PART I. HIGH FREQUENCY DOMAIN: PHYSIOLOGIC REQUIREMENT FOR PULSATILE DELIVERY

New Clock Mutations in *Drosophila*[a]

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INTRODUCTION

Attempts to understand the biochemistry of development have benefited enormously from the guidance of genetic studies.[21] The same eye toward genetic landmarks has recently led several groups of investigators to some of the molecules controlling stereotyped behaviors, including biological rhythms.[20,27] This has been particularly rewarding in the study of *Drosophila* biological rhythms, as mutations in several genes have strong effects on circadian behavior.

Circadian rhythms are easily monitored in *Drosophila*. For example, the flies show diurnal locomotor behavior when exposed to 24-h environmental cycles of light or temperature, and sustain alternating bouts of high and low activity when the environmental cycle is removed. In fact, circadian activity rhythms will arise spontaneously in young flies even if they have never been exposed to light or temperature cycles, attesting to the presence of an endogenous biological clock (unpublished data). Circadian rhythms can also be detected in *Drosophila* development, with eclosion (emergence of the adult from the pupal cocoon) tightly controlled by the biological clock. Fertilized eggs beginning development at different times of day will produce adults that eclose together, at or about dawn, when a light cycle is supplied. This developmental convergence will take place in the absence of a light or temperature cycle if the population is given synchronizing light pulses days earlier.

THE FIRST CLOCK MUTATIONS

These rhythms were first used to detect mutations 20 years ago by Konopka.[13] Using chemical mutagenesis, Konopka isolated three allelic mutations in *D. melanogaster* that either lengthened (to 29 h) or shortened (to 19 h) the circadian period, or eliminated circadian rhythms altogether. This new gene was given the name *per* (*period*) and it was recognized that for long (per^{l}) and short (per^{s}) mutants, corresponding shifts in period length were seen for both eclosion and locomotion.

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The same mutation series was found to have a more fundamental effect on timing; high-frequency wing-beat songs produced during courtship by male *Drosophila* are affected by each of the mutations.[14] The song is composed of components that are reiterated with a 1-min frequency in wild-type flies, yet per^s and per^l flies sing 40-s and 80-s songs respectively. Flies with the arrhythmic mutation, per^o, produce arrhythmic song. The correlated effects on high-frequency and circadian timed behaviors indicate that *per* may not contribute to an intrinsically circadian process at the molecular level.

The *per* gene has been isolated and sequenced.[3,11] The gene codes for a 1200-amino-acid protein that shows some similarity to vertebrate proteoglycans.[11,18] Point mutations have been discovered in each of the original mutant strains.[2,28] The per^o allele contains a single nucleotide substitution when compared to the wild-type gene sequence. The substitution generates a translational stop signal. Thus, the arrhythmic phenotypes of per^o flies can be attributed to production of a partial protein, one-third the length of the wild-type molecule. For per^s and per^l, single amino acid substitutions are predicted from DNA sequence analysis.

Changes in the period of a fly's rhythm can also come from altering the dosage of the *per* gene product.[2] A series of transgenic *Drosophila* strains have been produced, each of which makes a different level of *per* RNA. Through analysis of these lines it was determined that period length and abundance of the gene product are correlated, with high levels of *per* giving shorter period circadian rhythms and lower levels giving longer period length. If dose is lowered to a twentieth of that seen for wild type flies, the rhythm is lengthened from 24 to almost 40 h.[2] Possibly the behavioral defects found in per^s and per^l mutants are related to changes in activity or stability of the *per* protein.[2] Interestingly, there appears to be a circadian cycling in the level of the *per* transcript in some tissues.[7] It is not yet clear whether this cycling is a response or a contributing factor to the organization of the *Drosophila* clock.

