Box 2–2 Generating Mutations in Experimental Animals (continued)

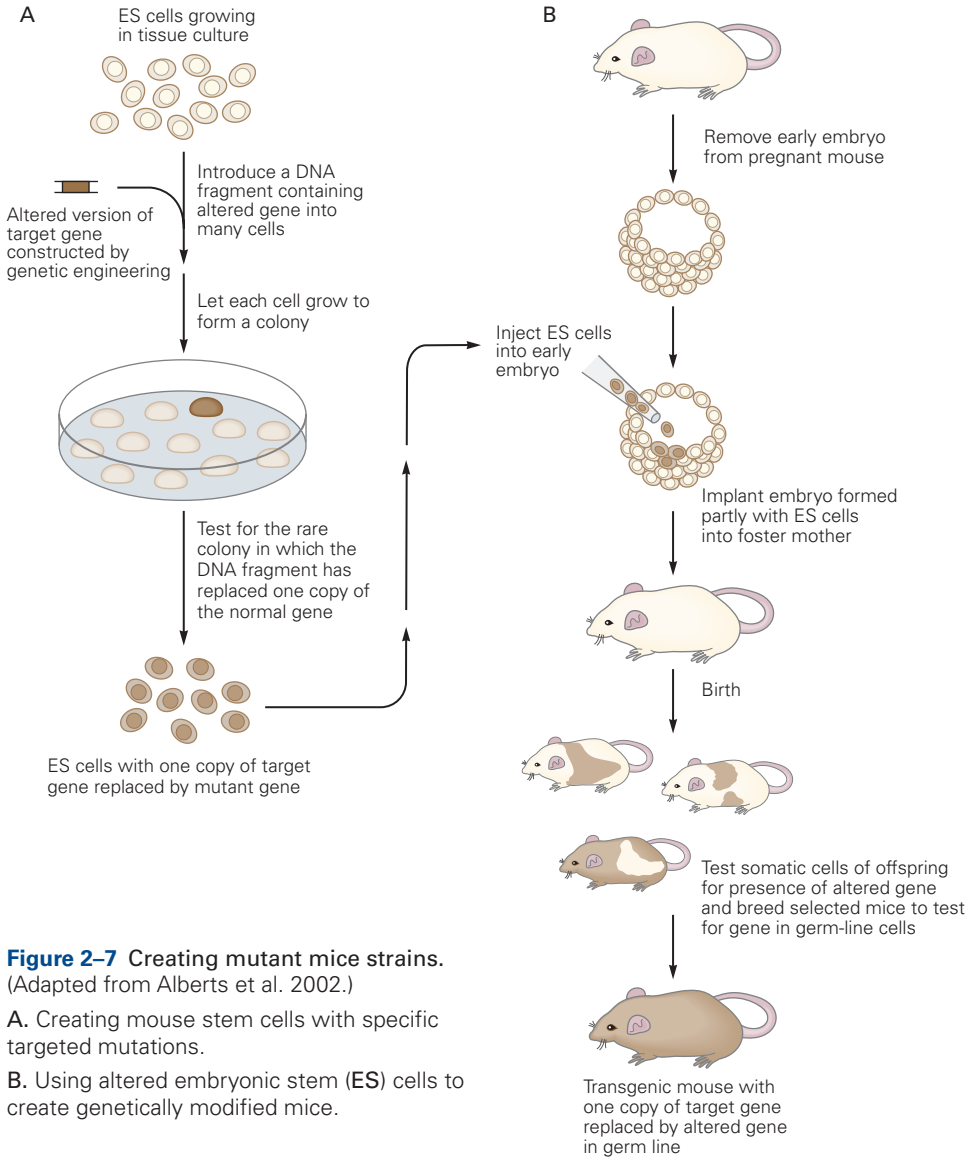

Figure 2–7 Creating mutant mice strains. (Adapted from Alberts et al. 2002.)

A. Creating mouse stem cells with specific targeted mutations.

B. Using altered embryonic stem (ES) cells to create genetically modified mice.

the tetracycline transactivator (tTA), which recognizes and binds to the tetO promoter. Expression of tTA can be placed under the control of a promoter in the mouse genome that normally drives gene transcription in only specific classes of neurons or specific brain regions.

When the two lines of mice are mated, some of the offspring will carry both transgenes. In these mice, the tTA binds to the tetO promoter and activates the downstream transgene. What makes the tTA transcription factor particularly useful is that it becomes inactivated when it binds certain antibiotics, such as tetracycline, allowing transgene expression to be regulated by administering antibiotics to mice. One can also generate mice that express a mutant form of tTA called reverse tTA (rtTA). This transactivator will not bind to tetO unless the animal is fed doxycycline. In this case, the transgene is always turned off unless the drug is given (Figure 2–9).

Altering Gene Function by RNA Interference and CRISPR

Finally, genes can be inactivated by targeting them with modern molecular tools. One such method is RNA interference, which takes advantage of the fact that most double-stranded RNAs in eukaryotic cells are routinely destroyed; the whole RNA is destroyed even if only part of it is double-stranded. By introducing a short RNA sequence that artificially causes a selected mRNA to become double-stranded, researchers can activate this process to reduce the mRNA levels for specific genes.

