New Clock Gene Cloned

A fruit fly gene called *timeless* holds new clues to the workings of the molecular clock that controls daily rhythms and behaviors

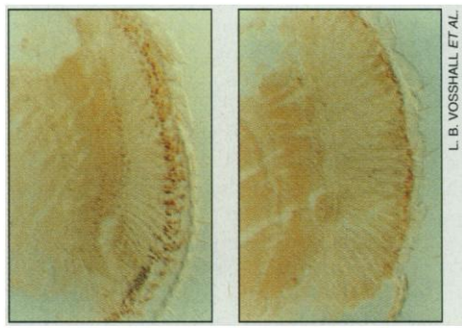
Inside the cells of organisms ranging from single-celled bacteria to human beings ticks a tiny biochemical clock that maintains the daily cycles of behavior known as circadian rhythms. By the time kept by this clock, plants know when to spread their leaves to the sun, fruit flies when to emerge from their pupae, humans when to drop off to sleep. Exactly how the clock keeps time is a mystery, however, as is the identity of most of the molecular wheels and gears that make it tick. Now, a research team from Rockefeller University, Harvard Medical School, and the University of Pennsylvania Medical Center has cloned a gene called *timeless* (*tim*), which makes a key protein component of the clock in the fruit fly *Drosophila melanogaster* (see pp. 805, 808, and 811).

The *timeless* gene is not the first clock gene to be cloned; it joins the *per* gene (for *period*) from fruit flies and the *frq* gene (for *frequency*) from the bread mold *Neurospora crassa*, both of which code for proteins that are central to those organisms' clocks. But with the cloning of *tim*, researchers can for the first time get a look at how two different proteins work together to form the internal mechanism of a single organism's clock. Clock researcher Joe Takahashi of Northwestern University calls the *timeless* work "the most important discovery since per was cloned."

The picture of how the PER and TIM proteins interact is far from complete, but already it seems that they work as a team to generate an oscillating cycle of activity in their own genes and probably other genes, which in turn set up daily rhythms in the fly's physiology and activity. That's a first step toward answering what Jay Dunlap of Dartmouth Medical School, whose lab identified and cloned the *frq* gene, describes as the three basic questions in circadian biology: "How does the clock itself run, how do you reset the clock, and how do you get output from the [clock] to regulate other things in the cell?"

What's more, researchers are hopeful that the same methods that they used to find the *timeless* gene may also help them root out the other clock components that they believe are still awaiting discovery. "The clock field is entering a really exciting time [in which] the initial molecular mechanisms are being uncovered," says Northwestern's Takahashi.

The first piece of the fruit fly clock to be identified was *per*, discovered in the early 1970s by Ron Konopka in Seymour Benzer's lab at Caltech. Later, researchers studying the first step leading to PER protein synthesis, the production of messenger RNA (mRNA) by the gene, found that *per* activity cycles up and down with a 24-hour period. This cycling alone was not sufficient to prove that PER is part of the basic clock mechanism. It could have just meant that *per* is controlled by the clock. But other information confirmed PER's central role in the clock. For example, mutations in the *per* gene alter a fly's circadian rhythms in any one of several critical ways, abolishing them altogether, for example, or making them longer or shorter.



Time stands still. PER protein normally accumulates in nuclei *(dots)* in fly heads *(left)*, but the *tim* mutation shuts down the process *(right)*.

Even though these studies taken together suggested that the cycling of *per* gene activity was at the core of the fruit fly clock mechanism, how the cycling was achieved was still a puzzle. Other studies showed that it is controlled, at least partly, by the PER protein itself. As the levels of *per* mRNA increase, cells produce more PER protein, which then goes into the nucleus and shuts down its own gene. That causes the mRNA and protein levels to drop and eventually releases the gene from its own self-imposed repression, allowing it to be active again.

A missing partner for PER

But that couldn't be the full answer, says Young. "Lots of genes autoregulate," he says, "but you don't get a clock out of them." Left on its own without other influences, a protein that can freely enter the nucleus and turn down expression of its own gene would not end up cycling in an endless rhythmic fashion, Young says, but would instead damp out concentration swings, reaching some relatively constant, intermediate level. That means that something else must interact with PER to maintain the cycling pattern.