Immunocytochemistry has been used to map the locations of cells producing the *per* protein in developing *Drosophila*.[15,22,23] A few cells in each segment of the embryonic nervous system make the protein, and later in development protein is found at highest levels in the brain, especially the eyes and optic lobes. The protein also is found in unexpected locations, including the pupal and adult gonads of both sexes, and in the embryonic and larval salivary glands. The ring gland complex makes *per* protein, which is of some interest given the role of related cells in the rhythmic release of eclosion hormone in other insects.[25] Surprisingly, the protein is not always found in the same subcellular compartment in these different tissues. The protein is cytoplasmic and perhaps located on cell surfaces in the embryonic nervous system and salivary glands. It is abundant in the cytoplasm of nurse cells in adult and pupal ovaries. The protein is associated with nuclei in the adult optic lobes, photoreceptor cells of the eye, and ring gland complex.[22,23] Certainly the protein may have somewhat different roles in the different cellular locations.

Defects in the physiology of single cells have now been recognized in *per* mutants and may provide clues to the mechanism by which the gene affects behavior. A decade ago it was discovered that salivary gland cells isolated from arrhythmic *per* mutants absorbed certain voltage-sensitive dyes with an unexpected pattern: synchronous absorption was seen in all cells of a wild-type gland, while cell-by-cell absorption was asynchronous for per^o tissue.[26] These early results have been extended in direct studies of intercellular junctional communication. Low levels of *per* protein synthesis, and arrhythmic and long-period phenotypes can be correlated with poor cell-to-cell communication across gap junc-

tions. Higher than wild-type levels of this communication have been observed in tissue isolated from *Drosophila* having short-period rhythms.[1] From these results it has been suggested that *per* may regulate biological rhythms through effects on gap junctions in the electrical synapse.[1] In any event the locus of the clock affected by the *per* mutant series may involve a group of cells rather than an intracellular process. Such a role also would be consistent with *per*'s effects on both high-frequency and circadian rhythms.

ADDITIONAL GENES INFLUENCE *DROSOPHILA* RHYTHMS

A strength of any genetic analysis of biological rhythms comes from the ability to ask what a list of key molecules might look like. Do proteins that play an essential role in the organization and function of biological clocks compose a special class that can be resolved by structural studies? Are there sites of action in the developing animal that are common to all of these molecules? Do the molecules interact to provide a functional pathway? What is the order of their expression and interdependencies? Are the molecules evolutionarily conserved, supporting the idea of a fundamental mechanism?

We are attempting to expand on the genetic and molecular work initiated at the *per* locus by identifying and isolating new genes that also have important roles in the *Drosophila* biological clock. Significantly, there have been powerful new procedures developed for mutating and recovering genes in *Drosophila* over the past few years.[4,19] Thus, although earlier genetic screens have recognized several genes playing a role in biological rhythms,[10,12] we will forego isolation of existing mutations, and focus instead on producing new mutants by current methods.

MUTATION AND RECOVERY OF NEW GENES

FIGURE 1 illustrates our mutagenesis and screening procedure for biological clock mutants. Mutagenesis involves the induced transposition of a genetically marked P element. These form a class of transposable DNAs that can integrate in almost any chromosomal region. P elements produce mutations at the sites of chromosomal entry by interfering with the normal regulation of target genes or by breaking up protein-coding DNA sequences.

Induced transposition occurs in flies carrying two complementing elements. One P element encodes the enzyme transposase, but is not responsive to its presence. The second element is deficient for the transposase, but responsive. The latter element relocates in response to introduction of the first element in a genetic cross. Although transposition occurs in only 30 to 50% of such crosses, movement of the element is directly monitored because the responding P element carries an eye-color gene that follows it to every new chromosomal location. Moreover, the chromosome receiving the transposed element is immediately determined by the pattern of segregation of the eye-color gene and the different chromosomes.[4,19] One special advantage of the system is that each transposition creates a single new mutation. A strain of mutant flies can be established corresponding to each mutation event and subsequently tested for inheritance of a behavioral abnormality. It is generally considered that a screen producing about 10,000 independent lines could include mutations in most *Drosophila* genes.