Another experimental tool is CRISPR, a method in which components of a bacterial immune system are deployed in nonbacterial cells to directly attack a selected DNA sequence. To target a gene with CRISPR,

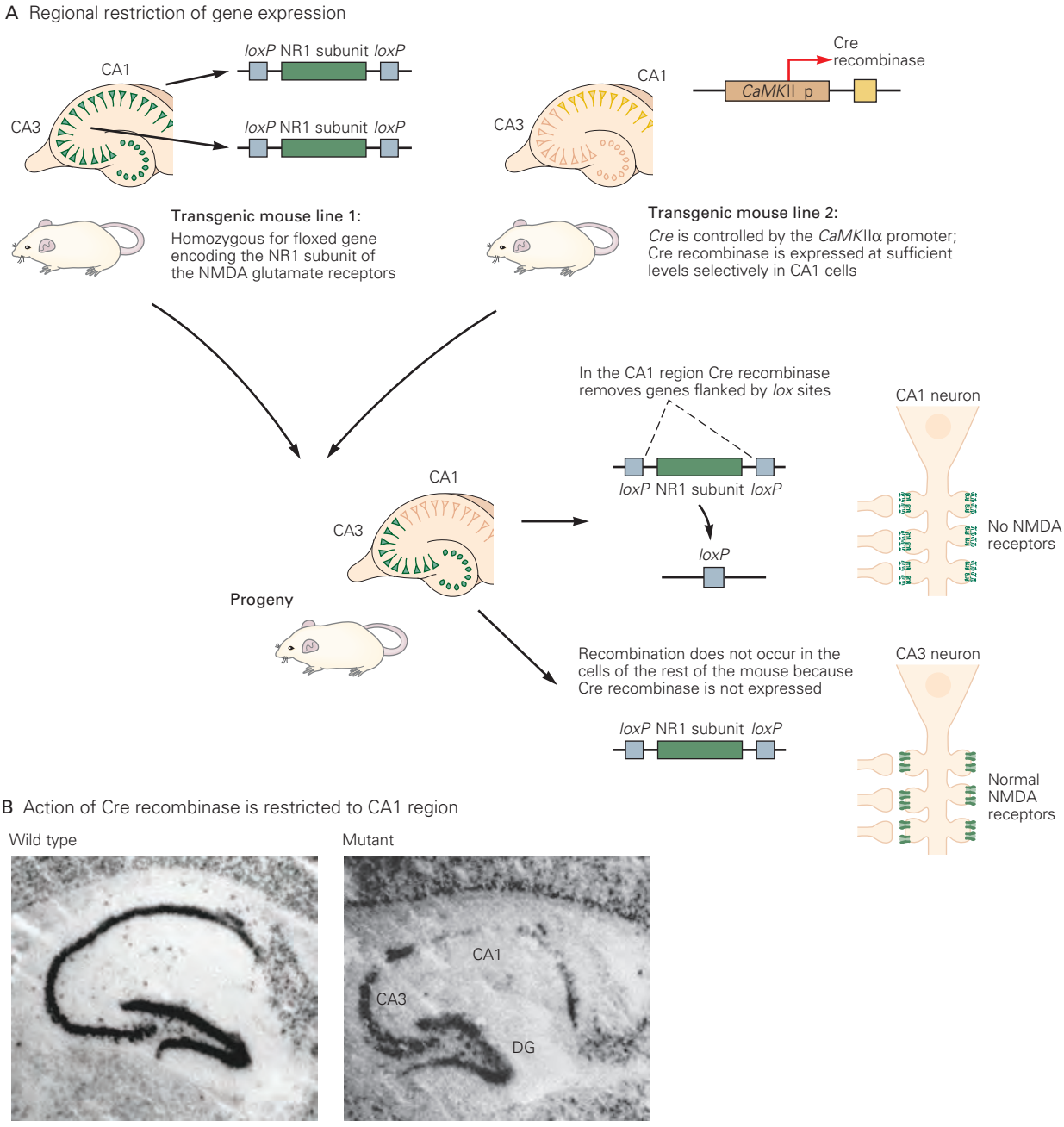

Figure 2–8 The Cre/loxP system for gene knockout in selective regions.

A. A line of mice is bred in which the gene encoding the NR1 subunit of the NMDA receptor has been flanked by loxP genetic elements (transgenic mouse line 1). These so-called "floxed NR1" mice are then crossed with a second line of mice in which a transgene coding for Cre recombinase is placed under the control of a transcriptional promoter specific to a cell type or a tissue type (transgenic mouse line 2). In this example, the promoter from the *CaMKIIα* gene is used to drive expression of the *Cre* gene. In progeny that are homozygous for the floxed gene and that carry the *Cre* recombinase transgene, the floxed gene will be deleted by Cre-mediated loxP recombination only in cell type(s) in which the promoter driving Cre expression is active.

B. In situ hybridization is used to detect mRNA for the NR1 subunit in hippocampal slices from wild-type and mutant mice that contain two floxed NR1 alleles and express Cre recombinase under the control of the CaMKIIα promoter. In the mutant mice, expression of the mRNA for NR1 (dark staining) is greatly reduced in the CA1 region of the hippocampus but remains normal in CA3 and the dentate gyrus (**DG**). (Reproduced, with permission, from Tsien, Huerta, and Tonegawa 1996.)

Box 2–2 Generating Mutations in Experimental Animals (continued)

a bacterial protein (typically but not always a protein called CAS9) is produced together with an engineered guide RNA that has sequence similarity with the target gene. The CAS9-guide RNA complex seeks out and cleaves the target sequence in the genome of the cell of interest. That initial cleavage can induce point mutations, insertions, and deletions at that site, and can also facilitate desired recombination or genetic replacement events. CRISPR tools are increasing in their sophistication and precision to the extent that they are now being considered for repair of hereditary mutations in people with severe inherited genetic diseases.

