PERSPECTIVES: CIRCADIAN RHYTHMS

Chronobiology—Reducing Time

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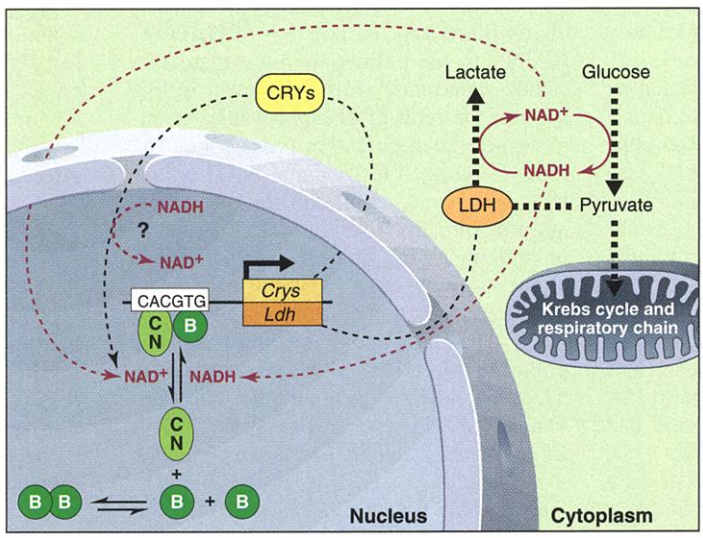
In mammals, physiology and behavior are subject to daily oscillations that are driven by an endogenous clock. The master clock (circadian pacemaker) resides in the suprachiasmatic nucleus (SCN) of the brain's hypothalamus. In the absence of external time cues, the SCN master clock generates cycles of approximately but not exactly 24 hours, and its phase must therefore be readjusted every day. This task depends on the retina, which detects changes in light intensity during the day's light-dark cycle (the photoperiod) and transmits this information to the SCN neurons.

Although the SCN is essential for circadian rhythmicity, circadian oscillators composed of many of the same proteins as the master clock seem to operate in most cells of the body. Under normal conditions, the SCN pacemaker synchronizes these peripheral clocks through neuronal and humoral signals. Intriguingly, if feeding is restricted in mammals, the phase of the oscillators in liver, kidney, heart, and pancreas becomes completely uncoupled from the phase of the SCN timekeeper. These findings clearly point toward an intricate interplay between metabolism and the circadian timing system (*1*). Two papers from the McKnight group, on pages 506 (*2*) and 510 (*3*) of this issue, report a fascinating discovery that may provide an unexpected molecular link between circadian oscillations and energy homeostasis.

Rutter *et al.* (*3*) reveal that the binding of two highly related master clock proteins, the transcription factors NPAS2 and Clock, to their DNA recognition sequences depends on the ratio of oxidized to reduced nicotinamide adenine dinucleotide (NAD and NADH, respectively). These molecules are essential components of the respiratory enzyme chain, and the ratio between them fluctuates according to changes in cellular metabolism.

Circadian clocks are thought to operate through interlocking feedback loops of gene expression (see the figure). The first mammalian clock gene identified, dubbed *Clock*, was isolated by positional cloning in a mutant mouse with altered circadian locomotor activity (*4*). Clock is a basic helix-loop-helix (bHLH) transcription factor that binds to E-box (CACGTG) DNA motifs when partnered with BMAL1, another bHLH protein required for circadian clock activity (*5*). The Clock:BMAL1 heterodimer stimulates the expression of other essential pacemaker components, such as the period proteins PER1 and PER2 and the cryptochromes CRY1 and CRY2. The CRYs repress the transcription of target genes switched on by Clock:BMAL1, and thereby establish a negative feedback loop in which *Per* and *Cry* gene expression switches off Clock:BMAL1 transcriptional activity (*6*). Cryptochromes carry a flavin adenine dinucleotide (FAD) cofactor and serve as circadian photoreceptors in plants and the fruit fly *Drosophila* (*7, 8*).



Metabolic states and circadian oscillators. The mammalian circadian feedback loop in gene expression is established by the BMAL1 (B) and Clock (C) transcriptional activator proteins and by the CRY transcriptional repressor proteins. In some brain regions, NPAS2 (N) substitutes for Clock. BMAL1 can bind to Clock or NPAS2, and these heterodimers activate transcription. Alternatively, BMAL1 can form homodimers with itself that do not activate transcription. The formation of the Clock:BMAL1 and NPAS2:BMAL1 heterodimers and their binding to DNA is stimulated by reduced NADH and inhibited by oxidized NAD. These heterodimers enhance the expression of the clock genes *Cry* and *Per* (not shown) and the clock output gene *Ldh*. CRY proteins repress Clock:NPAS2–mediated gene activation, possibly by oxidizing the NAD^+ cofactors associated with these proteins. Conceivably, the negative action of CRY proteins on Clock-NPAS2 could be reinforced by lactate dehydrogenase (LDH), which may increase the cellular concentration of NAD^+. (Only unphosphorylated NAD electron carriers are shown.)

