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Molecular clocks (joint Juan March/EMBO workshop) Madrid, May 10–12, 1999

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Introduction

Circadian rhythms in the physiology of plants and animals constitute key adaptations to survive day–night changes in the environment. In all species, an endogenous circadian clock directs these rhythms. A distinctive property of the clock is that it continues to function even under constant environmental conditions to generate an ~24 h (circadian) rhythm. The rhythm is synchronized (entrained) with the external day–night cycle by environmental signals, the most important of which is light. The clock seems to consist of three functional components: (i) an autonomous circadian pacemaker that generates the oscillation; (ii) an input pathway that synchronizes the pacemaker with the day–night cycle; and (iii) an output pathway whereby the pacemaker generates physiological rhythms.

During the past decade considerable effort has been made to identify clock components. The most successful approach has been based on mutant screens using *Arabidopsis*, cyanobacteria, *Drosophila*, *Neurospora* and, more recently, mice. A number of mutants have now been isolated and characterized in which clock outputs show altered timing. Genetic analysis has ultimately led to the cloning of genes involved in the function of the central pacemaker. It has become increasingly clear that the basic properties and organization of clock components are highly conserved even between these divergent species. Most of the clock genes identified encode transcription factors, which positively or negatively regulate their own transcription and thus constitute autoregulatory feedback loops. Additional clock components have been shown to regulate the intracellular localization, stability and transactivation potential of these transcriptional regulators.

The last 3 years have seen an avalanche of publications revealing new features of the circadian clock in the different model systems. For this reason, the ‘Molecular clocks’ meeting organized by the Juan March Foundation in Madrid, Spain, was particularly timely. It brought together many of the major players in the field and sought to discuss the common themes that are emerging from different avenues of clock research. The meeting specifically centered on a number of issues: (i) the way that light is perceived by and affects the function of the circadian clock; (ii) the first protein expression data for the various

vertebrate clock genes and clues as to how these genes are organized into regulatory networks; (iii) new insights into the function of the mammalian pineal gland; (iv) the emerging field of clock functions in peripheral tissues; and (v) new information on the components of the *Drosophila* circadian clock—one of the most extensively studied clock systems.

The effect of light

This section addressed the important issue of how light regulates the circadian clock and also seasonal variations in reproductive capacity in rodents. It is well established that enucleated rodents fail to entrain to phase shifting pulses of light, an observation that clearly points to the retina as being the principal site of photoreception for the circadian clock. The long-term goal of Russell Foster (London, UK) has been to identify which cells in the retina mediate this response and also to characterize the photopigment molecules that are involved. He has shown that circadian photoreception is unlikely to be a rod or cone function since photoentrainment by light, as well as the acute suppression of pineal gland melatonin synthesis, is still observed in transgenic mice that have specific and complete degeneration of rod and cone photoreceptors. Currently, the strongest candidates to be circadian photoreceptor cells appear to be retinal ganglion or amacrine cells. Foster also proposed several functional criteria that should be satisfied by proteins if they are involved in circadian photoreception. Namely, they should be a functional photopigment, they should be expressed within amacrine or ganglion cells within the retina, and their absorption spectra should match the action spectra measured with the rodless/coneless mice. Furthermore, mice bearing a targeted mutation of the circadian photopigment should have free-running circadian rhythms. This is an important issue given the recent results addressing the role of the cryptochrome genes *cry1* and *cry2* in the circadian clock (Griffin *et al.*, 1999; Kume *et al.*, 1999; Vitaterna *et al.*, 1999). Cryptochromes are members of the family of plant blue-light receptors and photolyases, and have now been identified in mouse and *Drosophila*. The phenotypes of mice bearing targeted mutations in the two *cry* genes were presented by Jan Hoeijmakers (Rotterdam, The Netherlands). *Cry1* mutant mice show accelerated free-running periodicity in locomotor activity, while *Cry2* mutants show a delay in this periodicity. However, *Cry1/Cry2* double mutants are completely arrhythmic, indicating that CRY proteins are in fact essential components of the endogenous pacemaker. Foster questioned the significance of the mammalian CRY proteins in circadian photoreception, since on many counts