RNA interference and CRISPR have great potential to increase the power of genetic analysis because they can be used on any species in which DNA or RNA can be delivered to cells, including animals that are not now used in classical genetic analysis, such as long-lived birds, fish, and even primates.

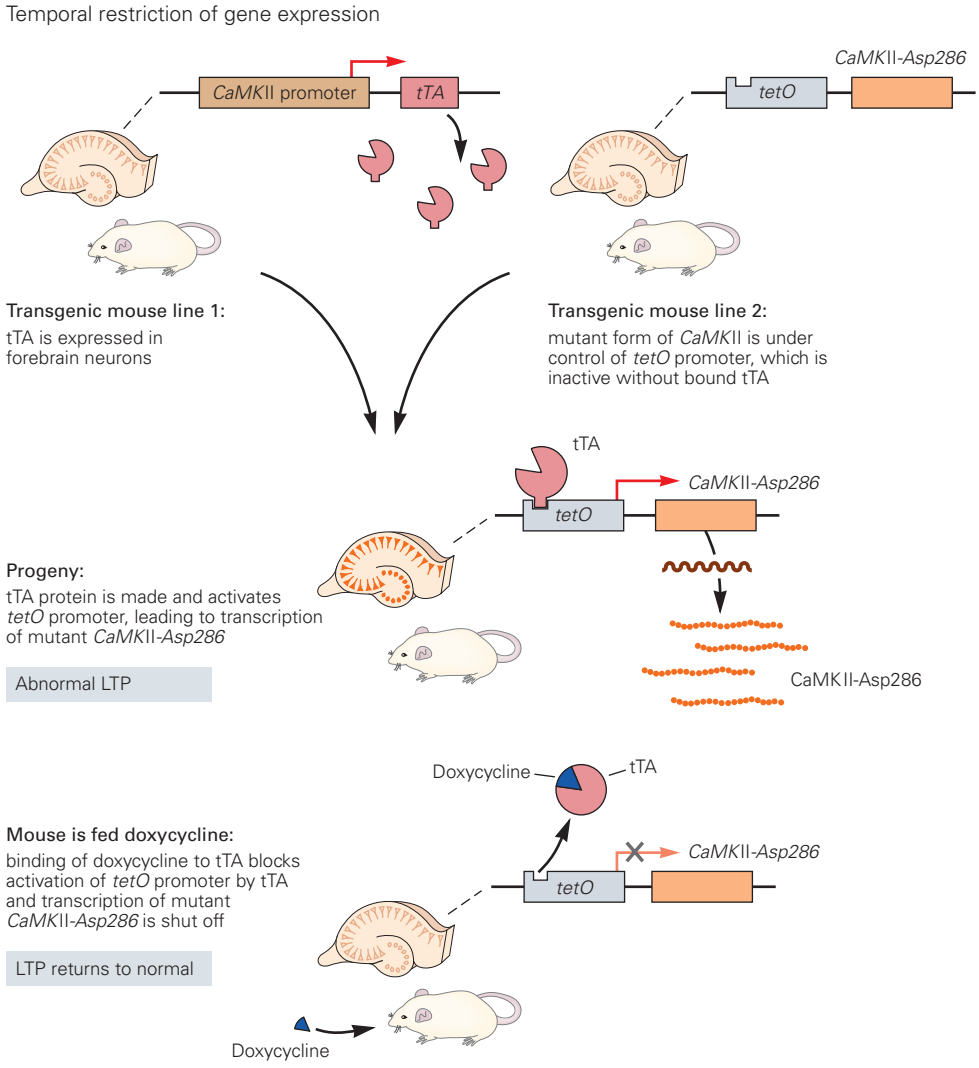

Figure 2–9 The tetracycline system for temporal and spatial regulation of transgene expression. Two independent lines of transgenic mice are bred. One line expresses, under the control of the CaMKIIα promoter, the tetracycline transactivator (**tTA**), an engineered protein incorporating a bacterial transcription factor that recognizes the bacterial tetO operon. The second line contains a transgene of interest—here encoding a constitutively active form of CaMKII (CaMKII–Asp286) that makes the kinase persistently active in the absence of Ca^{2+}—whose expression is under control of tetO. When these two lines are mated, the offspring express the tTA protein in a pattern restricted to the forebrain. When the tTA protein binds to tetO, it will activate transcription of the downstream gene of interest. Tetracycline (or doxycycline) given to the offspring binds to the tTA protein and causes a conformational change that leads to the unbinding of the protein from tetO, blocking transgene expression. In this manner, mice will express CaMKII–Asp286 in the forebrain, and this expression can be turned off by administering doxycycline to the mice. (Reproduced, with permission, from Mayford et al. 1996.)

Box 2–3 Introducing Transgenes in Flies and Mice

Genes can be experimentally introduced in mice by injecting DNA into the nucleus of newly fertilized eggs (Figure 2–10). In some of the injected eggs, the new gene, or transgene, is incorporated into a random site on one of the chromosomes. Because the embryo is at the one-cell stage, the incorporated gene is replicated and ends up in all (or nearly all) of the animal's cells, including the germline.

Gene incorporation is illustrated with a coat color marker gene rescued by injecting a gene for pigment production into an egg obtained from an albino strain. Mice with patches of pigmented fur indicate successful expression of DNA. The transgene's presence is confirmed by testing a sample of DNA from the injected animals.

A similar approach is used in flies. The DNA to be injected is cloned into a transposable element (P element). When injected into the embryo, the DNA becomes inserted into the DNA of germ cell nuclei. P elements can be engineered to express genes at specific times and in specific cells. Transgenes may be wild-type genes that restore function to a mutant or *designer genes* that alter the expression of other genes or code for a specifically altered protein.

