the loss of water vapour during transpiration. They consist of two guard cells (GCs) that delimit a pore (Fig. 1). Changes in the shape of the GCs, in response to turgor pressure, control the pore opening and closure, and hence gas exchange between the plant and the atmosphere. Gas exchange also depends on the spacing of stomata. As a general rule for all plant species, stomata never develop next to one another, but instead they are separated by a number of non-stomatal cells. A recent study has shed light on how the spacing of stomata is regulated in *Arabidopsis* [1].

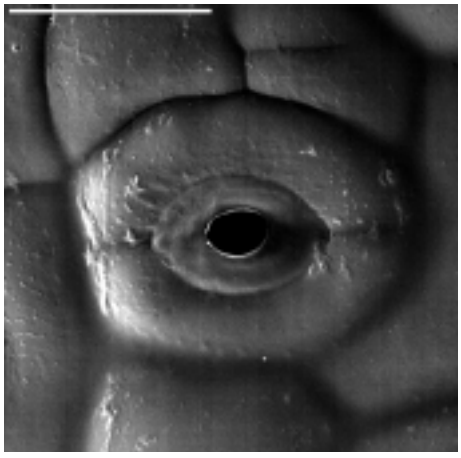

Fig. 1. CRIO-SEM of a stoma in *Arabidopsis*. Two guard cells delimit the stomatal aperture. Controlling the size of stomatal pore regulates gas exchange between the plant and the atmosphere. Scale bar: 10 μm.

In *Arabidopsis* leaf and cotyledon, stomatal development starts with an unequal cell division of a protodermal cell called a meristemoid mother cell (MMC) [2] (Fig. 2a). This cell division produces a small and triangle-shaped meristemoid and a neighbouring cell. These meristemoids are self-renewing cells, giving rise to new meristemoids. However, after a number of unequal cell divisions (from zero to three divisions), meristemoids lose their stem cell fate to become a rounded cell called a guard mother cell (GMC). The GMC undergoes a final, equal cell division that gives rise to the two GCs that form the stoma. These cell divisions in the stomatal pathway are precisely orientated in a spiral pattern that tends to place the stoma in the centre of the resulting multicellular complex (Fig. 2a).

The acquisition of MMC identity is prevented in cells that contact two stomata or meristemoids (Fig. 2b, top). However, many other epidermal cells can assume an MMC identity [2]. When MMC identity is assumed by a cell that makes contact with a stoma (or meristemoid), the MMC division is orientated so that the new meristemoid forms away from the

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