The real point in generating new mutations with transposable elements is that

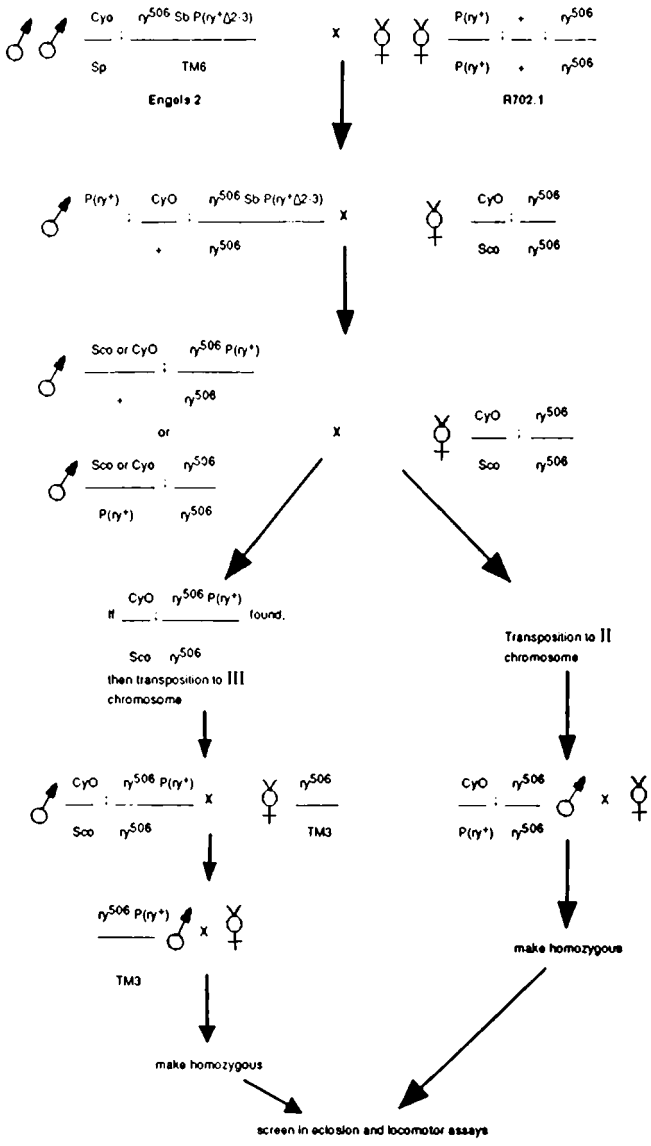

FIGURE 1. Genetic scheme to generate lines containing single P elements on autosomes. The P(*ry*$^+$) transposon in R702-1 was mobilized by providing P(*ry*$^+$ Δ2-3) as a source of transposase. The Engels 2 and the R702-1 strain have been previously described.[19,24] Homozygous lines were made by crossing appropriate siblings from each line. Relevant genetic symbols: *ry*^{506}—a third chromosome recessive mutation that leads to reddish brown eyes; P(*ry*$^+$)—a P element that carries the wild type *rosy* gene and can be mobilized, but does not express transposase; P (*ry*$^+$ Δ2-3)—a defective P element that expresses transposase, but cannot be mobilized itself (also carries a wild-type *ry* gene); *CyO*—a second chromosome balancer (represses recombination) that carries the dominant *Curly* wing mutation; *Sco*—a second chromosome dominant bristle mutation; *Sp*—a second chromosome dominant mutation that affects sternopleural bristles; *Sb*—a third chromosome dominant bristle mutation; *TM3*—a third chromosome balancer that carries several markers, including *ry* and *Sb*; *TM6*—a third chromosome balancer.