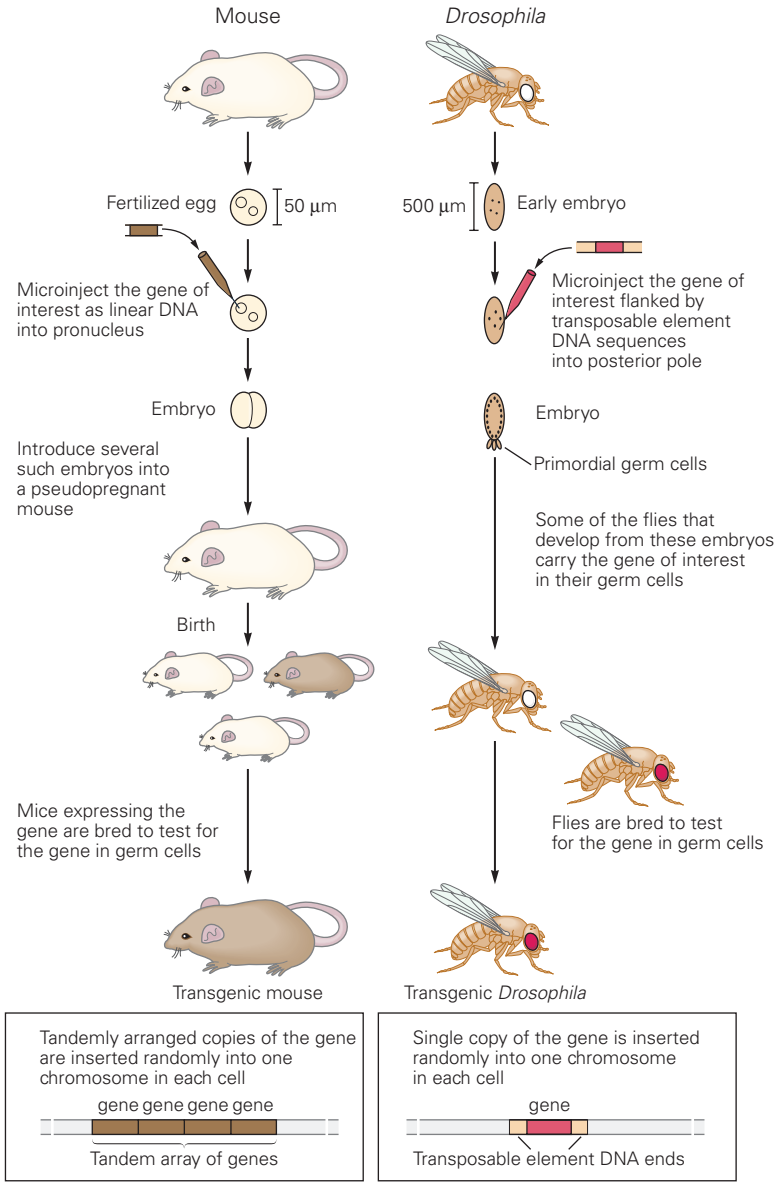

Figure 2–10 Generating transgenic mice and flies. Here the gene injected into the mouse causes a change in coat color, while the gene injected into the fly causes a change in eye color. In some transgenic animals of both species, the DNA is inserted at different chromosomal sites in different cells (see illustration at bottom). (Adapted from Alberts et al. 2002.)

We have a particularly complete picture of the genetic basis of the circadian control of behavior. An animal's circadian rhythm couples certain behaviors to a 24-hour cycle linked to the rising and setting of the sun. The core of circadian regulation is an intrinsic biological clock that oscillates over a 24-hour cycle. Because of the intrinsic periodicity of the clock, circadian behavior persists even in the absence of light or other environmental influences.

The circadian clock can be reset, such that changes in the day-night cycle eventually result in a matching shift in the intrinsic oscillator, a phenomenon familiar to any traveler recovering from jet lag. The clock is reset by light-driven signals transmitted by the eye to the brain. Finally, the clock drives effector pathways for specific behaviors, such as sleep and locomotion.

Benzer's group searched through thousands of mutant flies to look for rare flies that failed to follow circadian rhythms because of mutations in the genes that direct circadian oscillation. From this work emerged the first insight into the molecular machinery of the circadian clock. Mutations in the *period*, or *per*, gene affected all circadian behaviors generated by the fly's internal clock.

Interestingly, *per* mutations could change the circadian clock in several ways (Figure 2–11). Arrhythmic *per* mutant flies, which exhibited no discernible intrinsic rhythms in any behavior, lacked all function of the *per* gene, so *per* is essential for rhythmic behavior. *Per* mutations that maintained some function of the gene resulted in abnormal rhythms. Long-day alleles produced 28-hour behavioral cycles, whereas short-day alleles produced a 19-hour cycle. Thus *per* is not just an essential piece of the clock, it is actually a timekeeper whose activity can change the rate at which the clock runs.

The *per* mutant has no major adverse effects other than the change in circadian behavior. This observation is important because prior to the discovery of *per* many had questioned whether there could be true "behavior genes" that were not required for the physiological needs of an animal. *Per* does seem to be such a "behavior gene."

How does *per* keep time? The protein product PER is a transcriptional regulator that affects the expression of other genes. Levels of PER are regulated throughout the day. Early in the morning, PER and its mRNA are low. Over the course of the day, the PER mRNA and protein accumulate, reaching peak levels after dusk and during the night. The levels then decrease, falling before the next dawn. These observations provide an answer to the circadian rhythm puzzle—a central regulator appears and disappears throughout the day. But they are also unsatisfying because they only push the question back one step—what makes PER cycle? The answer to this question required the discovery of additional clock genes, which were discovered in flies and also in mice.

