Lighting up Biological Clocks

Genes from glowing organisms illuminate circadian rhythms

By JOHN TRAVIS

In the ocean, the brownish, single-celled alga *Gonyaulax polyedra* puts on an unusual light show. At night, it glows softly and emits bright flashes from time to time. But during the day, when these organisms aggregate near the ocean's surface and use sunlight to make needed chemicals through photosynthesis, their glow dims and they rarely flash.

In 1958, Harvard's J. Woodland Hastings discovered that *G. polyedra* doesn't shut down its night-light and flashbulbs simply because the sun rises. When placed in constant darkness, the algae maintain a nearly day-long light cycle.

This pattern of bioluminescence provides a striking visual example of a circadian rhythm, a built-in cycle of biological activity that tells an organism when it should perform a specific function. Driving these approximately 24-hour-long cycles are biological clocks, internal timekeepers that tick away in organisms as simple as bacteria and fungi and as complex as fruit flies, mice, and people. Among other duties, the circadian rhythms created by these powerful clocks determine when animals sleep or move about, honeybees collect nectar, plants unfurl their leaves, and molds send out spores.

Biological clocks are more than an academic curiosity, however. The study of the human clock, which resides in a tiny collection of brain cells called the suprachiasmatic nucleus, has become a pressing issue. Researchers know that jet lag and problems caused by working at night stem largely from the body's battle against its normal circadian rhythms. Certain depressions and sleep disorders may also result from defective clocks.

Despite their importance, biological clocks are poorly understood. What genes run the clock? How do they keep time? How do those clock genes govern other genes, so-called clock-controlled genes, whose cycles of activity create circadian rhythms?

To answer these questions, researchers have turned to *G. polyedra* for inspiration. As in many other species, including fireflies and glow worms, the alga's light show results from a simple chemical reaction. An enzyme, slightly different in every species but always called luciferase, produces light when it cleaves another molecule, often called luciferin.

Through adept genetic manipulation, researchers have now taken the DNA that makes luciferase and transferred it into plant, insect, and other species that don't naturally light up. In laboratories around the world, forms of life that Mother Nature never intended to glow are doing just that.

More important, researchers have performed the genetic transfer in such a way that the glow rises and falls in synchrony with an organism's biological clock. This novel laboratory maneuver is helping investigators screen for and analyze genes crucial to the clock and genes controlled by it. "It's an extremely powerful new tool," says Gene Block, director of the National Science Foundation's Center for Biological Timing at the University of Virginia in Charlottesville.

This clock-driven, science-crafted light show made its debut on the Charlottesville campus, where a group led by Steve Kay had long been interested in how biological clocks work in plants. But while investigators had unearthed clock genes in mice, hamsters, fruit flies, bread mold, and other organisms, they had not enjoyed similar success with plants. For plants, the best Kay and others had been able to do was isolate dozens of clock-controlled genes. They desperately wanted the genes that make up the gears and springs of the clock itself.

The first step toward finding a gene necessary for a particular function, most geneticists agree, is to find mutants—organisms that don't perform the function or that perform it abnormally. With this in mind, Kay and his colleague Andrew J. Millar transferred the firefly's *luciferase* gene into the genome of the tiny weed *Arabidopsis thaliana*. Before inserting the gene, however, they fused another piece of DNA to it. That DNA, a regulatory element called a promoter, belonged to a gene in the weed that the group knew followed a circadian rhythm, turning on during the day and off at night. By attaching this promoter to the gene *luciferase*, they hoped that the firefly gene would come under the influence of the weed's biological clock.

The effort was a resounding success, as the team reported in the Feb. 24 SCIENCE. When the researchers sprayed luciferin onto their luciferase-producing weeds, the plants emitted a green glow in step with the circadian rhythm of the promoter: The light starts building in the predawn hours, peaks in the morning, and subsides as night approaches.

Next, the team produced plants with defective clocks by soaking seeds from the genetically engineered weed in a chemical that causes DNA mutations. Most of the mutations affected genes unrelated to the plant's biological clock, but a few apparently damaged crucial clock genes. All the researchers had to do was visually monitor thousands of seedlings for ones that glowed at inappropriate times, a task that was automated with computers and videocameras. The plants "looked like little stars" in the petri dishes, says Kay.

Kay and his colleagues found 21 plants with defective biological clocks, the first time circadian rhythm researchers had been able to do this. Some of the mutants had cycles shorter than the plant's natural cycle, some longer. "Without [*luciferase*], they never could have found these rhythm mutants

in *Arabidopsis*," says Jeffrey C. Hall of Brandeis University in Waltham, Mass.

Kay's group now has to sift through the DNA of the mutant weeds to track down the genetic flaws that perturb the plant's biological clock. That challenging task is the main reason the team chose *A. thaliana*. Like the mouse and the fruit fly, this small weed has been well studied by geneticists; their accumulated knowledge should speed the search. The group has already pinpointed the approximate locations of the damaged DNA in a few of the seedlings.

Kay and his colleagues pioneered this *luciferase* technique, but they had some friendly competition along the way. An international team of investigators conducted a similar scan for clock genes in the genome of a cyanobacterium, a blue-green alga that photosynthesizes during the day, like *G. polyedra*. The team, which includes Susan S. Golden of Texas A&M University in College Station, Texas, Carl Johnson of Vanderbilt University in Nashville, and Takao Kondo of Japan's National Institute for Basic Biology in Okazaki, discussed its results in the Nov. 18, 1994 SCIENCE.

