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Fly Clocks

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I. Introduction

As a result of the combination of classic genetics, modern molecular biology, and a remarkably precise behavioral assay, studies in *Drosophila* have generated an explosion in our understanding of the basic molecular mechanisms of circadian rhythms. Furthermore, the identification of *Drosophila* clock genes has led to the isolation of the mammalian counterparts, including some from humans. It is probably only a matter of time before alterations in the structure or expression of these genes is intimately linked with diseases thought to be caused by clock defects. This chapter outlines the monumental contributions of forward genetics in mice as well as in flies to our understanding of the molecular basis of circadian rhythmicity.

Circadian rhythms are classically defined by a few core properties. First, they proceed under constant environmental conditions. Thus, daily behaviors, such as the sleep–wake cycle, may proceed in the absence of exposure to the solar cycle and thus are driven by an endogenous mechanism. When placed in an underground cellar isolated from all environmental or temporal cues, experimental subjects continue to experience a daily sleep–wake cycle [1]. Similarly

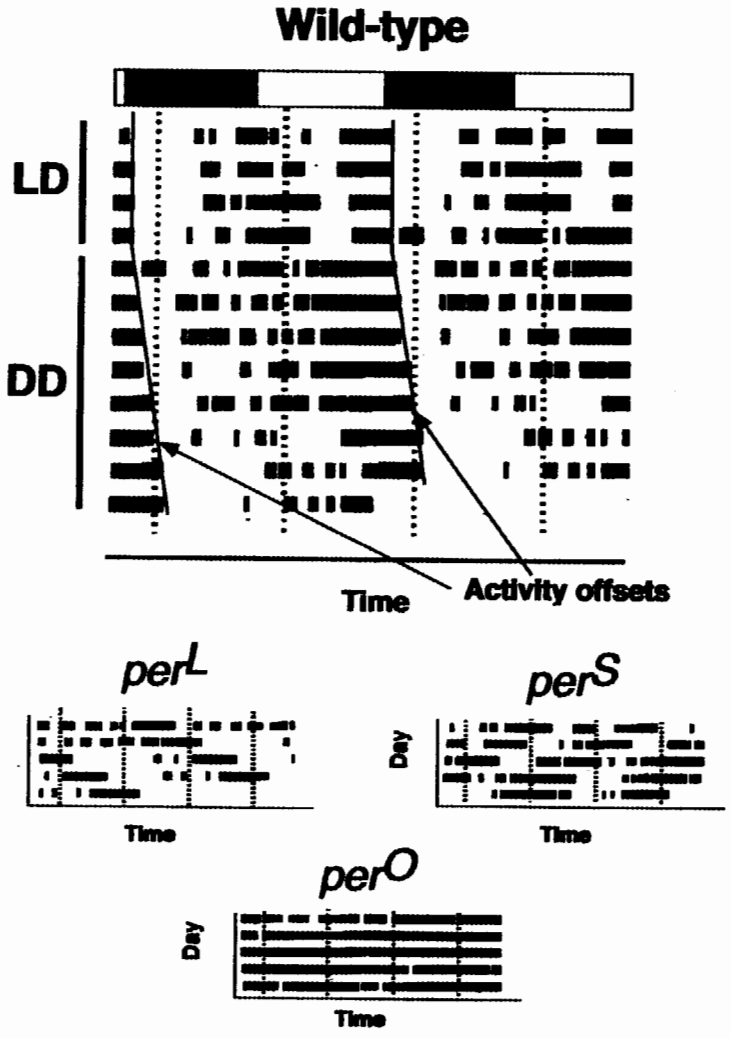

Figure 1 Actogram or activity plot from wild-type and *period* mutant fruit flies. For wild-type flies on the top actogram, 2 days of data are plotted on each line. Each dark horizontal bar reflects locomotor activity. Darker areas with more bars indicate times of higher activity; lighter areas with fewer bars indicate times of lower activity. Transitions from high activity to low activity, called activity offsets, are prominent. In constant darkness (DD) conditions, these offsets occur slightly later and later each subjective day, indicating a periodicity of slightly greater than 24 hr. Under 12-hr light–12-hr dark entrainment (LD), these offsets synchronize to the 24-hr LD cycle. In the actogram of per^L mutant and per^S mutant, activity offset occurs later and later for per^L and earlier and earlier for per^S in DD conditions, reflecting their internal periodicities. The actogram of per^O mutant reveals no apparent rhythmicity.

in the fruit fly (Fig. 1), a circadian rhythm of locomotor activity persists under constant darkness (DD) conditions.

Second, rhythmic phenomena display a periodicity of approximately, but not exactly, 24 hr; hence the term *circadian* (L. *circa* = approximate, *dia* = day). As a consequence, humans generally, but not exclusively, exhibit a sleep–wake rhythm which slightly exceeds 24 hr when isolated from temporal cues. These individuals will gradually go to sleep and wake up later and later with respect to external objective time. In fruit flies (Fig. 1), bouts of high activity also drift later and later with respect to external time, reflecting a slightly long-period clock. This consistent imprecision of clocks may contribute to the difficulty many people experience crawling out of bed on Monday morning after allowing themselves to follow their long-period clocks over the weekend.

Third, environmental signals are capable of resetting the clock. Light is probably the most dominant, and internal clocks are synchronized by the 24-hr solar cycle. As a result, most individuals generally wake up and go to sleep at about the same time every day. If fruit flies are maintained in 12-hr light–12-hr dark cycles (LD; see Fig. 1), their locomotor activity rhythm becomes synchronized to this external cycle. In addition, activity changes precede the environmental transitions, reflecting an anticipatory role of the clock. Keeping time rather than simply responding to external stimuli is crucial to fitness and survival throughout biology, as comparable clocks have been identified even in photosynthetic cyanobacteria.

Finally, the periodicity of clocks is largely invariant over a physiological range of temperatures. In stark contrast, the behavior of most proteins are dramatically influenced by temperature. This temperature compensation may be considered a necessary adaptation for poikilotherms to prevent ambient temperature changes from causing dramatic shifts in the phase of internal clocks. However, cultured rhythmic tissues from homeotherms are also temperature compensated [2]. Temperature compensation, therefore, appears to be a fundamental clock property; perhaps reflecting the benefits of a robust homeostatic system to defend against many environmental perturbations.