Emboldened by the success of the fly circadian rhythm screens, Joseph Takahashi began similar but far more labor-intensive genetic screens in mice in the 1990s. He screened hundreds of mutant mice for the rare individuals with alterations in their circadian locomotion period and found a single gene mutation that he called *clock*. When mice homozygous for the *clock* mutation are transferred to darkness, they initially experience extremely long circadian periods and later a complete loss of circadian rhythmicity (Figure 2–12). The *clock* gene therefore appears to regulate two fundamental properties of the circadian rhythm: the length of the circadian period and the persistence of rhythmicity in the absence of sensory input. These properties are conceptually identical to the properties of the *per* gene in flies.

The mouse *clock* gene, like the *per* gene in flies, encodes a transcriptional regulator whose activity oscillates through the day. The mouse CLOCK and fly PER proteins also shared a domain called a *PAS domain*, characteristic of a subset of transcriptional regulators. This observation suggests that the same molecular mechanism—oscillation of PAS-domain transcriptional regulation—controls circadian rhythm in flies and mice.

More significantly, parallel studies of flies and mice showed that similar groups of transcriptional regulators affect the circadian clock in both animals. After the mouse *clock* gene was cloned, a fly circadian rhythm gene was cloned and found to be even more closely related to mouse *clock*, than was *per*. In a different study, a mouse gene similar to fly *per* was identified and inactivated by reverse genetics. The mutant mouse had a circadian rhythm defect, like fly *per* mutants. In other words, both flies and mice use both *clock* and *per* genes to control their circadian rhythms. A group of genes, not one gene, are conserved regulators of the circadian clock.

Characterization of these genes has led to an understanding of the molecular mechanisms of circadian rhythm and a dramatic demonstration of the similarity of these mechanisms in flies and mice. In both flies and mice, the CLOCK protein is a transcriptional activator. Together with a partner protein, it controls the transcription of genes that determine behaviors such as locomotor activity levels. CLOCK and its partner also stimulate the transcription of the *per* gene. However, PER protein represses CLOCK's ability to stimulate *per* gene expression, so as PER protein

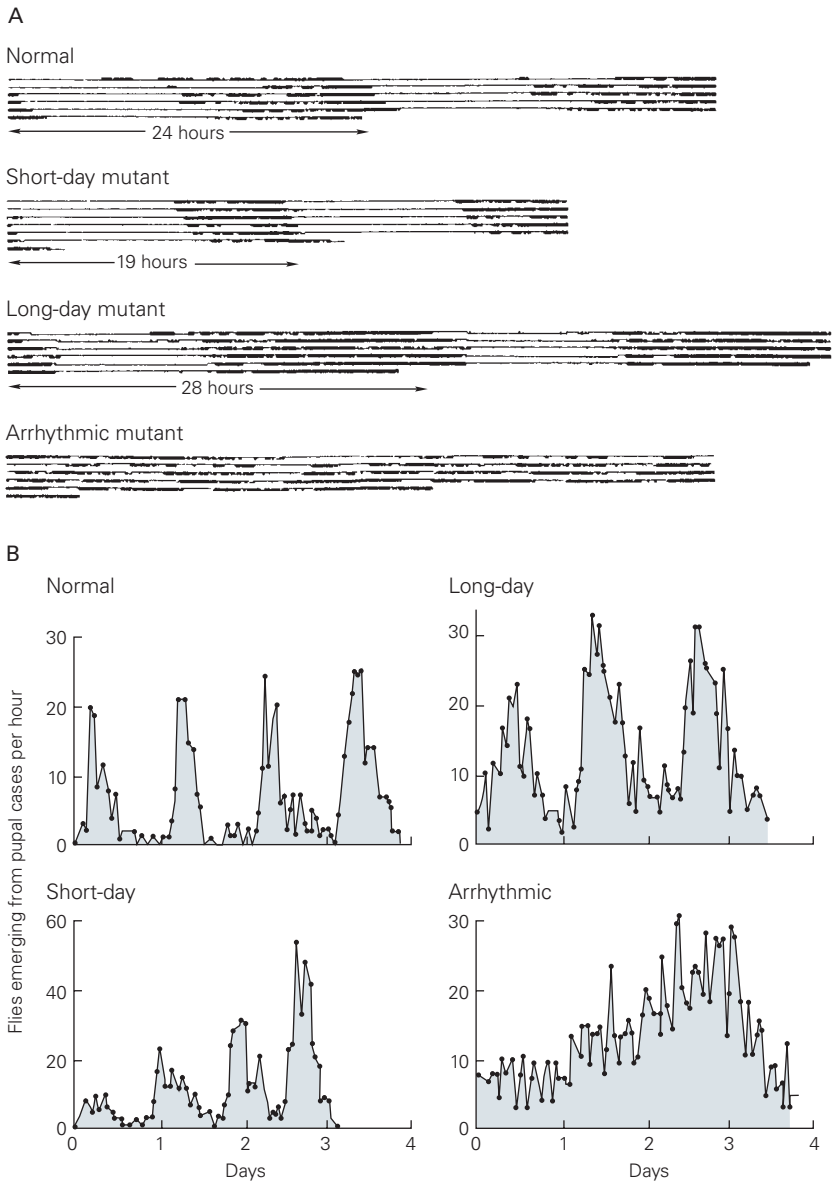

Figure 2–11 A single gene governs the circadian rhythms of behaviors in *Drosophila*. Mutations in the *period*, or *per*, gene affect all circadian behaviors regulated by the fly's internal clock. (Reproduced, with permission, from Konopka and Benzer 1971.)