NPAS2, a close relative of Clock, is expressed in several nuclei of the mouse forebrain. Like Clock, it binds to DNA as a heterodimer with BMAL1 (*9*). However, in contrast to Clock, NPAS2 is not expressed in the SCN. Hence, at first glance, it is not clear what part NPAS2 might play in the circadian timing system (*10*). To address this question, Reick *et al.* (*2*) screened a neuroblastoma cell line for target genes of the NPAS2:BMAL1 transcription complex. They identifed mRNA transcripts for several essential clock components, including PER1, PER2, CRY1, and BMAL1. Interestingly, the phase of *BMAL1* mRNA oscillation in mouse brains is virtually the opposite of that for *Per1*, *Per2*, and *Cry1* mRNAs. In keeping with these findings, NPAS2:BMAL1 heterodimers stimulate *Per1*, *Per2*, and *Cry1* gene expression, whereas they repress transcription of the endogenous *BMAL1* gene, thereby providing an explanation for the phase differences between these genes. Furthermore, in the forebrains of *NPAS2*-deficient mice, rhythmic *Per2* gene expression is abolished. Hence, NPAS2 has taken over the job of Clock in the circadian oscillators of these brain structures.

As Rutter *et al.* report (*3*), the screen for genes regulated by NPAS2:BMAL1 also identified the *LdhA* gene as a direct target of this transcription factor complex. *LdhA* encodes the A isoform of lactate dehydrogenase (LDH), an enzyme that catalyzes the reduction of pyruvate to lactate during anaerobic respiration. Under aerobic conditions, however, the bulk of pyruvate (which is the end product of glycolysis) has another destiny: It is taken up by mitochondria and enters the Krebs cycle (see the figure).

So how could McKnight's group get excited about a side reaction that has bored biochemistry students for decades? It is the NAD electron carriers participating in glycolysis and pyruvate reduction that captured the attention of these researchers. A combination of clever guesswork and great intuition prompted Rutter *et al.* (*3*) to examine the influence of NADH and NAD

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cofactors on the DNA binding of NPAS2 and its close relative Clock. When bound to BMAL1, both of these proteins recognize E-box motifs in DNA recognition sequences, but BMAL1 can also bind to DNA on its own (that is, as a homodimer). The authors used purified recombinant proteins and either unphosphorylated cofactors (NAD^+ and its reduced form NADH) or phosphorylated cofactors (NADPH and its reduced form $NADP^+$) at physiological concentrations. They report that reduced and oxidized NAD(P) electron carriers have inverse effects on the proportion of NPAS2:BMAL1 (or Clock:BMAL1) heterodimers to BMAL1:BMAL1 homodimers that bind to DNA. Although the heterodimeric interaction is greatly stimulated by NAD(P)H, it is strongly inhibited by $NAD(P)^+$. As a consequence, only BMAL1 homodimers (whose affinity for DNA is not affected by NAD cofactors) occupy E-box motifs at a low $NAD(P)H/NAD(P)^+$ ratio. Because BMAL1 homodimers are incapable of activating transcription, the susceptibility of the heterodimer to this redox potential establishes a molecular switch for activating the Clock:BMAL1 transcription complex.

Redox electron transfer through NADH and NAD may also provide an elegant way for cryptochromes to inhibit the activity of Clock and NPAS2. Preliminary experiments from the McKnight laboratory suggest that NPAS2 also binds to a heme cofactor (*11*). Conceivably, the interaction of CRYs with NPAS2 provokes electron transfer from NPAS2-associated NAD(P)H to CRY-associated FAD, and finally to the heme cofactor bound by NPAS2. This electron shuttle would convert NAD(P)H into $NAD(P)^+$ while conserving the redox state of CRY-associated FAD. The standard reduction potentials of NAD, FAD, and heme (a measure of the ease with which a molecule can be converted to its reduced form) are in perfect alignment with such a scheme. Depending on the stability of the change, binding of NPAS2 to DNA might be abolished.