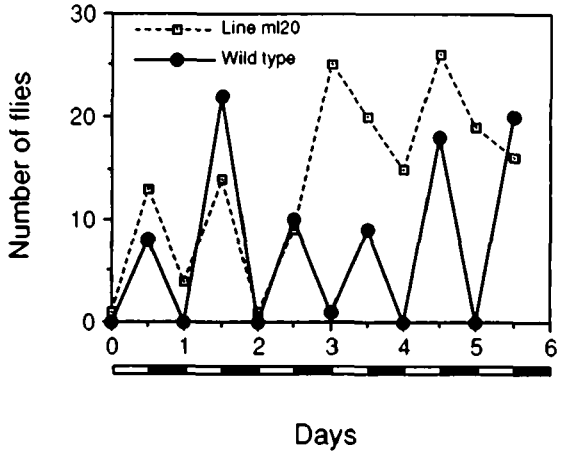

FIGURE 2. Primary eclosion screen of line *ml20* and a line showing wild-type behavior. Flies were kept in a 12 : 12 L : D cycle at 18°C. Emerging flies were collected 30 min before lights on and 2–3 h before lights off. Plotted points represent the number of flies that have emerged since the previous collection. Lights on and off are indicated by the white and black bars respectively.

they afford immediate molecular cloning of mutated chromosomal DNA. Sequences corresponding to the P element are used to recover the transposed element from its new location, along with segments of neighboring DNA that compose the *Drosophila* gene of interest. The recovery step was never a part of earlier schemes for generating new biological rhythm mutations. Changes in structure and expression of the target DNA can be mapped, making it possible to understand why the P element is causing the mutant phenotype. Wild-type DNA corresponding to the gene can be recovered by sequence homology.

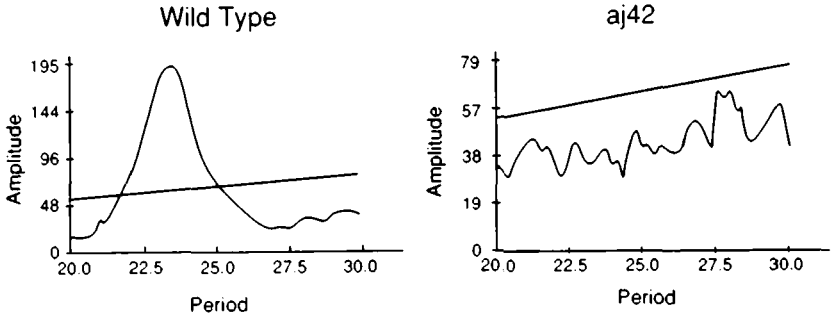

FIGURE 3. Locomotor activity in constant darkness in line *aj42* and a wild-type line. Flies were entrained to a 12 : 12 L : D cycle for 4 days and then activity of individual flies was monitored in constant darkness as described in the text. Periodograms shown here were generated by chi-square analysis of the raw data.[6] Significant periodicities are represented by values above sloping lines ($p \leq 0.05$). The period of the wild type fly was calculated to be 23.6 h.

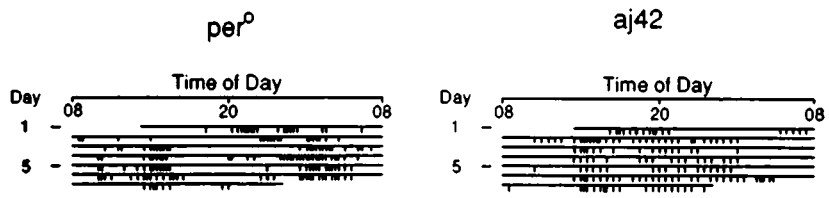

FIGURE 4. Locomotor activity in L : D cycling in *aj42* and per° flies. Flies were kept in a 12 : 12 L : D cycle (lights on hours 2–13) for 11 days. Activity was measured from day 5. Each horizontal line corresponds to 24 h, and deflections reflect activity (see text).

To isolate new clock mutations, two parameters of rhythmic behavior, eclosion and locomotor activity, are assessed for each transposition strain. For the eclosion screening, populations of several hundred larvae and pupae are raised in a light/dark cycle, typically L : D 12h : 12h for about five days immediately preceding eclosion. Emergence of adults is quantified by manual collection twice a day. Initially collections are made 30 min before dawn and 2 to 3 h before dusk in the presence of the LD cycle. Mutations altering period length should show aberrant entrainment of eclosion rhythms to produce a high frequency of night emergence. This is, in fact, what is seen for all three *per* mutations.[13] From eclosion screening, candidate mutations can be subsequently analyzed for effects on period length in the absence of an environmental cycle.