they do not satisfy the criteria for the mouse circadian photopigment. A cryptochrome homolog has also been identified in *Drosophila* and was presented by Michael Rosbash (Waltham, MA). In this case, however, transgenesis and studies in cultured cells seem to confirm a role for CRY in *Drosophila* circadian photoreception (Ceriani *et al.*, 1999). Interestingly, the *cry* gene has also been isolated as part of a random screen for *Drosophila* clock mutants. R.Stanewsky (Resensburg, Germany) performed a mutagenic screen using *period* promoter–luciferase reporter transgenic flies in which he identified a *cry* mutant by virtue of its lack of oscillation in *period* gene expression. Finally, Michael Menaker (Charlottesville, VA) presented new data addressing the regulation of reproductive function in response to day length. He proposed that the retinal photoreceptors that mediate photoentrainment of the clock are not necessarily those responsible for the reproductive response to light. This comes from studies where bright, white light was used to damage the retina of albino hamsters. In these animals, there was a normal photoentrainment to light–dark cycles, but the reproductive response was that associated with animals maintained under constant darkness.

Vertebrate clockworks

The first clock gene to be identified in a vertebrate as the result of a genetic screen was the *Clock* gene, identified by Joe Takahashi (Evanston, IL). This transcriptional regulator functions as a heterodimer with BMAL1. A feature shared by both these transcription factors is the PAS domain, a structural motif involved in protein–protein interactions. The PAS domain takes its name from the first three members of the PAS domain family: *Drosophila* PER gene, mammalian Arnt, and Sim, the product of the fly *single-minded* gene. Both CLOCK and BMAL1 also incorporate a basic helix–loop–helix (bHLH) domain, which has been implicated in DNA binding as well as mediating dimerization. Homologs of *Clock* and BMAL1 have also been identified in *Drosophila*, and furthermore one *Neurospora* clock gene, WC (white collar) 1, was reported by Jay C.Dunlap (Hanover, NH) to display sequence similarity with the mammalian BMAL1 protein. Paolo Sassone-Corsi and Nicholas S.Foulkes (Strasbourg, France) reported two BMAL homologs with different transcriptional activation function as well as a *Clock* gene homolog in the zebrafish (Cermakian *et al.*, 2000). In contrast to the non-oscillating expression of mouse *Clock*, zebrafish *Clock* mRNA shows a strong circadian rhythm of expression in many tissues.

Mammalian homologs of the *Drosophila period* and *timeless* genes have recently received a lot of attention. To date, three homologs of *period* (*mper1*, *mper2* and *mper3*) and one homolog of *timeless* (*mtim*) have been identified in the mouse. Remarkably, the basic function of the mammalian *per* and *tim* genes shows many similarities with that of their fly counterparts (Zheng *et al.*, 1999). Thus, for example, Marina Antoch (Evanston, IL) reported that CLOCK is a positive regulator of *mper1* expression, as determined by examining the expression of *mper* genes in the *Clock* mutant mice. C.Weitz (Boston, MA) also presented *in vitro* data demonstrating that the *mper1* gene promoter is upregulated by CLOCK and BMAL. He also

showed the results of a yeast two-hybrid analysis consistent with a physical interaction between mPER and mTIM proteins.

The recent availability of specific antibodies for mammalian PER and TIM should now provide us with important additional insight into the function of these proteins. Their ability to form a complex and thereby change their subcellular localization is a key facet of their function in *Drosophila*. Several presentations included new protein data obtained using these antibodies. Steven Reppert (Boston, MA) has studied the intracellular localization of the mPER1, 2 and 3 proteins in transfected COS cells. While mPER1 and 2 seem to be distributed between cytoplasm and nucleus, mTIM was predominantly nuclear and mPER3 predominantly cytoplasmic. He presented evidence that the mPERs can heterodimerize in certain combinations and thereby change their subcellular localization. However, he did not visualize the direct mPER:mTIM interaction predicted by C.Weitz. Michael Hastings (Cambridge, UK) described the patterns of PERs and TIM protein expression during the circadian cycle in the suprachiasmatic nucleus (SCN), the site of the central circadian pacemaker in mouse (Hastings *et al.*, 1999). His immunohistochemical analysis revealed that the mPER and mTIM proteins were predominantly localized in the nuclei during the entire circadian cycle. Also, while the levels of mPER protein altered as predicted from the well documented changes in mRNA expression, mTIM protein levels did not fluctuate.

