BIORHYTHMICITY

New Clues Found to Circadian Clocks—Including Mammals'

If you have ever struggled out of bed the morning after flying eastward across several time zones, you've felt what happens when you try to buck the strong rhythms kept by your internal clock. Exactly how those daily, or circadian, rhythms are generated is a mystery under intense study. Until recently, clock researchers had only three clock components to go on: two proteins from fruit flies and one from a bread mold. Studies of those proteins had yielded a model of how those organisms' clocks may work, but the whole field eagerly awaited a glimpse into the circadian clock of a mammal, to see if our clocks have a similar mechanism. Now, that waiting has been rewarded.

In a pair of papers in today's issue of *Cell*, Joseph Takahashi and his colleagues at Northwestern University in Illinois report that they have cloned the first clock gene from mice. That discovery, combined with two new genes from the bread-mold clock—reported just 2 weeks ago in *Science* (2 May, p. 763) by a team led by Jennifer Loros and Jay Dunlap at Dartmouth Medical School—suggests that the mammalian clock indeed resembles those of simpler organisms. All three genes contain a feature known as a PAS domain, which is beginning to look like a common theme in clock proteins.

Besides providing what circadian-rhythm researcher Michael Rosbash of Brandeis University calls "a molecular link" between the mammalian clock and those of lower organisms, the trio of new genes also adds to the growing picture of how all these clocks may work. The three previously reported clock genes all code for proteins that oscillate in abundance, regulating their own expression by building up over a 24-hour period until they turn their genes off and start the cycle over. The new mouse and bread-mold genes may represent a new component equally essential to the clock mechanism: a protein that drives production of the oscillating proteins, in essence keeping the clock ticking.

The Loros team found that the genes that it is studying, known as *white collar-1* and *-2* (*wc-1* and *-2*), turn on the transcription of *frequency* (*frq*), which codes for an oscillating protein in the bread mold *Neurospora crassa*. The Takahashi team's gene, called *Clock*, also appears to be a transcriptional activator, although its target isn't known. "This was almost the best possible result we could have imagined," says Takahashi. The *Clock* gene "has all these motifs that allow you to figure out what class of protein it is."

Until now, research on mammalian clocks had gone slowly. The fruit-fly clock gene, *period* (*per*), was discovered more than 20 years ago, followed by *Neurospora's frq* in 1978 and the second fruit-fly clock gene, *timeless* (*tim*), in 1994. All of those genes had been cloned. But efforts to clone mammalian clock genes based on similarity to those genes had failed, spurring Takahashi to begin searching from scratch for clock genes in mice.

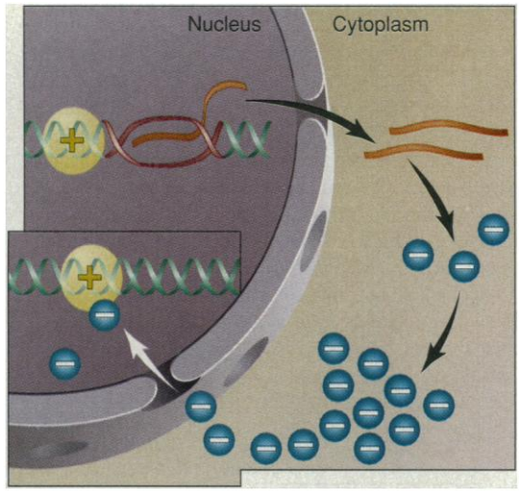


ILLUSTRATION: K. SUTLIFF

Winding the clock. Positive gene activators (+), such as WC-1, WC-2, and possibly CLOCK, turn on genes that make proteins (▭) needed for clock function. These proteins eventually move back to the nucleus and shut off their own genes.

His group, including Fred Turek and Lawrence Pinto, also at Northwestern, screened mice in which mutations had been chemically induced by William Dove at the University of Wisconsin, Madison. The researchers looked for animals that showed a disturbance in their daily patterns of running on their exercise wheels. When kept in total darkness, a mouse with a normal clock keeps a precise 23.7-hour cycle of alternate rest and running. Takahashi's team found a mutation that stretched that cycle to 25 hours in mice with one copy of the mutant gene, and to 27 to 28 hours in animals with two copies. Indeed, these latter animals completely lost their rhythmicity after 2 weeks in the dark, implying that a part in their internal clocks was broken.