Our primary screen now includes analysis of locomotor activity. Individual flies from the different mutagenized strains are placed in 5 mm × 50 mm transparent glass cuvettes. For each tube a far red light emitting diode and phototransistor are placed on opposite sides. In this arrangement a moving fly breaks the light beam as it travels along the length of the cuvette. A complete array of these sensors can monitor 200 flies at a time by continuous computer sampling. Typically, the behavior of 5 or 6 flies from a single transposition line is followed for about a week. Spectral analysis is performed to locate new mutants.[6]

Examples of two new lines, obtained by the coupling of these screens, are shown in FIGURES 2–4. In preliminary screens, strain *m120* shows exaggerated night emergence in the eclosion tests (FIGURE 2), but locomotor analysis indicates a wild-type period length of 24 h (data not shown). Segregation of the P-element-linked genetic marker indicates linkage to chromosome 2 (Sehgal, Price, and Young, in preparation). The split behavior of the strain is quite different from that of arrhythmic *per* mutants, and raises the prospect of separating functions required for control of locomotor activity and eclosion.

Strain *aj42* shows a much stronger effect on circadian organization (Sehgal, Price, and Young, in preparation). There is no evidence for any degree of entrainment of eclosion in the presence of a light cycle (data not shown). When locomotor activity is examined in continuous darkness there is again no evidence for rhythms (FIGURE 3). However, in the presence of a 12h : 12h L/D cycle a striking inversion of locomotor behavior is seen; flies show nocturnal rather than the wild-type diurnal behavior (FIGURE 4; Sehgal, Price, and Young, in preparation). Genetic mapping experiments indicate that the *aj42* mutation is linked to chromosome 2 and is recessive.

Aside from the inversion of activity levels in relation to light cycles, the pattern of locomotor behavior observed for *aj42* mutants is comparable to *per*°. In

the latter flies, arrythmia is seen in eclosion and locomotor behavior in continuous darkness. Locomotor activity is gated in *per*^{o} flies by light/dark cycles, and transitions from activity to inactivity are coincident with the light/dark transitions (FIGURE 4; Sehgal, Price, and Young, in preparation). Further support for the conclusion that the behavior patterns reflect environmentally driven behavior, rather than the unmasking or reinforcement of underlying circadian rhythms, may come from alignment of activity cycles with imposed noncircadian light cycles.

It may be useful to compare the behavior patterns of *aj42* and *per*^{o} to those of a biological rhythm mutant altering the development of functional connections between the eye and brain (*disconnected*).[5] In contrast to *per*^{o} and *aj42*, rhythmic behavior observed in *disco* flies during light cycling suggests residual activity of a circadian pacemaker. Activity cycles emerge out of register with the environmental cycle indicating that the flies have the ability to anticipate the coming light transition or measure time from the prior transition. Thus, genetic analyses of the *Drosophila* circadian system seem to be revealing several levels of function available for further dissection.

SOME CLOCK MUTATIONS ARE ASSOCIATED WITH PROMINENT CHANGES IN NEUROANATOMY

Mutations of the *per*, *m120*, and *aj42* loci show no visible effects on adult fly morphology. As indicated above, some mutations affecting expression of circadian rhythms have strong effects on the development of the fly.[5] Mutations affecting the anatomy of the eyes and optic lobes are of special interest, as they seem to be associated with aberrant locomotor activity rhythms at high frequency.[5,8,9] To supplement our direct screening for clock mutants we also have begun an ordered examination of these morphological mutants. These studies continue to suggest an important role for the visual system in the organization of circadian rhythms.