The unexpected relationship between circadian clock proteins and redox potential adds a new wrinkle to chronobiology research. If the NAD interaction domains of the Clock and NPAS2 proteins can be narrowed down to a minimal peptide sequence, it may become possible to engineer proteins that cannot bind to NAD(P)H cofactors. The activity of such proteins in cells and intact animals then could be investigated, hopefully providing validation for the clock-redox connection observed in the test tube and enabling fundamental questions to be addressed. For example, is redox potential involved in light- or food-induced phase shifting, and in the operation of the circadian oscillator? The balance between reduced and oxidized NAD cofactors in the cell cytoplasm depends on the intracellular concentrations of fuels (such as glucose), oxygen, and LDH, and this balance is itself subject to daily oscillations (*12, 13*). It is also noteworthy that the NPAS2:BMAL1 heterodimer has as a potential target LDH, an enzyme that influences cellular redox potential and thereby changes the activity of its activator. Such a negative feedback loop could contribute to circadian clock activity, promoting LDH from a routine metabolic enzyme to an integral clock component.

Some years ago, it was proposed that NAD cofactors formed the gears of the circadian clock of *Euglena gracilis* (*14*). In this unicellular phytoflagellate, NAD^+ levels oscillate, and the addition of NAD^+ or $NADP^+$ to the culture medium results in steady-state phase shifts. However, in this system, the ratio of phosphorylated to unphosphorylated cofactors (rather than the ratio of reduced to oxidized NAD cofactors) is believed to participate in the generation of circadian oscillations. It is worth mentioning in this context that NADPH is about three times as efficient as NADH in stimulating the binding of NPAS2 to DNA (although phosphorylated and unphosphorylated NAD electron carriers have the same redox potential). The relevance of these observations to Clock:BMAL1 activity and to general clock activity can also be tested, at least in cultured cells.

The unexpected connection between NAD balance and Clock protein activity discovered by McKnight and colleagues could revolutionize our notions of circadian oscillators and circadian phase signaling to peripheral clocks. It will be fascinating to study the relationship between cellular redox states and circadian timing through future experiments in vivo.

References

1. J. A. Ripperger, U. Schibler, *Curr. Opin. Cell Biol.* **13**, 357 (2001).
2. M. Reick, J. A. Garcia, C. Dudley, S. L. McKnight, *Science* **293**, 506 (2001); published online 5 July 2001 (10.1126/science.1060699).
3. J. Rutter, M. Reick, L. C. Wu, S. L. McKnight, *Science* **293**, 510 (2001); 5 July 2001 (10.1126/science.1060698).
4. D. P. King *et al.*, *Cell* **89**, 641 (1997).
5. M. K. Bunger *et al.*, *Cell* **103**, 1009 (2000).
6. K. Kume *et al.*, *Cell* **98**, 193 (1999).
7. P. F. Devlin, S. A. Kay, *Plant Cell* **12**, 2499 (2000).
8. P. Emery, R. Stanewsky, J. C. Hall, M. Rosbash, *Nature* **404**, 456 (2000).
9. J. A. Garcia *et al.*, *Science* **288**, 2226 (2000).
10. L. P. Shearman, M. J. Zylka, S. M. Reppert, D. R. Weaver, *Neuroscience* **89**, 387 (1999).
11. S. L. McKnight, personal communication.
12. J. L. Robinson, S. Foustock, M. Chanez, B. Bois-Joyeux, J. Peret, *J. Nutr.* **111**, 1711 (1981).
13. Y. G. Kaminsky, E. A. Kosenko, M. N. Kondrashova, *Int. J. Biochem.* **16**, 629 (1984).
14. K. Goto, D. L. Laval-Martin, L. N. Edmunds Jr., *Science* **228**, 1284 (1985).

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PERSPECTIVES: EVOLUTION

The Cambrian Explosion Exploded?

Richard Fortey

The beginning of the Cambrian period, some 545 million years ago, saw the sudden appearance in the fossil record of almost all the main types of animals (phyla) that still dominate the biota today. To be sure, there are fossils in older strata, but they are either very small (such as bacteria and algae), or their relationships to the living fauna are highly contentious, as is the case with the famous soft-bodied fossils from the late Precambrian Pound Quartzite, Ediacara, South Australia.

Consequently, it has been concluded that exceptional evolutionary activity over 10 million years or so at the base of the Cambrian generated ancestors of most of the living phyla and maybe many other "failed phyla" besides (*1*). Other paleontologists have questioned whether such rapid evolution is possible and have instead postulated a phylogenetic "fuse"—an extended period of evolutionary genesis that has left little or no fossil record (*2*). So just how explosive was the Cambrian evolutionary "explosion"?

Support for a phylogenetic fuse is provided by the discovery of a true crustacean in early Cambrian strata from Shropshire, England, reported by Siveter *et al.* on page 479 of this issue (*3*). This fossil phosphatocopid "ostracod" is preserved extraordinarily well, with all its delicate limbs cast in calcium phosphate, allowing it to be assigned to the crustaceans with confidence. Very few fossils of this great antiquity reveal so

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