II. Circadian Rhythms in Human Health and Disease

Given the ubiquitous nature of circadian clocks, circadian biologists are puzzled that the role of rhythms in human health and disease appears to be underappreciated. Circadian clocks greatly influence the rates of myocardial infarction, which occurs far more commonly in the morning than at other times of the day [3]. This predictable temporal pattern of variation has implications for the dosing of cardiac medications [4]. Blood pressure also varies significantly with the time

of day, which has implications for the clinical diagnosis of hypertension [5]. Finally, airway pressure in asthmatics appears to increase significantly during the night, leading to the institution of nocturnal dosing of antiasthmatic medications [6].

In addition to the circadian influence on diseases, certain disorders, called circadian dysrhythmias, are thought to be due to primary defects in the central clock mechanism. The most obvious manifestations of a disturbed circadian clock are sleep disorders. People with delayed sleep phase syndrome (DSPS) have great difficulty going to sleep at night and difficulty waking in the morning. People with advance sleep phase syndrome (ASPS) have the opposite problem; that is, difficulty staying awake in the evening with consequent early morning awakening. These patients presumably manifest long-period clock and short-period endogenous clocks, respectively. Recently, families have been identified with an inherited form of ASPS [7].

Jet lag results from the limited ability of circadian clocks to reset rapidly to new external time. Rapid jet travel across several time zones and the consequent desynchronization of internal clocks from external time results in the pathology of this disorder. Furthermore, the typically long (>24 hr) endogenous human periodicity explains why most people experience less jet lag when traveling westward. In this direction, we have to lengthen our internal period to adapt to the new environmental cycle. Traveling eastward requires a shortening of our already >24-hr period and is therefore more difficult. This subjective impression of jet travelers has been confirmed more objectively by analyzing retrospectively the performance of professional baseball teams as a function of their direction of travel [8].

A less obvious and somewhat more controversial notion is that clock defects lead to pathological mood states, such as bipolar disorder and seasonal affective disorder. Seasonality, or changes in behavior with the season, is thought to be mediated in part by circadian clocks; they are capable of telling the season by sensing changes in day length. In many people, extreme seasonality can lead to "winter depression" or seasonal affective disorder. In fact, the therapeutic efficacy of light for winter depression is correlated with its ability to advance the clock [9]. In addition, bipolar disorder, a far more common disease, may also be due to disrupted clocks. It is intriguing in this context that the mood stabilizer lithium (whose mechanism of action remains unknown) also has clock-resetting properties at therapeutic doses [10].

III. A Mammalian Clock: The Suprachiasmatic Nucleus

Much of the circadian work during the last three decades has been devoted to the identification and analysis of the anatomical substrate of the mammalian clock, the suprachiasmatic nucleus (SCN). This pacemaker locus consists of a large collection of hypothalamic neurons (approximately 10,000) located just above the optic chiasm. This region was first identified as a direct target of retinal projections and its function was confirmed in lesion experiments [11,12]. An elegant series of lesion and transplantation rescue experiments solidified the role of the SCN not only in being required for rhythmicity but also in being sufficient to determine rhythmic properties. One of the key experiments resulted from the serendipitous discovery of a spontaneously mutant short-period hamster named *tau* [13]. In lesion/transplantation experiments, the period of the rescued animal reflected the genotype of the donor tissue, virtually proving that the SCN was the seat of the mammalian clock [14]. The discovery of *tau* marked the beginning of clock genetics in mammals. However, the absence of genetic markers in the hamster has hindered cloning efforts.

The SCN not only mediates rhythmic behaviors but also manifests rhythmic physiological properties. For example, this group of neurons exhibits 24-hr metabolic rhythms in intact animals as measured by 2-deoxyglucose uptake [15]. In dissociated cell culture, SCN neurons exhibit circadian regulation of spontaneous activity even when electrically isolated. Rhythmicity is therefore a property of individual SCN neurons [16]. Despite the work of many SCN biologists, little was known about the molecular underpinnings of these intracellular rhythms.

IV. Circadian Rhythms Are Influenced by Genes: Behavioral Genetics and the *period* Mutants

The application of classic genetics in model organisms such as *Drosophila* led to the first major insights into the molecular basis of circadian rhythms. During the late 1960s, Konopka, a graduate student in Benzer's laboratory at the California Institute of Technology, initiated genetic screens in *Drosophila* to find mutants with altered or missing rhythms [17]. It had been observed by fruit fly biologists, who routinely select young virgin female flies for mating crosses, that adult flies tend to emerge from their pupal case at dawn. This rhythm persists even under constant dark conditions, and is thus circadian [18]. Like pioneers earlier in the century who had screened mutagenized flies for altered eye colors or other morphological phenotypes, Konopka and Benzer devised a simple screen to identify adults that emerged or eclosed at the "wrong" time; that is, at times when wild-type flies do not normally emerge. They identified three mutant strains (see Fig. 1): One had a long period of 29 hr under constant darkness conditions, a second had a short period of 19 hr, and a third had no measurable rhythmic features. All three mutant phenotypes mapped to a single locus on the X chromosome, termed *period* (*per*). Interestingly, these mutants did not have any other obvious defect in behavior and physiology. Therefore, the *period* gene appeared

to be dedicated to clock function. The success of this very simple approach was due in part to the precision of the eclosion assay, which could easily detect the difference between strains with 24- or 29-hr periods (approximately 20% difference). Few behavioral assays have this magnitude of precision. Second, the short generation time (10 days) as well as abundant genetic tools in *Drosophila* made it an ideal organism for a classic genetic attack on this enigmatic behavior.

Subsequent genetic screens have assayed the circadian locomotor activity of individual flies. This assay has since been miniaturized and automated for high-throughput analysis. Single flies are placed into small transparent glass tubes and inserted into monitors, which have infrared beams perpendicular to the length of the tube. The infrared light is not visible to the adult fly to prevent a light influence on clock functions. Each time the fly crosses the infrared beam, one activity unit is measured. Activity levels as a function of time are then analyzed to produce a period measurement.

V. Circadian Clocks Consist of Circadian Transcriptional Feedback Loops: The *period* Gene

Prior to the era of clock gene cloning, circadian rhythms were largely viewed as an emergent property of neuronal populations [19]. The cloning and analysis of the *period* gene reformulated the circadian clock as an intracellular transcriptional feedback loop at its center. Konopka and Benzer were clearly ahead of their time, as the requisite molecular tools to clone the *period* gene did not emerge until the early 1980s. The cloning and sequencing of *per* revealed that the period-altering *per* mutants contained missense mutations only. The arrhythmic *per* allele contained a premature stop codon, a gratifying result considering the clockless phenotype [20,21]. However, neither the cloning nor the sequencing of *per* led to any immediate functional insight, as it was a pioneer protein with no obvious relatives. During these early days, it was proposed that *period* may be a cell surface molecule; consistent with notions of coupled oscillator models [22,23]. But subsequent noncircadian work identified a substantial homology with the basic helix–loop–helix transcription factor, *single-minded* (*sim*). However, *per* itself does not contain any canonical DNA-binding domains, which made the relationship with transcription factors less than certain [24].