A. Locomotor rhythms in normal *Drosophila* and three strains of *per* mutants: short-day, long-day, and arrhythmic. Flies were shifted from a cycle of 12 hours of light and 12 hours of dark into continuous darkness, and activity was then monitored under infrared light. Thick segments in the record indicate activity.

B. Normal adult fly populations emerge from their pupal cases in cyclic fashion, even in constant darkness. The plots show the number of flies (in each of four populations) emerging per hour over a 4-day period of constant darkness. The arrhythmic mutant population emerges without any discernible rhythm.

accumulates, *per* transcription falls (Figure 2–13). The 24-hour cycle comes about because the accumulation and activation of PER protein is delayed by many hours after the transcription of *per*, a result of PER phosphorylation, PER instability, and interactions with other cycling proteins.

The molecular properties of *per*, *clock*, and related genes generate all properties essential for circadian rhythm.

1. The transcription of circadian rhythm genes varies with the 24-hour cycle: PER activity is high at night; CLOCK activity is high during the day.

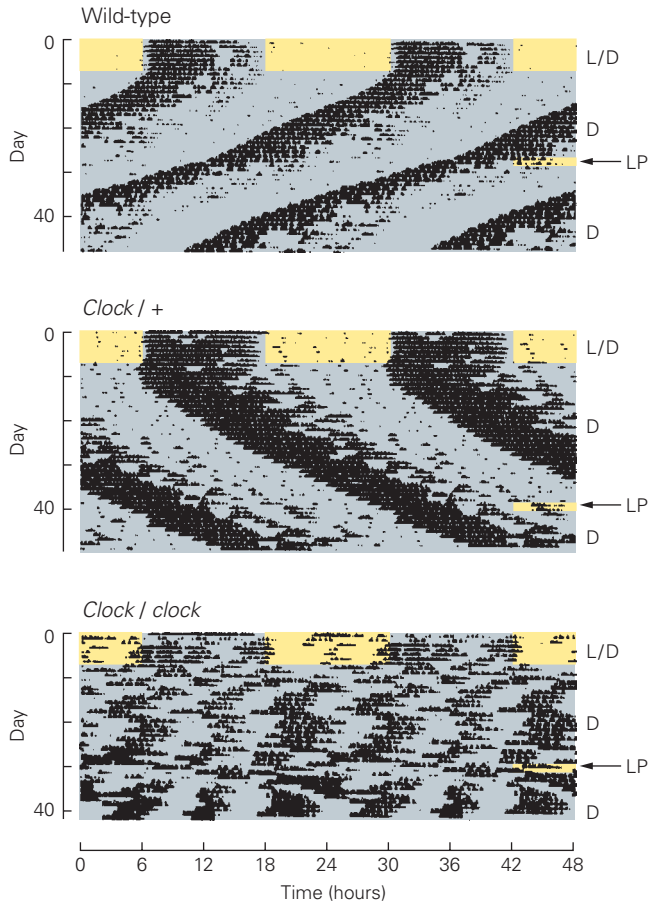

Figure 2–12 Circadian rhythm regulation by the *clock* gene in mice. The records show periods of locomotor activity by three animals: wild-type, heterozygous, and homozygous. All animals were kept on a light-dark (L/D) cycle of 12 hours for the first 7 days, then transferred to constant darkness (D). They later were exposed to a 6-hour light period (LP) to reset the rhythm. The circadian rhythm for the wild-type mouse has a period of 23.1 hours. The period for the heterozygous *clock*/+ mouse is 24.9 hours. The homozygous *clock*/*clock* mice experience a complete loss of circadian rhythmicity on transfer to constant darkness and transiently express a rhythm of 28.4 hours after the light period. (Reproduced, with permission, from Takahashi, Pinto, and Vitaterna. 1994. Copyright © 1994 AAAS.)

2. The circadian rhythm genes are transcription factors that affect each other's mRNA level, generating the oscillations. CLOCK activates *per* transcription and PER represses CLOCK function.
3. The circadian rhythm genes also control the transcription of other genes that in turn affect many downstream responses. For example, in flies, the neuropeptide gene *pdf* controls locomotor activity levels.
4. The oscillation of these genes can be reset by light.

The detailed elucidation of this molecular clock mechanism was recognized by the 2017 Nobel Prize in Physiology or Medicine, awarded to Jeffrey Hall, Michael Rosbash, and Michael Young.

The same genetic network controls circadian rhythm in humans. People with advanced sleep-phase syndrome have short 20-day cycles and an extreme early-to-bed, early-to-rise "morning lark" phenotype. Louis Ptáček and Ying-hui Fu found that these individuals have mutations in a human *per* gene. These results demonstrate that genes for behavior are conserved from insects to humans. Advanced sleep-phase syndrome is discussed in the chapter on sleep (Chapter 44).

Natural Variation in a Protein Kinase Regulates Activity in Flies and Honeybees

In the genetic studies of circadian rhythm described earlier, random mutagenesis was used to identify genes of interest in a biological process. All normal individuals have functional copies of *per*, *clock*, and the related genes; only after mutagenesis were different alleles generated. Another, more subtle question about the role of genes in behavior is to ask which genetic changes may be responsible for behavioral variation among normal individuals. Work by Marla Sokolowski and her colleagues led to the identification of the first gene associated with variation in behavior among normal individuals in a species.