Team members located the responsible gene using two different approaches. They mapped it to chromosome 5, and after narrowing its location by using genetic tricks, they found two genes in that region. Sequencing then revealed a mutation in one, suggesting that it was *Clock*. In an independent effort, the researchers introduced into the mutant mice progressively smaller pieces of DNA from the large region known to contain the gene—seeking the smallest piece that would correct the mutation and restore a normal rhythm.

Both approaches zeroed in on the same gene; when the team sequenced it, they found that it codes for a protein that has PAS domains—a motif first identified in PER, and subsequently found in WC-1 and WC-2. "PAS now appears to be a signature motif that is cropping up in clock genes," says clock researcher Steve Kay of the Scripps Research Institute in La Jolla, California.

But the strongest evidence that the CLOCK protein plays a central role in the mouse clock, says Harvard Medical School researcher Charles Weitz, is the Takahashi team's finding that loading up mice with extra copies of the normal gene speeded up their circadian rhythms. Based on that, "I am really positive about CLOCK being a central part of the oscillator," adds Kay. One of the hallmarks of a clock protein, he explains, is that "the more you build [it] up, the faster you go around the loop."

Clues as to how the CLOCK protein may take part in the oscillator come from its structure, which bears two hallmarks of proteins that regulate genes: a sequence known as a basic helix-loop-helix (bHLH), which enables a protein to pair up with another protein and bind to a gene, and a glutamine-rich domain specific to gene-activating proteins. The mutant form of the CLOCK protein is missing part of that glutamine-rich activating region, an indication that the region is essential to CLOCK's function.

The idea that CLOCK cooperates with other proteins to activate gene expression "fits nicely" with the group's previous genetic findings, says Weitz. Just one copy of the mutant form of *Clock* is enough to lengthen a mouse's daily rhythm—but the researchers found that if the copy is deleted, rather than mutated, the animal's clock runs normally. That means the mutant protein actually interferes with the normal protein's function. And the gene's structure suggests it may do so by competing with the normal protein for binding with its as-yet-unknown partner. A complex containing a mutant CLOCK protein might sit down on a gene but would not turn it on.

The idea that CLOCK regulates the expression of some other clock component also fits well with the general notion of how clocks seem to work, at least in *Drosophila* and *Neurospora*. PER, TIM, and FRQ are all proteins made in increasing amounts during the daily cycle, eventually reaching levels at

which they feed back to shut off their own genes. That causes the levels of the proteins to drop, and the genes to turn back on. This oscillator, controlled by rising and falling protein levels, could be how the clock keeps time, but it also needs the equivalent of a power source: a gene activator that drives the expression of genes such as *per*, *tim*, and *frq* when the proteins aren't shutting them off. Such a gene driver might be part of the oscillator itself if its activity is repressed each day when the other proteins turn their genes off.

Loros and Dunlap's team at Dartmouth has shown that WC-1 and -2 drive the *frq* gene, and there is new evidence suggesting that a CLOCK-like protein may activate *per* in fruit flies. Paul Hardin's team at the University of Houston recently found that the *per* gene includes a DNA sequence known to serve as the binding site for a still-unidentified protein with a bHLH motif—the same motif found in CLOCK. That suggests, says Hardin, that there could be a mouse counterpart of CLOCK driving *per* expression. That, in turn, has fueled speculation that CLOCK may drive a *per*-like gene in mice.

That's just one of the speculations fueled by the new results. There is also the issue of the PAS sequence, which has now been found in more than a dozen proteins, most of them transcription activators and some of them clock genes. It has several apparent functions, and it isn't clear which of them is crucial in clocks. But its presence points to a possible evolutionary link, suggesting that clock mechanisms—which evolved to deal with daily light-dark cycles—may have arisen from light-responsive proteins in primitive organisms. Besides their role in *Neurospora*'s clock, WC-1 and WC-2 are regulators of all light-responsive genes in the mold; moreover, a number of light-responsive proteins with no known clock function—found in algae, bacteria, and higher plants—have PAS-like sequences, suggesting they share a common ancestor with clock proteins (*Science*, 2 May, p. 753).

Beyond all this speculation, researchers are looking forward to using the new genes as an opening for testing their hypotheses. With *Clock* in hand, researchers finally have a handle on the mammalian clock; they can now search for other components by looking for proteins that CLOCK interacts with and genes that it activates. Takahashi notes that there is no proof yet that CLOCK is a central part of the oscillating mechanism of a mouse's timepiece; by checking whether CLOCK activity levels rise and fall, and how manipulations of it affect mouse rhythms, researchers will learn whether it is a key component. And perhaps one day, when the molecular parts of the mammalian clock have all been discovered, jet-lagged travelers will know which molecular knob to turn to reset it.