This confusion was resolved by the observations that (1) *per* RNA cycles with a 24-hr period, (2) missense mutants which cause short- and long-period phenotypes alter in a parallel manner the period of the RNA cycling, and (3) the arrhythmic *per* allele abolishes *per* RNA cycling [25]. In addition, transient induction of *per* results in stable shifts in the phase of the clock [26]. Taken together, these observations indicate that *per* protein regulates the levels of its own RNA.

This feedback appears to be largely transcriptional. Genomic regions 5′ to the *per* coding region, the putative *per* promoter, mediate cycling of a reporter RNA, indicating that *per* RNA oscillations are at least in part mediated by transcriptional control [27]. In addition, PER levels, its phosphorylation state and nuclear localization all undergo rhythmic changes [28–31]. The general view is that *per* transcription rises during the day leading to rises in *per* RNA. PER then undergoes a set of posttranscriptional events, which results in translocation into the nucleus and inhibition of its own transcription (Fig. 2). This idea of a *per*-based circadian transcriptional feedback loop is the central organizing principle for the field of molecular rhythms. A large fraction of research during the last decade in flies and more recently in mammals has been devoted toward advancing our understanding of this molecular cycle.

VI. Molecular Mechanisms of Timekeeping Delays in the Feedback Loop: The *timeless* Gene

The cloning of the second circadian rhythm gene in *Drosophila*, the *timeless* (*tim*) gene was not reported until a full decade after *per*; a testament to the difficulty of the problem. Nonetheless, *tim* was initially identified by classic behavioral genetics as a recessive arrhythmic mutant [32]. To this day, *timeless* remains somewhat enigmatic owing to a lack of substantial sequence similarity with any well-studied gene family. However, *timeless* RNA and protein cycle with 24-hr periods like *per*. Arrhythmic *tim* mutants abolish both *per* and *tim* RNA cycling, and arrhythmic *per* mutants abolish RNA cycling from both genes, indicating reciprocal feedback regulation [32,33]. Taken together with similar observations from other organisms, rhythmic expression and feedback regulation appear to be general features of clock genes.

To generate a free-running oscillation, there must be one or more delays between the synthesis of RNA and proteins and feedback onto transcription of genes. Delays are crucial to keeping time and are distinguishing characteristics of circadian feedback systems. In the absence of delays, a feedback system does not oscillate and ultimately comes to a static equilibrium. Therefore, a major question for circadian biologists is what are the molecular mechanisms that delay *period* protein's negative feedback on its own transcription. The positional cloning of *tim*, allowed the first molecular forays into the delay problem. Initial work on PER focused on a region of sequence similarity with *Drosophila single-minded* (*sim*) and the aryl hydrocarbon nuclear translocator (ARNT), known as the PAS (*per*-ARNT-*sim*) domain. In fact, the PAS domain mediates homotypic and heterotypic PAS–PAS interactions in vitro and is capable of coimmunoprecipitating PAS proteins from fly head extracts, indicating a potential role in protein dimerization [34,35]. The in vivo relevance of the PAS domain to *period*

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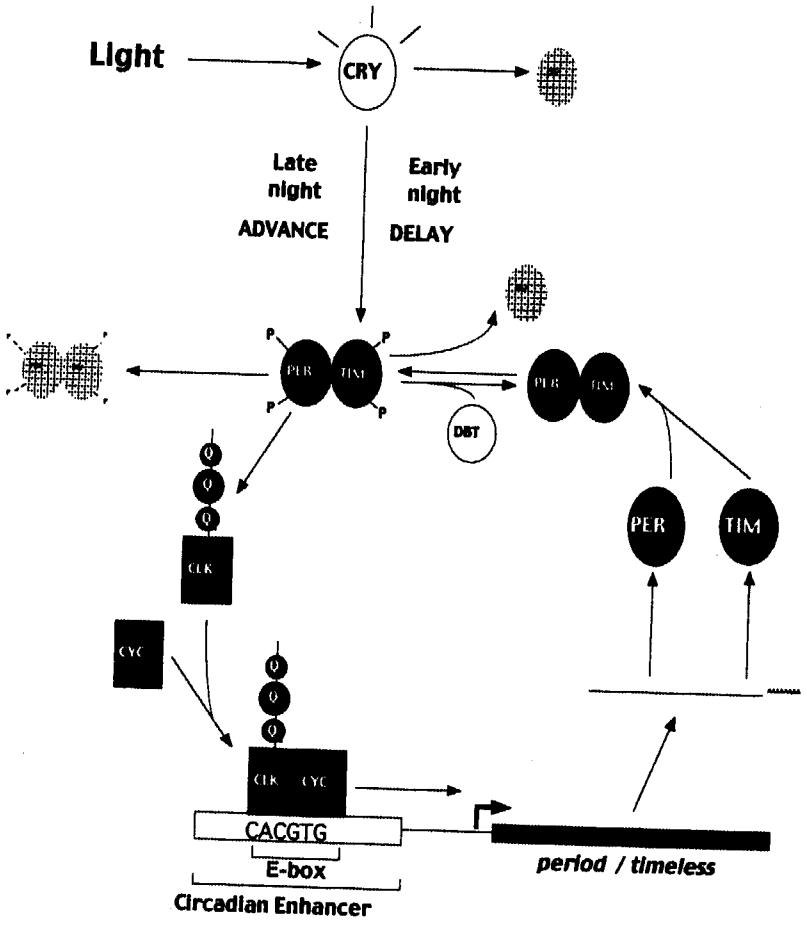