The pineal gland

The pineal gland is the site of synthesis of the hormone melatonin. It is light sensitive in lower vertebrates and has been termed the ‘third eye’; however, during mammalian evolution it has apparently lost this photoreceptive function. New results presented by Michael Menaker show that neonatal rat pineal cells maintained in culture do appear to be light responsive in terms of acute light suppression of melatonin synthesis. His findings also suggest that establishment of functional sympathetic innervation is at least in part responsible for the loss of photosensitivity *in vivo*. Solomon H.Snyder (Baltimore, MD) has been identifying genes that are differentially expressed day and night in the rat pineal gland using a subtractive PCR-based method. By this approach, his group has already demonstrated that mRNAs encoding serotonin *N*-acetyl transferase (AANAT) and PINA (an alternatively spliced transcript of the Wilson’s disease gene) are strongly upregulated during the night in the rat pineal gland. Most recently, they have revealed that expression of Patched-1 (PTC-1) is also strongly increased during the night in the pineal gland (Borjigin *et al.*, 1999). PTC-1 is a tumor suppressor homolog of the *Drosophila* segment polarity gene *patched* that acts as a receptor for *hedgehog* during embryonic development. The functional significance of this observation is still unclear, especially since they failed to detect *hedgehog* mRNA expression in this system (Borjigin *et al.*, 1999). Finally, José Naranjo (Madrid, Spain) presented data showing that expression of DREAM, a novel calcium-binding transcription factor that mediates transcriptional regulation directly in response

to calcium levels, also oscillates with a day–night rhythm in the pineal gland.

Clocks at the periphery

An exciting new development in chronobiology has been the discovery in both invertebrates and vertebrates of circadian oscillators in many different peripheral tissues, previously not implicated as possessing a circadian pacemaker function. How they are regulated and their functional significance are key questions, which are now being addressed in a number of different systems. The zebrafish *Clock* and BMAL gene homologs were reported to show robust rhythms of expression in a range of different tissues (Paolo Sassone-Corsi and Nicholas S.Foulkes, Strasbourg, France). The oscillation of *Clock* gene expression in the zebrafish continues in *ex vivo* organ cultures of the heart and kidney, and furthermore this rhythm appears to be directly light entrainable (Whitmore *et al.*, 2000). This is very similar to the situation in *Drosophila* where multiple, photoentrainable, peripheral oscillators have already been demonstrated by the analysis of *period*–luciferase transgenic flies. The functional significance of these fly peripheral clocks has been investigated further by Paul Hardin (Houston, TX). He has been able to show that a circadian clock in the fly's antenna may be responsible for determining the circadian response of electrical activity induced by odorant stimuli (Krishnan *et al.*, 1999).

The work of Ueli Schibler (Geneva, Switzerland) has extended the peripheral clock observation further by showing that circadian oscillations in gene expression can even be induced in immortalized fibroblast cell lines by differential serum treatment. Furthermore, the serum-induced rhythms of gene expression can also be induced in response to the activation of various intracellular signaling pathways. The implication of these findings is that all cells may contain the regulatory pathways that underlie the function of the circadian clock. Schibler's results should therefore provide important insight into the regulation of peripheral clocks. In addition, the potential to study clock regulatory mechanisms in cell lines provides a powerful tool to study ultimately circadian clock function *in vitro*.

Missing pieces in the clock puzzle

A fundamental limitation of the current approach of screening for clock mutants in *Drosophila* is that mutations affecting the clock that also confer lethality will not be detected. With this in mind, instead of screening for mutants, Michael Young (New York, NY) has used a differential display PCR approach to isolate *Drosophila* cDNAs that are differentially expressed day and night and that may therefore also constitute clock components. One of the clones they have characterized is *vri*, a transcription factor previously identified by virtue of its function during development. The protein VRI has homology with DBP, a transcription factor that oscillates in the liver and SCN. The expression of *vri* is synchronous with that of *per* and *tim*, and is low in *Clock* mutant flies. This suggests that *vri* is regulated by and may even be a part of the transcriptional regulatory loops formed by the other clock proteins.

Overall perspectives

This meeting in Madrid served to reinforce the idea that circadian clocks in species as diverse as *Neurospora*, *Drosophila* and mice are really very similar at many different levels. This is good news for clock researchers since conclusions made using any one of these model systems may well be relevant to our understanding of the circadian clock in other systems. These basic similarities in circadian clock organization are also consistent with the idea that the clock evolved extremely early during evolution. Its subsequent conservation may well be a testimony to its importance for all living things. With luck, the first years of this millennium will bring us closer finally to understanding the significance and workings of the circadian clock.

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