FIGURES 5 and 6 show some of our results stemming from the analysis of the *Drosophila* mutant *glass*. Extreme alleles produce sharply reduced compound eyes that are missing photoreceptor cells, and the optic lobes of the brain are degenerate.[16,17] Molecular studies have shown that the gene produces a protein

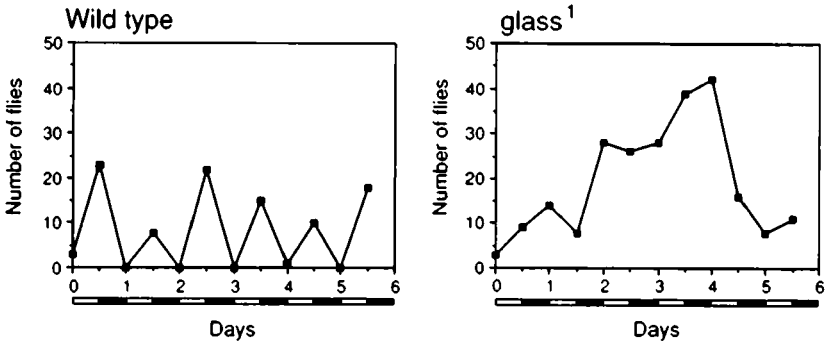

FIGURE 5. Temporal profiles of eclosion of wild type (Canton-S) and *glass*^{1} flies. Eclosion behavior was measured in L : D cycling as explained in FIGURE 2 legend.

bearing significant homology to a class of transcription factors that use certain spaced cysteine and histidine amino acid residues to bind zinc.[17] It has been proposed that the *glass* protein may control the development of photoreceptor cells, with degeneration of the optic lobes stemming from secondary effects of their absence. Support for this conclusion comes from studies showing that optic lobe defects are seen in wild-type tissue in response to abnormal development of an apposed *glass* eye.[16]

Flies carrying any of three mutant *glass* alleles, gl^1, gl^2, and gl^3, display a range of effects on locomotor activity and eclosion rhythms. The allele gl^1 shows strongest effects, producing arrhythmic eclosion (FIGURE 5; Vosshall and Young,

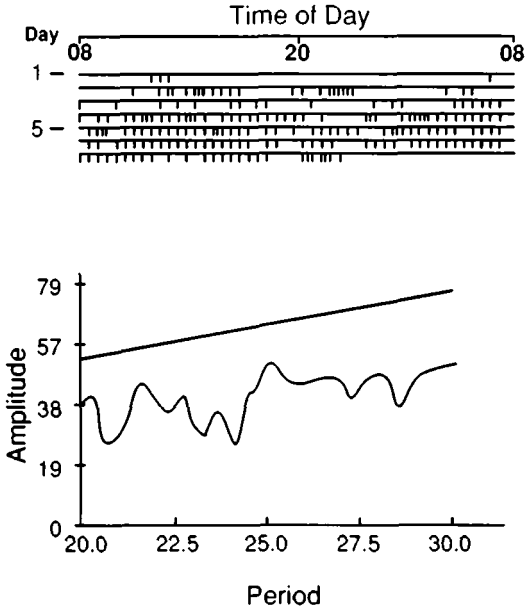

FIGURE 6. Representative records of locomotor activity from a *glass*^{1} fly. Fly was entrained to a 12 : 12 L : D cycle. Subsequently, free-running locomotor behavior (in constant darkness) was plotted as described in legends to FIGURES 3 and 4. No detectable rhythm is found in *glass*^{1} actogram (top) or periodogram (bottom).

in preparation) and locomotor activity (FIGURE 6; Vosshall and Young, in preparation). As in the case of arrhythmic *per* mutants and *aj42* mutants, no evidence for circadian pacemaker action is revealed in constant darkness or when light/dark environmental cycles are supplied (unpublished observation). Like *per*^{o}, activity levels are modulated in an L/D cycle (unpublished observation). Again, the response of these mutants to noncircadian environmental cycles should be especially informative.

Transgenic flies have been examined for rescue of wild-type rhythmicity. In preliminary experiments, segments of cloned *glass* DNA that encode the zinc-finger protein and restore photoreceptor development also restore wild-type bio-

logical rhythms (Vosshall and Young, in preparation). Nevertheless, one hypomorphic *glass* allele produces a severe eye phenotype without affecting biological rhythms (Vosshall and Young, in preparation). This suggests that the transcription factor encoded by *glass* may function outside the eye to control development of cells involved in the generation of *Drosophila* biological rhythms.

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