Larvae of *Drosophila* vary in activity level and locomotion. Some larvae, called rovers, move over long distances (Figure 2–14). Others, called sitters, are relatively stationary. *Drosophila* larvae isolated from the wild can be either rovers or sitters, indicating that these are natural variations and not laboratory-induced mutations. These traits are heritable; rover parents have rover offspring and sitter parents have sitter offspring.

Sokolowski used crosses between different wild flies to investigate the genetic differences between rover and sitter larvae. These crosses showed that the difference between rover and sitter larvae lies in a single major gene, the *for* (forager) locus. The *for* gene encodes a signal transduction enzyme, a protein kinase activated by the cellular metabolite cGMP (cyclic guanosine 3′,5′-monophosphate). Thus this natural variation in behavior arises from altered regulation of signal transduction pathways. Many neuronal functions are regulated by protein kinases such as the cGMP-dependent kinase encoded by the *for* gene. Molecules such as protein kinases are particularly significant at transforming short-term neural signals into long-term changes in the property of a neuron or circuit.

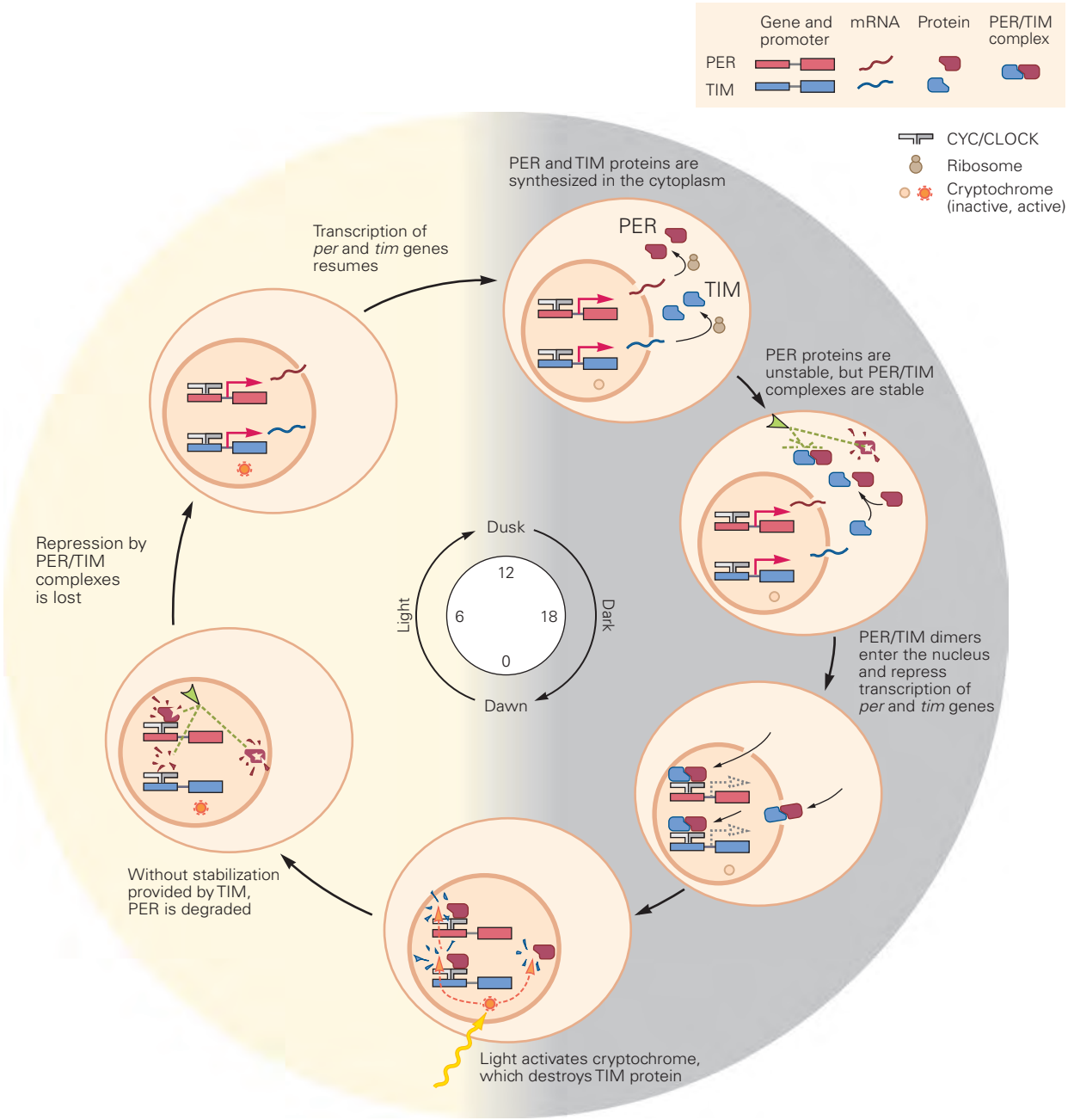

Figure 2–13 **Molecular events that drive circadian rhythm.** The genes that control the circadian clock are regulated by two nuclear proteins, PER and TIM. These proteins slowly accumulate and then bind to one another to form dimers. Once they form dimers, they enter the nucleus and shut off the expression of circadian genes including their own. They do so by inhibiting CLOCK and CYCLE, which stimulate the transcription of *per* and *tim* genes.