Researchers have wondered whether TIM might be that "something else" ever since Michael Young's group at Rockefeller University identified its gene several years ago by screening fruit flies for new mutations that upset their circadian rhythms. The mutant strain they identified had a normal *per* gene, but subsequent findings suggested that *tim* influenced how that *per* gene functioned. Young's team found that *tim* mutations abolish the cycling of *per* mRNA, and prevent PER from accumulating in cells and moving into the nucleus (*Science*, 18 March 1994, pp. 1603 and 1606).

"That was very exciting," recalls Young. "It suggested that ... perhaps the target of the *timeless* protein was somehow the PER protein," and that TIM was controlling PER's location and levels in the cell. To learn more about *tim*, the Rockefeller team immediately began to hunt down the gene, sifting through DNA cloned from the chromosomal region to which the mutation had been mapped, looking for likely candidates.

While that effort was proceeding, Young and his former postdoc Amita Sehgal, who by then had her own lab at the University of Pennsylvania, began a collaboration with Charles Weitz of Harvard Medical School to take a totally different tack in the search for new clock genes from flies, a search they hoped would also snare *tim*. Whereas Young's group had found the *tim* mutation by searching through mutagenized flies for those with aberrant behavioral rhythms, Weitz's team began an approach based on the assumption that other components of the clock mechanism would interact directly with PER. They used a method called the yeast two-hybrid screen, in which one protein serves as "bait" to fish out genes based on their ability to produce protein products in the yeast cells that bind to the bait protein.

With PER as bait in the fishing expedition, the collaborators hoped to pull out other genes whose protein products not only interact with PER, but are central to the clock. And *timeless* was foremost in their minds. "An explicit goal of this [project] was that if *timeless* does interact at the protein level directly with PER, we'd stand a damn good chance of finding it," says Weitz. "And that's just what happened."

Using these two different approaches, the teams zeroed in on *timeless* early this year. In Young's lab, postdoc Michael Myers found a gene that, in *tim* mutant flies, contained a disabling deletion, but in normal flies did not. That suggested the gene was *tim*, and the deletion was the mutation that disrupted rhythms in *tim* mutants. And when Sehgal looked at the levels of the gene's mRNA from living flies, she found that it cycles up and down once every 24 hours, just like the *per* mRNA. "That is what nailed it," says Sehgal. "We had a gene that had this deletion in the mutant, and it cycled." The Sehgal and Young groups were convinced they had found *tim*.

Meanwhile, Weitz postdoc Nicholas Gekakis had screened 20 million clones of DNAs copied from fruit fly mRNAs and had found 48 whose protein products bound to PER in the yeast assay. Young's group sent Weitz the *tim* clone, and Gekakis found that 16 of his 48 clones had the same DNA sequence, meaning they were all clones of *tim*. "We converged [on the *tim* gene] from different routes entirely," Weitz says. And a bonus of the yeast approach was that it showed that TIM binds directly to PER.

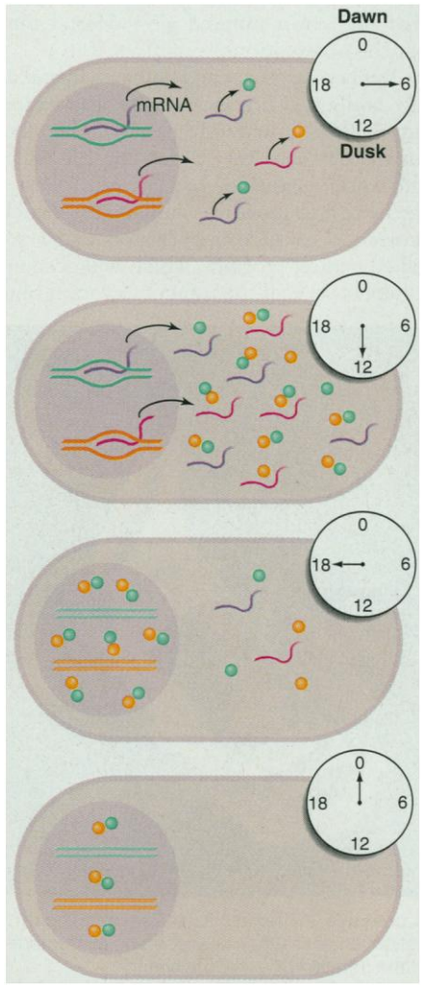
Building a model

The next question was: How might the TIM and PER proteins contribute to the running of the circadian clock? The growing collection of data is beginning to suggest an answer. The *per* and *tim* RNAs cycle with exactly the same period, and Sehgal and Adrian Rothenfluh-Hilfiker in Young's lab found that mutations in *per* upset *tim* mRNA cycling, just as mutations in *tim* had been found to upset per mRNA cycling. Those findings suggest that under normal circumstances TIM and PER somehow work together to turn down both of their own genes.