–Marcia Barinaga

MALARIA RESEARCH

How the Parasite Gets Its Food

Malaria is a notoriously tenacious infection. One reason is the *Plasmodium* parasite's ability to sequester itself inside red blood cells, where it is protected from attack by the immune system and many drugs. Once there, however, the parasite faces a problem common to fugitives: how to get food. Red blood cells, which are little more than sacks of hemoglobin, cannot provide all the nutrients *Plasmodium* needs. But new results are helping to explain exactly how the parasite imports sustenance from outside the cell.

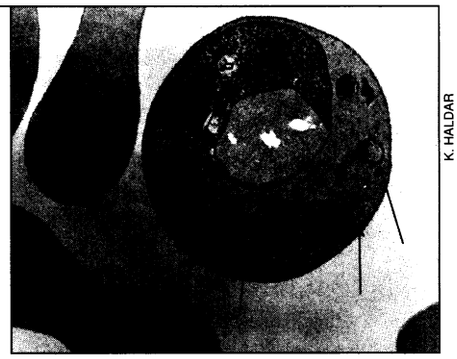
Researchers have suspected for several years that *Plasmodium* acquires at least some of its nutrients through a complex series of membranous tubules and vesicles that it constructs throughout the red blood cell shortly after taking up residence there. But while the structure of this network suggested that it might be a transport system, direct evidence for that had been hard to come by—until now, that is.

In work described on page 1122, biochemists Kasturi Haldar, Sabine Lauer, and Nafisa Ghori of Stanford University, with Pradipsinh Rathod of the Catholic University of America in Washington, D.C., have found that a chemical that disrupts the membrane network prevents the parasite from importing vital nutrients such as protein-building amino acids. The result is "the best evidence so far" that the membranes are an import system, says malaria researcher Barry Elford of Oxford University in the United Kingdom. The finding also suggests that drug researchers might take advantage of the system by designing antimalarial compounds that can sneak in with the essential nutrients.

The current work is an outgrowth of a previous discovery by Haldar and her colleagues. Almost 2 years ago, they showed that a chemical called PPMP, which prevents the parasite from forming a membrane component called sphingomyelin, disrupts the formation of the entire network—causing the tubules to become fragmented or constricted. To see whether this breakdown interferes with the network's proposed transport function, the team exposed both normal and PPMP-treated infected red blood cells to a dye called Lucifer yellow. In the control cells, the dye was distributed throughout the membrane network and in the parasite itself, but the PPMP-treated cells took up very little dye.

The researchers then went on to probe whether the chemical has a similar effect on the transport of nutrients. In a series of experiments, they exposed control cells and treated cells to several building blocks of nucleic acids and to glutamate, an amino acid used to build proteins—all with radioactive labels. When they measured how much radioactivity appeared in the two types of cells, they found that PPMP reduced accumulation of the nutrient molecules by as much as 91%. The team also found an even larger drop-off—up to 98%—in the amount of the imported substances actually used by the parasite to build DNA or proteins. The difference between the two figures suggests that while PPMP-treated cells were able to take up some of the molecules, they were unable to deliver them to the parasite, says Haldar.

Although the membrane-blocking compound itself might seem like an obvious drug candidate, Haldar says cutting the supply lines would kill the parasite slowly, giving it time to find alternate import routes and develop resistance. A better strategy might be to take advantage of the network to deliver drugs to the parasite. Indeed, while the network may block some drugs, a few compounds do seem to travel through it. The team found that blocking the network with PPMP also blocked the uptake of an experimental drug—a slightly modified nucleotide precursor that disrupts *Plasmodium*'s DNA synthesis. PPMP seemed to block uptake of the lethal compound by about 90%, enabling many cells to survive treatment with the drug.



K. HALDAR

The fugitive. The malaria parasite's network of tubules (small arrows) may import nutrients.

In fact, the new findings may help explain some drug-related mysteries, says Rathod: "It's always been puzzling why some modified nutrients are effective and why some very close analogs are ineffective." Some sort of selection mechanism in the membrane network may protect the parasite from certain drugs, he says. The team hopes to figure out those mechanisms in future experiments. "If you understand the permeability and specificity," says Rathod, "you can design drugs that take advantage of them."

–Gretchen Vogel