PER protein is highly unstable; most of it is degraded so quickly that it never has a chance to repress CLOCK-dependent *per* transcription. The degradation of PER is regulated by at least two different phosphorylation events by different protein kinases. When PER binds to TIM, PER is protected from degradation. As CLOCK drives more and more *per* and *tim* expression, enough PER and TIM eventually accumulate that the two can bind and stabilize each other, at which point they enter the nucleus where their own transcription is repressed. As a result, *per* and *tim* mRNA levels fall; thereafter, PER and TIM protein levels fall and CLOCK can (once again) drive expression of *per* and *tim* mRNA. During daylight, TIM protein is degraded by signaling pathways that are regulated by light (including cryptochrome), so PER/TIM complexes form only at night. The CLOCK protein induces PER and TIM expression but is inhibited by PER and TIM proteins.

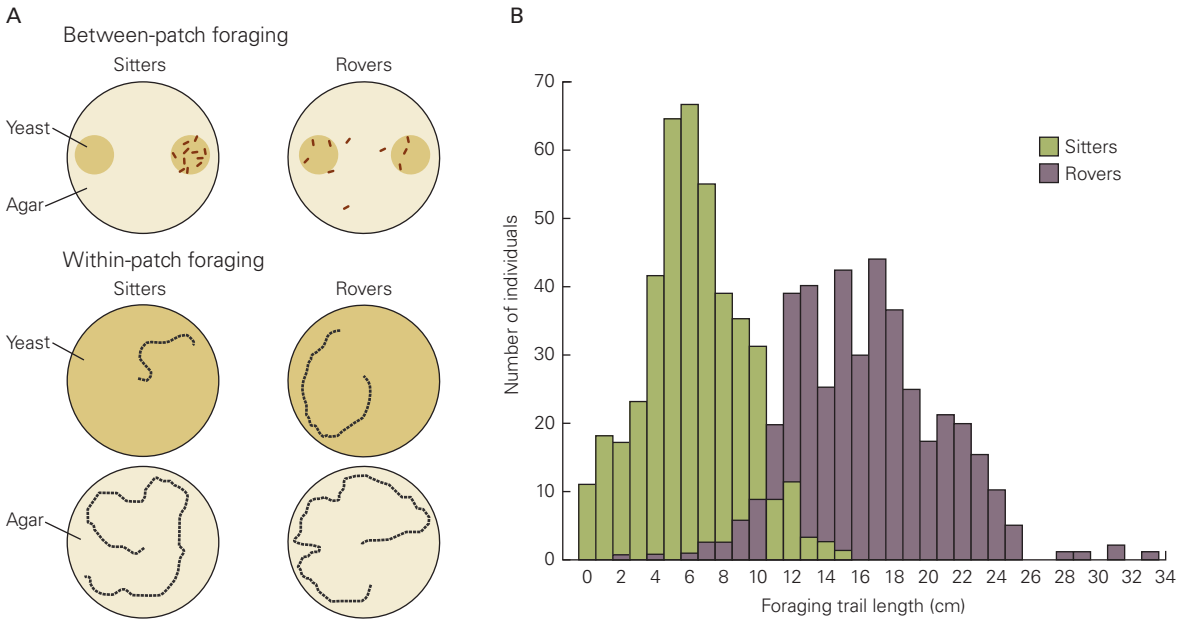

Figure 2–14 Foraging behavior of *Drosophila melanogaster* rover and sitter larvae differs while feasting on patches of yeast. (Reproduced, with permission, from Sokolowski. 2001. Copyright © 2001 Springer Nature.)

A. Rover-type larvae move from patch to patch, whereas sitter-type larvae stay put on a single patch for a long time. When foraging within a single patch, rover larvae move about more than sitter larvae. On agar alone, rover and sitter larvae move about equally.

B. While foraging within a patch of food, rovers have longer trail lengths than sitters (trail lengths were measured over a period of 5 minutes).

This difference in foraging behavior maps to a single protein kinase gene, *for*, which varies in activity in different fly larvae.

Why would variability in signaling enzymes be preserved in wild populations of *Drosophila*, which typically include both rovers and sitters? The answer is that variations in the environment create pressure for balanced selection for alternative behaviors. Crowded environments favor the rover larva, which is more effective at moving to new, unexploited food sources in advance of competitors, whereas sparse environments favor the sitter larva, which exploits the current source more thoroughly.

The *for* gene is also found in honeybees. Honeybees exhibit different behaviors at different stages of their life; in general, young bees are nurses, while older bees become foragers that leave the hive. The *for* gene is expressed at high levels in the brains of active foraging honeybees and at low levels in the younger and more stationary nurse bees. Activation of cGMP signaling in young bees can cause them to enter the forager stage prematurely; this change could be induced by an environmental stimulus or the bee's advancing age.

Thus the same gene controls variation in a behavior in two different insects, but in different ways. In the fruit fly, variations in the behavior are expressed in different individuals, whereas in the honeybee, they are expressed in one individual at different ages. The difference illustrates how an important regulatory gene can be recruited to different behavioral strategies in different species.

Neuropeptide Receptors Regulate the Social Behaviors of Several Species

Many aspects of behavior are associated with an animal's social interactions with other animals. Social behaviors are highly variable between species, yet have a large innate component within a species that is controlled genetically. A simple form of social behavior has been analyzed in the roundworm *Caenorhabditis elegans*. These animals live in soil and eat bacteria.

Different wild-type strains exhibit profound differences in feeding behavior. Animals from the standard laboratory strain are solitary, dispersing across a lawn of bacterial food and failing to interact with each other. Other strains have a social feeding pattern, joining large feeding groups of dozens or hundreds of animals (Figure 2–15). The difference between these strains is genetic, as both feeding patterns are stably inherited.

The difference between social and solitary worms is caused by a single amino acid substitution in a single gene, a member of a large family of genes involved in