To regulate the genes, PER apparently must first accumulate in the cytoplasm until something triggers it to move to the nucleus. Moreover, the Young group showed last year that the accumulation of PER and subsequent migration to the nucleus seem to be blocked in mutants lacking a functional TIM protein. That finding, together with the evidence that the two proteins bind to each other, led the collaborators to propose that the binding of the two proteins may play a role in the timing of PER nuclear entry, and thus in the circadian cycle itself.

Support for that idea comes from experiments in which Weitz's group studied the interaction between TIM and a mutant form of PER, known as PER^L (where L stands for long) because the mutation lengthens the circadian period in flies. They found that PER^L binds to TIM protein less well than normal PER. That could explain why the cycle is longer in the per^L mutants, Weitz says. If the affinity between TIM and PER is reduced, PER and TIM would have to reach higher levels before they bind to each other, causing a delay in the entry of PER to the nucleus.

All this has led Sehgal, Weitz, and Young to propose a model in which the PER protein is relatively unstable when it's first made in the cytoplasm. As a result, the protein molecules accumulate slowly, until they run into TIM proteins, which are being made at the same time. The two proteins, according to the model, then bind to one another, forming stable dimers that enter the nucleus. There they shut down the expression of their own genes, either directly or indirectly, and may affect other genes as well.



SOURCE: AMITA SENGAL

Clockwork. The *per* and *tim* genes give rise to proteins *(yellow and blue dots)*, which form dimers that may enter the nucleus to shut down their own genes. That causes protein levels to drop, and the cycle begins again.

Clock researchers find this scenario attractive because it may explain how feedback of a protein on its own gene can generate an oscillating clock, rather than simply settling at a constant intermediate level of gene expression. If PER needs to bind to TIM before it can enter the nucleus, its entry into the nucleus would be delayed, says Young—which would "guarantee that the protein shows up after it's too late to undo the transcription that's already been done." That delay, says University of Virginia circadian rhythm researcher Steve Kay, would seem to be "an important checkpoint for circadian regulation." Factors such as the rate at which PER and TIM accumulate in the cytoplasm, and the length of time they remain active in the nucleus, would work to set the period of oscillation at 24 hours.

As appealing as the model is, the authors of the papers, as well as others in the field, caution that it has many unproven elements. For example, researchers have only just begun to study the TIM protein itself, and so have not yet shown whether its concentration and location in flies change during the circadian cycle the way the model predicts. From the present experiments in yeast and test tubes, "there is every expectation that [TIM and PER] actually make physical contact" in flies, says Brandeis University circadian rhythm researcher Michael Rosbash, but "there are as yet no fly biochemistry experiments that look at TIM protein." Such in-fly results apparently will not be long in coming, though. The Rosbash and Young groups have work presently under review for publication showing that the PER-TIM interaction is indeed vital to circadian rhythms in flies.

Even if the model does hold up, the puzzle of circadian clocks would still have many gaping holes to be filled. "One major link that really needs to be defined is how the PER and *timeless* proteins feed back on their own [genes]," says Northwestern's Takahashi. Most researchers think that they probably don't do this by binding the DNA directly, because neither PER nor TIM has the DNA-binding domains characteristic of proteins that directly regulate gene expression. So there may be DNA binding proteins in the nucleus that must cooperate with PER and TIM to turn down the genes.

PER and TIM and their unknown nuclear partners probably regulate other, unknown genes as well, and these genes may hold the answer to one of the three questions of circadian biology mentioned by Dunlap: how output from the clock sets up circadian rhythms. The first step in that output probably involves the rhythmic regulation of a battery of genes, whose products contribute to the behavioral and physiological rhythms.

"We are really inching our way toward putting a lot of pieces of the jigsaw down," says Kay of the University of Virginia. With *tim* fitted into its place, some of the other missing pieces will undoubtedly fall into place as well. And the picture being pieced together will undoubtedly reveal more and more about the molecular gears and pendulums that make the circadian clock tick.

–Marcia Barinaga