Figure 2 The fly circadian clock. The circadian rhythm genes *period* (*per*) and *timeless* (*tim*) are rhythmically transcribed by the CLOCK (CLK) and CYCLE (CYC) bHLH-PAS transcription factors. CLK and CYC associate with each other and bind to rhythmically regulated E-boxes (CACGTG) in the *per* and *tim* promoters. Q, polyglutamine tracts thought to be important for activation function and deleted in the *Jrk* allele of *Clock*. PER and TIM proteins are able to physically associate with each other, become progressively phosphorylated and translocate to the nucleus to inhibit activation by the CLK–CYC complex. PER and TIM proteins become degraded, thus reinitiating a cycle. DOUBLETIME (DBT), a kinase, appears to play a crucial role in PER phosphorylation and degradation. Light is perceived by CRYPTOCHROME (CRY) which associates with TIM, resulting in degradation of both proteins. In the early night (ZT 15), degradation of TIM slows TIM accumulation, thus delaying the clock. ZT, Zeitgeber time where experiment is done under 12-hr light:12-hr dark conditions. ZT 0, lights on and ZT 12, lights off. In the late night (ZT 21), degradation of TIM speeds TIM degradation, thus advancing the clock.

function is buttressed by the identification of a missense mutation in the PAS domain of a period-lengthening allele of *per* (per^L). Therefore, a (PAS-containing?) PER-interacting factor may impose a delay on PER-mediated feedback. *timeless* not only behaves like *period* in its temporal pattern of expression, but it also appears to be intimately intertwined with *period* both genetically and biochemically. In addition to its effects on *per* RNA, *tim* is also required for nuclear localization of PER protein [36]. Coexpression of PER and TIM in *Drosophila* tissue culture cells results in the mutually dependent nuclear localization of these two proteins [37]. They interact strongly in the yeast two-hybrid assay, and their contact is seriously disrupted by the per^L PAS mutation [38]. These in vitro interactions are supported by both genetic and biochemical experiments in vivo. An allele-specific suppressor of per^L has been identified in *tim*, and although TIM does not contain a PAS domain, it represents the most potent in vivo partner of PER in coimmunoprecipitation experiments [39,40]. Thus, non-PAS proteins can directly interact with PAS-containing proteins. A required interaction between PER and TIM for nuclear entry may impose an additional temporal delay between the appearance of PER and transcriptional feedback.

VII. How Do Molecular Clocks Become Synchronized to External Light–Dark Cycles?

Circadian clocks do not simply free-run in contrived constant darkness conditions but must function in natural light–dark environments. In addition, they must synchronize or entrain to the external environment. How does the clock, that is, the PER–TIM cycle, become synchronized to the light–dark cycle? A significant step forward came with the observation that TIM protein levels, unlike those of PER, are suppressed within minutes of exposure to light [40–43]. Therefore, reduction in TIM protein levels may explain in part how the molecular cycle adjusts to changes in the external light regimen. In addition, the TIM light response appears to explain the clock response to brief light pulses (see Fig. 2). For decades circadian biologists used the resetting of the clock phase by short light pulses (minutes) as an experimental paradigm to assess both the state of the clock and its photoreceptive pathways [44]. The administration of light pulses at different times of day (in otherwise constant darkness) causes a phase advance during late night, phase delays during early night, and no changes during subjective day. This phenomenon correlates well with TIM protein levels and their response to light. When TIM levels are falling during the advance zone, a light pulse would hasten the disappearance of TIM and advance the clock. When TIM levels are rising during the delay zone, a light pulse would slow the appearance of TIM and delay the clock. Finally, when TIM levels are very low, light pulses

cause little or no phase shifts. Thus, TIM appears to play a role in the running of the clock as well as in responding to light.

VIII. Did Nature Create Different Clocks for Different Organisms?: The Search for Mammalian Clock Gene Homologs

One of the most striking observations from the last two decades of molecular biology is the high degree of evolutionary conservation in gene structure and function even between unicellular organisms and humans. Consider the *Drosophila eyeless* transcription factor, which is required for proper eye development [45]. Remarkably, ectopic expression of *eyeless* leads to the formation of ectopic eyes. Mutations in the human homolog Pax6 lead to aniridia, the absence of the iris muscle which controls pupil size [46]. Despite the obvious anatomical differences between the structure of the *Drosophila* and human eyes, the ectopic overexpression of the human homolog in flies also results in the ectopic development of *Drosophila* eyes [47]. Therefore, *eyeless* and Pax6 not only share structural similarity at the sequence level and parallel functional roles in visual organ development, these functions can be transferred between these divergent species. Given this and other similar observations, it was widely believed that there should be a mammalian ortholog of *per* and that its identification would serve as a molecular entree into the study of mammalian circadian rhythms. However, despite attempts by several laboratories, no such molecules were convincingly identified. Perhaps early developmental pathways or housekeeping (e.g., basal transcription or splicing) functions are conserved, but complex brain functions such as circadian rhythms may have arisen independently between the simple fly and complex mammals.

IX. Konopka and Benzer in the Mouse: Mammalian Forward Genetics

Given the dearth of progress in the cloning of mammalian homologs, a forward behavioral genetic approach in mice was initiated. Inspired by the work of Konopka and Benzer, mutagenized populations of mice were screened for circadian rhythm phenotypes as assayed by rhythmic wheel-running behavior. Such a screen had obvious technical obstacles to overcome; primarily the relatively poor state of the mouse as a useful organism for forward genetics. In addition to the long generation times and maintenance costs, the density of genetic markers, especially in relation to *Drosophila*, made positional cloning of induced single–base pair mutations especially difficult. Despite these challenges, there were many arguments in favor of screening for rhythmic phenotypes in mice. The

wheel-running assay is very precise, facilitating the detection of subtle phenotypes. Furthermore, it had been observed that nearly every circadian rhythm mutant in *Drosophila* exhibited a semidominant phenotype [17]. Assuming a similar situation in the mouse, an F1 screen for dominant phenotypes would eliminate the need for additional backcrosses to homozygose-induced mutations. Finally, the advance of the Human Genome Project and the advent of transgenic technologies in mammals facilitated the identification and functional analysis of candidate clones once mutant phenotypes were finely mapped.

These advantages proved instrumental in the identification of a mutant, *Clock*. Heterozygotes displayed a slightly long period and homozygotes had even longer periods grading into arrhythmicity [48]. A variety of defects in general physiology could lead to arrhythmic behavior, but the slight period alterations of the heterozygote suggested that this mutation may have disrupted a central timekeeping component [49]. The subsequent effort to map and clone *Clock* revealed that it contained a PAS domain, placing it in the same family as *per* [50,51]. However, *Clock* also contained a basic helix–loop–helix domain (bHLH), previously shown in other DNA-binding transcription factors to play a role in direct protein–DNA contacts and protein–protein dimerization. As expected, *Clock* RNA is expressed in the SCN, but its levels do not oscillate with circadian time [50]. Although *Clock* was not a true ortholog of *per*, their PAS domains indicated family ties and raised the possibility that the fly and mammalian circadian systems were more well-conserved than suggested by the failure of previous cloning efforts.

