Circadian timekeeping: Loops and layers of transcriptional control

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The organization of biological activities into precise daily cycles is nearly universal, present in forms as diverse as cyanobacteria, fungi, plants, and animals. As was first shown in 1729 by de Mairan for daily leaf movements in a plant (1), such rhythms are generated internally, persisting in constant environmental conditions with a period close to one day. The underlying oscillatory machinery is referred to as a circadian clock, “circadian” meaning “approximately one day.” Circadian clocks throughout phylogeny share fundamental properties (2), the two most important being temperature compensation, without which clock accuracy would suffer at the hands of fluctuations in ambient temperature, even in homeotherms (3), and entrainment to light–dark cycles, the process by which input from photoreceptors keeps circadian clocks set precisely to local time. By virtue of these fundamental properties, circadian clocks measure both elapsed time and local time, allowing temporal control over physiological processes and anticipation of daily environmental challenges and opportunities. It is likely that this predictive power has been the evolutionary driving force responsible for the ubiquity of circadian clocks (4).

In recent years, this fascinating and profound problem of biological timekeeping has begun to yield its secrets at a quickening pace, progress in large part propelled by the cloning and characterization of the first three circadian clock genes, period (per) (5, 6) and timeless (tim) (7–9) from Drosophila and frequency (frq) (10, 11) from the fungus Neurospora (reviewed in ref. 12). As yet no clock gene has been cloned from any vertebrate, though the identification of hamster (13) and mouse (14) circadian clock mutants indicates that genetic dissection of vertebrate circadian clocks is possible. At present it is unclear how the treasure trove of knowledge about the anatomy, physiology, and formal properties of vertebrate circadian clocks relates to the emerging molecular picture of the Drosophila and Neurospora clocks, described below. On the basis of circumstantial evidence, it is widely expected that at least the general principles will be conserved. In a paper presented in this issue, Green and Besharse (15) have boldly pursued this expectation in a screen for clock-controlled genes in the Xenopus retina, within which is a well-characterized circadian clock. Their identification of a novel clock-controlled gene, encoding the putative transcriptional regulator nocturnin, opens up a promising new line of molecular attack on the vertebrate circadian clock.

Striking similarities in the functions (but not sequences) of the cloned Drosophila and Neurospora clock genes point to a general mechanism for circadian clocks, summarized in the following simplified scheme. The core of the clock is at least in part constituted by a negative-feedback loop of clock gene transcription. During the phase of the cycle when clock genes are active, clock gene transcripts accumulate and, after a lag of 4–8 hr, the clock proteins accumulate in the cytoplasm and are subsequently transported to the cell nucleus. [In Drosophila, the period and timeless proteins are required to form a heterodimeric complex to gain admission to the nucleus (8, 16), functionally coupling the two clock genes into a common feedback loop.] Once within the nucleus, the clock proteins directly or indirectly repress transcription of their own genes, resulting in a rapid decline in the levels of the short-lived clock gene transcripts. After an interval of 12 hr or so, the clock proteins in the nucleus are degraded, leading to derepression of the clock genes, renewed accumulation of their transcripts and protein products, and so on, with a period of 24 hr. A key feature of the clock as a transcriptional feedback loop is that both the mRNA and protein products of the core clock genes exhibit a circadian oscillation in abundance, with mRNA and protein peaks offset. Clock resetting is likely accomplished by a rapid alteration in one or more of the cycling components acting within the feedback loop: exposure to light leads to the rapid induction of the frequency transcript in Neurospora (17) and to the rapid disappearance of immunoreactive timeless protein, presumably reflecting proteolysis, in Drosophila (18–21). In each case the effect of light on the cycling clock molecule is sufficient, in principle (22), to account for entrainment to light–dark cycles.

How do circadian clock gene expression rhythms fall into a handful of stereotyped
phase and amplitude patterns (33). (As consummate specialists in photosynthesis, cyanobacteria have hitched their fate to the light-dark cycle, so in retrospect global control of cyanobacterial physiology by a circadian clock is not so surprising.) Further studies showed that one particular rhythmic pattern common to a subset of genes could be ascribed to the activity of one type of sigma transcription factor (34). Thus if each of several different sigma factors were controlled by the circadian clock with a different rhythmic pattern, each sigma factor could then confer its exclusive rhythmic pattern on the expression of a large number of specific target genes (34). This example illustrates what might turn out to be a central principle in circadian timekeeping—by placing a circadian clock at the top of a hierarchy of gene expression, a variety of timing signals can be conveyed differentially to defined banks of genes.

No doubt with transcriptional loops and hierarchies in mind, Green and Besharse (15) set out to identify genes that exhibit a circadian rhythm of expression within the *Xenopus* retina, using for their experiments cultured *Xenopus* eye cups. This preparation has several advantages—the clock within it functions for extended periods in culture and can be entrained to light–dark cycles; during the course of experiments clock function can be verified by monitoring a known output, the rhythmic release of melatonin; and the clock has been localized to specific identifiable cells, photoreceptors, not yet the case for other vertebrate clocks.

Starting with cultured eye cups maintained on a light–dark cycle, they removed retinas every 6 hr for 2 days. Using RNA extracted from the retinas, they performed differential display PCR to screen large numbers of transcripts for a daily pattern in expression. One rhythmic transcript was identified that proved to be specific to photoreceptors. Its abundance was 20-fold higher at night than during the day, and its rhythm of expression persisted, albeit with decreased amplitude, in eye cups kept in constant darkness, proving that the rhythm was under the control of an endogenous clock. Nuclear run-on experiments demonstrated a robust circadian rhythm of transcriptional initiation in constant darkness, proving that clock control was transcriptional. Because of its nighttime expression, the predicted protein product has been named “nocturnin.” Interestingly, the deduced nocturnin amino acid sequence shows significant homology to CCR4, a non-DNA-binding transcriptional coactivator in yeast, and it contains a sequence shows significant homology to CCR4, a non-DNA-binding transcriptional coactivator in yeast, and it contains a motif at its N terminus similar to a leucine zipper dimerization domain, features which suggest a transcriptional regulatory function. Nocturnin is apparently a member of a conserved family of vertebrate proteins, since several different but highly related human sequences were identified in a search of the expressed sequence tag data base.

Green and Besharse (15) make a strong case that the novel gene they have discovered is a clock-controlled gene expressed in an identified vertebrate circadian clock cell. Working backward to determine the proximate cause of nocturnin gene transcriptional rhythms will provide a challenging but exciting opportunity, unique at present, to follow an intracellular pathway into a vertebrate clock. As a potential transcription factor, it is plausible that nocturnin is deployed in a clock-controlled hierarchy of gene expression, especially since specialized processes in photoreceptors, such as disc shedding and synthesis of phototransduction components, are known to be under clock control. It is also plausible that nocturnin acts within the transcriptional feedback loop related to the clock. These two tenets can be distinguished experimentally, but it will require identification of multiple independent outputs of the clock and an efficient means of transfecting *Xenopus* photoreceptors. For example, if nocturnin acts within the loop, then expressing it constitutively should disrupt or alter all clock functions, whereas if it acts on an output pathway, then at least some clock functions should remain unaffected. Identification of a mouse homolog would greatly facilitate the analysis. Could nocturnin be the first vertebrate clock component to be identified? Time will tell if nocturnin tells time.