Instead of stealing the spark of the firefly, this group stole the *luciferase* gene from a bioluminescent bacterium. The enzyme produced by this gene is different from the one the firefly makes; it generates light when it interacts with a gaseous organic molecule, not when it acts on luciferin.

To bring this *luciferase* gene under the sway of the blue-green alga's biological clock, the investigators joined it to the promoter of a gene used only during photosynthesis. "It's a very well-behaved circadian promoter," says Golden.

She and her colleagues then chemically mutated their genetically engineered organisms and recorded when the cyanobacteria lit up. After looking at 150,000 colonies of the blue-green algae, they identified 17 that either didn't follow a normal 24-hour circadian rhythm or that displayed an abnormal bioluminescent rhythm over that period. The collaboration is now trying to track down the genes responsible for those mutants.

In more recent research, the investigators attempted to make a complete tally of the clock-controlled genes in the cyanobacterium. They inserted the light-producing gene at random throughout the organism's genome. Whenever *luciferase* landed near a clock-controlled gene, they reasoned, that gene's promoter should also turn the neighboring *luciferase* on and off in a rhythmic manner.

To their surprise, almost every *luciferase* they inserted produced a circadian bioluminescent rhythm, indicating that the cyanobacterium's biological clock rules the activity of hundreds of genes. "It suggests there is more global and pervasive control [by the clock] than we thought," says Johnson.

At a meeting of the Endocrine Society in Washington, D.C., this June, Hall described the latest *luciferase* trick. A number of years back, when Kay's group had just begun working with the gene, Hall heard about the effort and wondered if the technique could aid his research into the biological clock of the fruit fly. This clock causes the insects to follow a well-defined circadian pattern of rest and activity.

Hall and others investigating the fruit fly clock concentrate their efforts largely on *per* (short for *period*), a gene thought to be an integral part of the insect's clock mechanism. Mutations in *per* quicken, slow, or destroy altogether the fly's circadian rhythm. Bolstering the classification of *per* as a clock gene, not merely a clock-controlled gene, is the fact that *per* appears to contain the instructions for building a transcription factor, a protein that regulates the activities of other genes.

The activity of *per* normally cycles up and down in a clear circadian rhythm; its production of protein peaks at night and bottoms out during the day. But the limited research techniques available have stymied further insight into *per's* role in the clock, asserts Hall.

In the past, he explains, scientists had to study *per* by establishing large fruit fly populations whose clocks were in synchrony. They would then periodically select a group from the population and grind up the insects' heads to gauge *per's* activity, a challenging and labor-intensive procedure requiring them to measure small amounts of protein or messenger RNA produced by *per*. Such experiments use up insects fast, says Hall. The circadian rhythms of individual flies also quickly fall out of sync, rendering *per* assays of groups almost meaningless, he adds.

Frustrated by these difficulties, Hall asked Kay whether they could join the firefly *luciferase* gene to *per* and simply watch the clock gene in action. "Andrew and I had already been joking for a long time about making [the fruit fly] into a firefly," says Kay. The notion remained largely a topic of barroom conversation, says Hall, until he heard about an Australian research group that had attached *luciferase* to a promoter activated by high temperatures and inserted the union into the fruit fly genome.

The Australian team ground up the resulting flies, added luciferin to the mix, and saw the telltale glow. But, says Hall, shaking his head in disbelief, the Australians never looked at live insects.

He quickly requested the Australians' few remaining live insects and went to Kay's lab in Virginia to put the flies to the test. Observing the insects with the same cameras used in Kay's plant research, they turned up the heat on the flies and sprayed them with luciferin. Then "Steve began screaming, 'We've got hot spots.' Their heads literally glowed," recalls Hall.

With that proof, the two groups quickly moved to link *luciferase* to *per's* promoter and then transferred that construct into a strain of fruit fly. Simply by feeding these genetically altered flies a constant diet of luciferin, Hall, Kay, and their colleagues now have in hand insects that glow brighter and dimmer as they pass through the ups and downs of *per's* daily cycle. By placing a fly in a test tube and supplying it with luciferin-laced food, they can use automatic light-recording devices to follow *per's* activity for days, if not weeks.

In one of their first efforts, the investigators documented the circadian rhythms of 1,500 individual flies, an avalanche of data they could never have gathered through traditional *per* assays. "The simplicity of the technique is one of its powers. You can now do experiments routinely. The only impediment is your imagination," says Hall.

Investigators, for instance, plan to watch how *per* responds to the various external stimuli that influence the fly's biological clock. Light has a strong impact on biological clocks, helping insects and other organisms keep accurate time (see p.111).

The ability to take measurements frequently throughout *per's* 24-hour cycle has already revealed a subtle upturn in the gene's activity not long before it reaches its lowest point. This second peak, says Hall, will force investigators to reconsider their models of how the *per* cycle is produced.

In the future, fruit flies may glow in more than one color. Investigators have recently found a second possible clock gene, *timeless*, which produces a protein that appears to interact with *per's*. Hook *timeless* up to a *luciferase* gene that produces light at a slightly different wavelength than *per's*, and watch the interplay of the two genes, suggests Hall.

Block thinks the technique will even find a home in studies of mammals. Fiber-optic cables inserted into a mouse's suprachiasmatic nucleus might convey images of glow rhythms produced by mammalian clock or clock-controlled genes married to *luciferase*, he says.

The enzyme luciferase, agree many clock investigators, provides a crucial advance in the way they conduct research. "Many of the questions in science are straightforward. You make leaps by applying a new technology to them," notes Jay Dunlap of Dartmouth Medical School in Hanover, N.H. Glowing fruit flies offer illuminating proof of that sentiment. □