X. Nature Does Not Create New Ways of Making Clocks: The Genome Projects and the Cloning of New Evolutionarily Conserved *period* Genes

More than 10 years after the cloning of *Drosophila per*, two independent groups reported the cloning of a putative human ortholog (*hPer*). Using a series of degenerate oligonucleotides complementary to the PAS domain of *Drosophila per* (*dper*), *hPer* was isolated by polymerase chain reaction (PCR) [52]. As part of the Human Genome Project, another group discovered *hPer* while sequencing cDNAs from chromosome 17 [53]. Several previously identified genes had PAS domains and therefore limited homology to *Drosophila per*. However, this putative human *per* had homology with *dper* thoughout its full length [52,53]. The analysis of mouse homologs revealed that mouse *per* (*mPer*) not only is expressed in the SCN, but its transcript also oscillates with a 24-hr period in constant darkness [52,53]. Interestingly, *mPer* cycling in this nocturnal (night-active) animal is antiphase to *per* cycling in the diurnal (day-active) *Drosophila*. Furthermore, changes in the lighting regimen which shifted the behavioral circadian rhythm

similarly shifted the RNA cycle phase [52,53]. Thus, mammalian *per* not only had structural similarity with *Drosophila per*, but also executed similar functions based on its expression profile.

Genome database searches identified two additional mammalian *per* homologs, *mPer2* and *mPer3*, both of which cycle in phase with *mPer1* in the SCN [54–56]. Further study distinguished the three mammalian *per* homologs from fly *per* and from each other. The most notable difference is that *mPer1* and *mPer2*, but not *mPer3* or *dper*, are rapidly induced by short light pulses [54,57,58]. However, this response is circadianly gated. As mentioned above, the ability of light to phase shift or reset circadian clocks is dependent on the circadian time at which the light pulse is administered. In other words, the circadian clock itself regulates the efficacy of light-induced resetting. Therefore, *per* induction by light correlates well with the ability of light to phase shift the clock, implicating this molecular response in light-mediated phase shifts. However, there is currently no genetic evidence which demonstrates a requirement of *per* induction for light-mediated clock resetting.

Recent genetic evidence suggests that an autoregulatory feedback loop underlies the mammalian clock. Deletion of a portion of the PAS domain of *mPer2* in vivo results in short periods grading into arrhythmicity, indicating a functional role in the central pacemaker [59]. In addition, the cycling levels and amplitude of *mPer1* and *mPer2* RNAs is substantially reduced, suggesting the presence of feedback regulation. Thus, the notion of feedback loops, established in *Drosophila*, can be extended to mammals.

XI. Getting Cyc'd about Clocks: Flies and Mammals Share Circadian Transcription Factors

Given the penchant of transcription factors to heterodimerize, it was thought that the *period* PAS domain may represent an interface for protein–protein contacts with a transcriptional activator. Unlike the other PAS family members, the *period* gene does not contain a canonical DNA-binding basic helix–loop–helix domain. Thus, the heterodimerization model remained speculative. Studies of the *Drosophila period* promoter identified a 69–base pair circadian enhancer which requires the presence of an E-box (CACGTG) to confer high-amplitude cycling to a reporter gene. Such E-boxes are known binding sites of bHLH-PAS transcription factors [60]. Thus, it was proposed that the PER-PAS domain may serve as a protein–protein contact to inhibit the activity of an undefined bHLH-PAS transcription factor. Concurrent with the mapping and cloning of *Clock*, rhythm mutagenesis screens in flies identified three novel complementation groups distinct from *per* and *tim*. One group was initially defined by a mutant named *Jrk*. This semidominant mutant displayed a slight period alteration with substantial arrhythmicity as a heterozygote. *Jrk* flies were completely arrhythmic as homozygotes [61]. In addition, *per* and *tim* transcription, RNA levels, and protein levels were pegged at trough levels in homozygous *Jrk* flies [61]. A second group, called *cycle*, shared many similarities with *Jrk*. As a homozygote, *cycle* also abolished locomotor rhythms and severely reduced *per* and *tim* transcription, RNA, and protein levels [62]. Given their phenotype, the two genes appeared to encode important positive components for the transcripton of clock genes, perhaps even bHLH-PAS transcription factors. A search of the ever-expanding expressed sequence tag (EST) database for new members of the *Drosophila* bHLH-PAS fam-

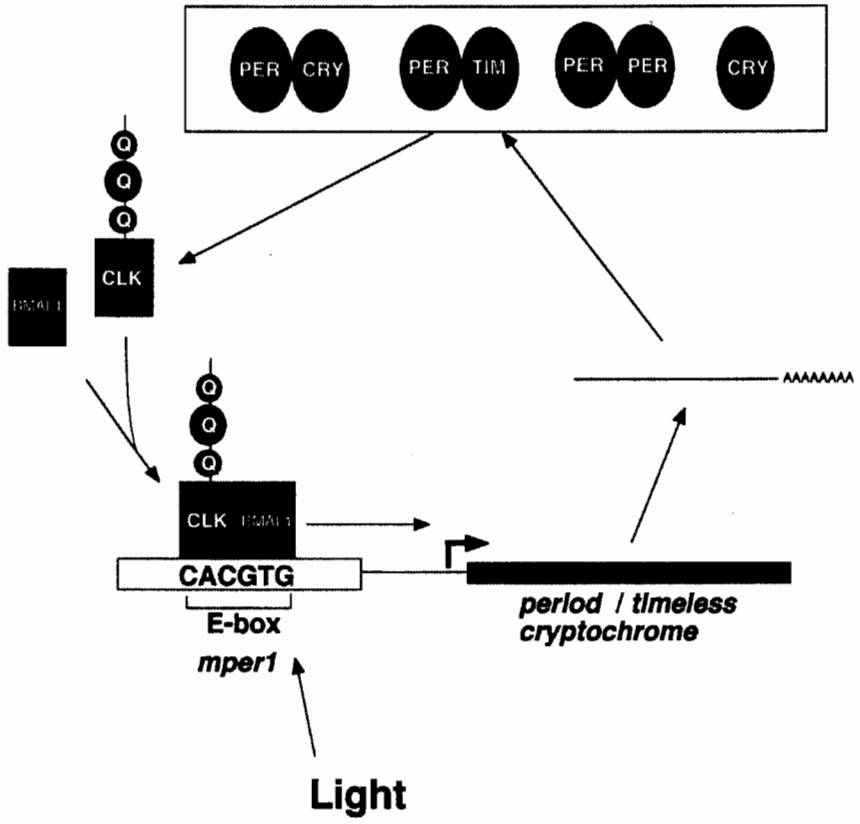

Figure 3 The mammalian circadian clock. CLOCK and the mammalian homolog of BMAL1 activate transcription from E-boxes. The *per*, *cry*, and *tim* (in part) homologs are rhythmically expressed. Line with AAAAAA indicates polyA+ mRNA. Various combinations of PER, TIM, and CRY homologs can feedback and inhibit transcription of the CLOCK–BMAL complex. CRY appears to be the most potent inhibitor.

ily identified two novel members: one, *Drosophila Clock* (*dClk*), displayed striking homology with mouse *Clock* and chromosomally colocalized with *Jrk*. The sequencing of *dClk* in *Jrk* identified a premature stop codon removing much of the glutamine-rich C terminus presumably involved in transcriptional activation [61]. This truncated form is predicted to behave as a dominant negative transcription factor, which is consistent with the dominant phenotype observed in flies. The other novel bHLH-PAS gene showed striking homology with the mammalian gene *BMAL1* and colocalized with *cycle*. Sequencing also revealed a premature stop codon in *cycle* mutant flies, which is consistent with its null phenotype.

A search for the partner of mouse CLOCK using a two hybrid approach also identified BMAL1 [63]. Furthermore, these proteins interact and activate from E-boxes in the *mPer1* promoter. *Drosophila Clock* and *cycle* were also cloned by homology and were shown to interact with each other, bind to functionally relevant E boxes (CACGTG) in the *per* and *tim* promoters, and transcriptionally activate *per* and *tim* [64]. Thus, the CLK : CYC heterodimer appears to be the positive transcription factor that transcribes *per* and *tim*.

In addition, coexpression of *per* and *tim* modestly inhibit (about two- to threefold) the activity of the CLK : CYC complex in cell culture [64]. Consistent with feedback inhibition, PER and TIM proteins coimmunoprecipitate with dCLK in fly head extracts at times of falling transcription rates [65]. In fact, a mammalian *tim* homolog has also been identified and may also participate with PER homologs to feedback on CLOCK-BMAL1–mediated transcription (Fig. 3) [66,67]. These complementary studies in flies and mice revealed the central transcriptional components of the circadian feedback loop and solidified the evolutionary connection between flies and mice. They also demonstrate the growing synergy between classic genetic methods and genome projects, which appear to have replaced to some extent the traditional role of individual molecular biologists.

XII. The Postranslational Clock: The *doubletime* Kinase

Although the current view of circadian clocks has focused on the role of transcription, there is accumulating evidence that posttranscriptional mechanisms also contribute and may even be the predominant means of maintaining self-sustaining oscillations. They also may contribute to the delays that are so important to the molecular feedback cycle. In *Drosophila*, promoterless *per* transgenes are capable of rescuing both RNA cycling and behavioral rhythms in a null *per* genetic background [68]. Nuclear run-on analysis in these flies clearly demonstrated an absence of transcriptional cycling, implying a reliance on posttranscriptional regulation [69]. In addition, there is direct evidence that posttranslational control regulation is also important. For example, antibodies to PER and TIM proteins

reveal that they undergo progressive changes in mobility with respect to time of day [30,40–43]. These mobility changes appear to be due to changes in the phosphorylation state, as they are reversed by phosphatase treatment. The time of the lowest mobility (most phosphorylation) immediately precedes the disappearance of the protein, suggesting that phosphorylation may be a signal for activation of the proteolytic machinery. Furthermore, short- and long-period alleles of *per* appear to affect the magnitude and timing of these phosphorylation changes, suggesting, but certainly not proving, that phosphorylation may be a functional element of the clock [30]. Similar rhythmic changes in the phosphorylation state have been observed in the product of the *Neurospora* circadian rhythm gene, *frequency* [70]. However, genetic evidence demonstrating the importance of these phosphorylation changes in PER was lacking until the identification of the relevant kinase, the *doubletime* gene.

Like *per*, *tim*, *Clock*, and *cyc*, *doubletime* was originally identified in behavioral screens [71]. Short-period, arrhythmic, and long-period alleles as well as a homozygous lethal allele were identified. In fact, the lethal *doubletime* allele remains the only clock gene mutant with a developmental phenotype, suggesting that there may be more circadian gold to be mined among mutants with more pleiotropic phenotypes. The *doubletime* (*dbt*) gene encodes a serine–threonine kinase highly homologous with the mammalian casein kinase I-epsilon [72]. Short- and long-period alleles shift both *per* and *tim* RNA and protein profiles, including their phosphorylation states [71]. In the null homozygous lethal mutant, PER levels were examined in homozygous viable larvae (only the adults are dead). High PER levels in these larvae indicate that *doubletime* may regulate PER stability, which is consistent with previous correlations between phosphorylation state and protein disappearance [71]. In addition, *doubletime* protein (DBT) physically associates with PER in cotransfection experiments [72]. Thus, DBT appears to be involved in PER phosphorylation, which may be required for PER degradation. Although genetic and biochemical data circumstantially link *dbt* with *per*, there is no direct evidence that *per* is a phosphorylation target of *doubletime*. Nonetheless, forward genetics in the fruit fly and the subsequent cloning of *doubletime* has provided the first molecular handle on the role of phosphorylation in the circadian clock. As day follows night, the cloning of this fly rhythm gene will be followed by knockout of the mammalian counterpart to address its potential role in rhythms.

XIII. A Clockwork Blue: The Blue Light–Sensitive *cryptochrome* Genes and the Eyes of the Clock

Mammals and insects appear to utilize physiologically distinct photoreceptive pathways for circadian entrainment and phase shifting. In mammals, eyes are

essential, but circadian photoreception does not appear to require the presence of retinal rods or cone photoreceptor cells [73,74]. However, the action spectrum of phase-shifting light correlates well with those of vitamin A–based opsin photoreceptors [75]. Thus, it has been proposed that an unconventional opsin may mediate the phase-shifting effects of light. These action spectra, however, do not exclude a role for multiple photoreceptors, or multiple types of photoreceptors, that is, nonopsins, which in combination produce this action spectrum. In fact, it may even be the case that rod and cone–based photoreception is sufficient for phase shifting, but its lack of requirement may reflect functional redundancy in the light input pathway. Differences between the action spectrum of rodless and coneless mice and their wild-type counterparts may reveal the relative roles and photopigments in each compartment. Indeed, recent data suggest that nonopsins participate in phase shifting. For example, light applied to the back of the knee of human subjects can cause phase shifts [76]. The authors proposed a heme-based mechanism. However, these striking results have not been reproduced in humans and other mammals and therefore remain controversial [77]. In addition, a class of blue light photoreceptors, called cryptochromes, have been implicated as potential photoreceptors. Although lacking repair activity, cryptochromes are homologous to blue light–sensitive DNA repair enzymes [78]. Nonetheless, they mediate blue light–sensitive photoresponses in plants, such as phototropism [79]. In mammals, cryptochromes are found both in the SCN and in the retinal ganglion and inner nuclear cell layers; the latter are parts of the retina remaining in rodless, coneless mice [80]. Intriguingly, the expression of one of these genes, *mCry1*, is under circadian control in the SCN [80]. Given their potential function and expression pattern, it was proposed that cryptochromes may be responsible for light-mediated phase shifting. In vivo deletion of the *mCry2* gene results in slightly lengthened periods, and, in contrast to prediction, increased photosensitivity [81]. Nonetheless, these mice do display a reduced light induction of *mPer1* RNA [81]. In addition, the double knockout of both *mCry1* and *mCry2* is completely arrhythmic, suggesting a role in the central pacemaker [82]. Consistent with these genetic data, both mCRY1 and mCRY2 are able to repress CLOCK-BMAL1–mediated transcriptional activation strongly when cotransfected in cultured cell lines [83]. The current data therefore support a role of *mCry* in the transcriptional feedback loop itself, but do not rule out the possibility that *Cry* may also function in photoreception. For example, light induction of *per* RNAs might be mediated by cryptochromes. Thus, bona fide mammalian circadian photoreceptors remain unknown and an object of current investigations.

On this issue of circadian photoreception, the mammalian picture is blurred but the fruit fly appears much clearer. In *Drosophila*, a difference in the phase-shifting action spectrum with mammals suggested the presence of a non–retinal-based photopigment [84,85]. These beliefs were buttressed by experiments demonstrating that depletion of vitamin A from flies did not abolish their phase-shift response, implicating a photoreceptor more blue shifted than mammalian

photoreceptors. Using a novel luciferase-based screen, a mutation in a *Drosophila cryptochrome* gene ($dcry^b$) was identified which displayed impaired entrainment and abolished phase responses to light pulses [86]. Furthermore, overexpression of *cry* resulted in increased circadian photosensitivity [87]. Similar to plant cryptochromes, dCRY protein is degraded within minutes in response to exposing flies to light (87). In apparent contrast to mammals, these alterations in *cry* function had no apparent effect on free-running periodicity; placing *cry* within the input pathway and firmly outside the central pacemaker.

Despite crippled *cry* function, $dcry^b$ flies are still able to entrain to 12-hr light, 12-hr dark cycles. This entrainment can be impaired but not completely abolished by mutations in a phospholipase C protein involved in the visual (opsin)–based phototransduction cascade, indicating a second phototransduction pathway [86]. Therefore, as in mammals, vitamin A–based photoreception may also be involved in *Drosophila* circadian photoreception. The remaining entrainment capability implies that there may even be a third circadian phototransduction pathway. Finally, *dcry* itself seems to be under robust circadian control at the RNA level, but such control is low or absent at the protein level [87]. RNA cycling may reflect the importance of newly synthesized dCRY protein in phase shifting. Alternatively, the peak of RNA during the morning may ensure sufficient dCRY protein during the daylight hours when it is degraded. RNA cycling may even be an epiphenomenon, a reflection of common transcription factors which contribute both to expression in circadian tissues and to circadian regulation itself.

CRY may also have light-independent functions on circadian pacemaker gene macrometabolism in flies, not unlike the mammalian model [86]. Despite an absence of period differences between flies with and without *dcry*, there are light-independent alterations in *per* and *tim* expression under temperature cycling constant darkness conditions. Indeed, dCRY directly interacts with TIM in both cultured cells and in yeast two-hybrid assay, although the latter is dramatically dependent on light exposure [88]. Therefore, dCRY appears to be biochemically intimate with pacemaker components but not required for their function, at least under laboratory conditions. The intimate association with TIM may merely reflect a role in phase shifting.

In sum, forward genetics has identified a strong fruit fly candidate for a circadian photoreceptor. Further studies will be required to see if the close ties between flies and mammals in the structure and function of clock components breaks down in the *cryptochrome* family.

XIV. Clocks Are Everywhere!

Since the discovery of the suprachiasmatic nucleus, lesion and transplantation experiments have supported a model in which the circadian kingdom is a dictatorship: the SCN directing otherwise passive peripheral tissues. The more recent

identification of clock genes and the analysis of their spatial and temporal expression patterns reveals that the role of the SCN is more akin to the conductor of an orchestra: keeping the far-flung organs in synchrony with each other. First, the expression of clock genes in *Drosophila* is not restricted to a handful of clock-related neurons [89]. Several nonneural tissues also rhythmically express clock genes, suggesting circadianly related functions [90]. Flies containing *per* promoter transgenes fused to a luciferase reporter (*per-luc*) exhibit cycling bioluminescence when fed on luciferin-containing media [91]. Surprisingly, peripheral tissues from these *per-luc* flies autonomously cycle in culture. In fact, these cultured oscillators autonomously entrain to light–dark cycles, indicating the presence of independent photoreceptive pathways. Some of these concepts extend to mammals. Like the fly rhythm genes, *mClock* is transcribed in several nonneural tissues [50]. In addition, rat *per* mRNAs cycle in these peripheral tissues like the heart and lungs. Oscillatory molecular cycling is therefore not exclusive to the SCN [92]. In fact, circadian oscillation of the *per* genes is evident in a rat-1 fibroblast cell line after serum shock [93]. This immortalized cell line has been maintained in culture for over 25 years, yet retains much of the circadian machinery. Thus, fruit fly genetics has led to a reformulation of our view not only of molecular mechanisms but also of organism-level organization of circadian systems.

XV. Clocks and Outputs

How do core pacemaker elements biochemically link with output behaviors, such as the sleep–wake cycle? Several putative genes in circadian output pathways have been identified based on the cycling of their expression at the RNA level. In fact, virtually the entire genome is under circadian control in photosynthetic cyanobacteria [94]. In *Drosophila*, random sampling of transcripts suggests a much smaller but substantial fraction of cycling transcripts, probably between 1% and 5% [95]. Cycling at the RNA level has implicated transcription factors in mediating output. As *Clock* encodes the first bona fide transcription factor identified in the circadian pacemaker, it has been proposed to mediate the cycling of these putative output RNAs in both flies and mammals. In mammals, the mRNA from the vasopressin gene is circadianly regulated [96]. The vasopressin promoter contains a putative CLOCK E-box binding site, CACGTG [97]. In homozygous *Clock* mutant mice, vasopressin mRNA levels are reduced. Furthermore, in tissue culture experiments, CLOCK activates vasopressin gene expression in an E-box–dependent manner. These data are consistent with the hypothesis that CLOCK directly binds to E-boxes in the vasopressin promoter and mediates its rhythmic transcription. However, the sufficiency or requirement of this promoter E-box has not been demonstrated in vivo, and the tissue culture

experiments do not prove that the activation of CLOCK on the vasopressin promoter is biochemically direct.

In addition to transcriptional mechanisms, posttranscriptional mechanisms may be important for mediating rhythmic output gene expression. One *Drosophila* output gene, *lark*, appears to be circadianly regulated by a posttranscriptional mechanism. *lark* Was initially identified as a mutant with a late phase of eclosion [98]. However, the absence of a circadian locomotor aberration suggested that the central pacemaker is unaffected in these mutants, placing *lark* on an output pathway. Unlike other output genes, however, LARK protein does cycle but *lark* RNA does not [99]. Therefore, posttranscriptional mechanisms must underly this circadian cycling. In fact, *lark* itself encodes an RNA-binding protein, suggesting that a downstream RNA targets in the eclosion output pathway may be nontranscriptionally regulated [100]. One such candidate is the gene *crg-1* whose mRNA cycles in phase with *per* and *tim* [101]. However, at the level of transcription, it does not cycle, suggesting that the mRNA cycling is due to changes in stability not synthesis [69]. It remains to be seen if *crg-1* is a direct target of LARK.

A theoretical concern with the transcriptional output model is that high amplitude transcriptional cycling may be attenuated by the stability of output mRNA and protein products. Most reporter genes that demonstrate RNA cycling when fused to circadian promoters do not demonstrate cycling at the protein level, reflecting protein stability [102]. Thus, posttranscriptional and even posttranslational mechanisms may be the best way to ensure that pacemaker oscillations are transferred out of the clock. These putative posttranslational changes in output have yet to be identified. However, mutations in protein kinase A abolish locomotor rhythms but not eclosion rhythms, implicating phosphorylation changes in a subset of output pathways [103].

What is the function of cycling gene expression in mammalian peripheral tissues? There are a variety of clock-driven physiological and pathological processes. It remains to be seen, of course, how pacemaker proteins link to disease-related pathways in these tissues. The identification of pacemaker genes in flies and mammals as well as their downstream targets should further the understanding of clock-controlled processes, such as the sleep–wake cycle.

XVI. An Ode to Forward Genetics and Gene Discovery

The strength of the forward genetic approach is its ability to identify genes in the absence of any hypothesis or notion of how a system operates. The experience of forward genetics in flies and in mice demonstrates the successful application of this approach. However, this experience can be extended especially now with an $n = 6$ in flies, and suggests an even more important fruit of this genetic labor.

At the outset of Konopka and Benzer's endeavor, one might have hypothesized that some fraction of mutants would only indirectly affect clock function but would not participate directly in the clock mechanism. Put more experimentally, one might clone clock genes with no direct biochemical relationship one to another. One might also have hypothesized that another fraction of mutants would affect some general function like transcription, which participates only indirectly in circadian rhythm generation by participating in nearly all processes required in a living cell.

Thirty years after Konopka and Benzer's original work, the geneticists now have hard evidence to counter this pessimistic view; we are now six for six and counting. In each case, further functional analysis of the cloned rhythm genes demonstrated that they encode central components that directly interact in biochemically meaningful fashions. In addition, none of the genes encodes a general component, such as RNA polymerase II. Are circadian geneticists simply lucky? A tenable argument perhaps after the discovery and analysis of *period* but less plausible after *timeless.* After analysis of several genes in both flies and mammals, this seems unlikely. By screening for rhythm phenotypes in adult flies, the probability of identifying a mutation that affects the clock only very indirectly appears to be low. Perhaps such a mutant probably affects other developmental processes and therefore would not live to be assayed. Similarly, mutations in genes such as RNA polymerase II, which may play a direct role but also have more general functions, would be so essential for orchestrating the intricacies of early development that a mutant would be unlikely to live to adulthood. It is therefore no accident that the genetics selected for central clock components are largely dedicated to the assayed phenotype. With the completion of the full *Drosophila* genomic sequence, the forward genetic approach should be even more powerful as an arm of genome-wide functional analysis.

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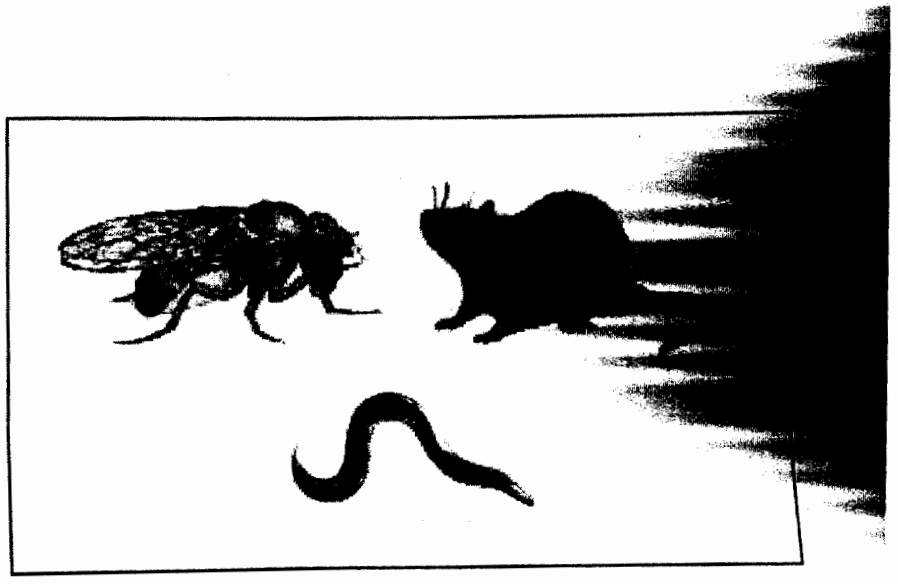

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