Principles and Problems Revolving Round Rhythm-related Genetic Variants

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Much of what is known about the regulation of circadian rhythms has stemmed from the induction, recognition, or manufacture of genetic variants. Such investigations have been especially salient in chronobiological analyses of *Drosophila*. Many starting points for elucidation of rhythmic processes operating in this insect entailed the isolation of mutants or the design of engineered gene modifications. Various features of the principles and practices associated with the genetic approach toward understanding clock functions, and chronobiologically related ones, are discussed from perspectives that are largely genetic as such, although intertwined with certain neurogenetic and molecular-genetic concerns when appropriate. Key themes in this treatment connect with the power and problems associated with multiply mutant forms of rhythm-related genes, with the opportunistic or problematical aspects of multigenic variants that are in play (sometimes surprisingly), and with a question as to how forceful chronogenetic inferences have been in terms of elucidating the mechanisms of circadian pacemaking.

What'dya say we bust up this joint?
Rodney Dangerfield (1980)

INTRODUCTION

Figuring out how circadian rhythms are regulated from within various organisms, ranging from microbes to mammals, has often been underpinned by genetic variants. These items have either been created or applied, or both. “Creation” is not always involved; because some rhythm variants, especially in *Drosophila*, are naturally occurring, as we shall see—over the course of a treatment that will discuss *chronogenetic* issues as such. Many such conceptual matters have arisen from genetic analysis of rhythms in *Drosophila*, which will figure most prominently in the piece, although analogous cases stemming from chronogenetic studies of mammals are also mentioned. Molecular mechanisms of circadian pacemaking, and the neural substrates of biological rhythms in animals, will not be objects of much focus, although these matters will come into play. Indeed, many molecular-chronobiological and chrononeurogenetic investigations of animals have been permitted by the generation or recognition of variants with abnormal circadian phenotypes.

THE POWER AND PROBLEMS ASSOCIATED WITH MULTIPLE ALLELES AT RHYTHM- RELATED GENETIC LOCI

The all-time classical rhythm mutants in *Drosophila melanogaster* are the three that were induced at the *period* (*per*) locus (Konopka and Benzer 1971). Each of them was independently isolated; thus, the separate mutations did not have to be allelic, but they turned out all to map to one X-chromosomal locus and failed to complement one another (see below).

The first such mutant found was *per*⁰ (now called *per*⁰¹), based on aperiodic adult emergence (also known

as eclosion). Later, *per*⁰¹ flies were shown also to exhibit arrhythmicity for adult behavior (Konopka and Benzer 1971). This mutant was isolated in 1968 (for the history, see Weiner 1999). The induction by chemical mutagenesis of *per*⁰¹ prompted extension of the study, which was published 3 years later by R.J. Konopka and S. Benzer, under the title “Clock mutants in *Drosophila*.” However, an arrhythmic genetic variant need not have been altered in a clock gene, one whose functions subserve ongoing circadian pacemaking. Instead, *per*⁰¹ could have been brain damaged such that the neural substrates of the rhythmic attributes formed abnormally or not at all. In this regard, it is well known that physical destruction of a pacemaker structure in the mammalian brain leads to arrhythmic locomotion (see, e.g., Weaver 1998), which also can result from neuroanatomical injury caused by a mutation (see, e.g., Scheuch et al. 1982).

For *Drosophila*'s part, the isolation of two further *period* mutants was crucial, for they displayed altered circadian cycle durations, 5 hours shorter or longer than the normal approximately 24 hours (Konopka and Benzer 1971). These *per*^S and *per*^{L1} (née *per*^L) eclosion mutants, by their original definition, were revealed also to be adult locomotor variants. They “comapped” with each other and with *per*⁰¹ and were found to be allelic by virtue of non-complementation. For example, *per*^S/*per*⁰¹ heterozygotes exhibited substantially shorter periods than do *per*^S/*per*⁺ flies. The latter display about 21.5-hour periodicity, signifying semidominance, and *per*⁰¹/*+* flies “run” slightly but consistently long, about 24.5 hours as opposed to about 24 hours (see, e.g., Smith and Konopka 1982). But *Drosophila*, whose only apparent functional *period* allele is *per*^S, exhibit 19–20-hour rhythms (Konopka and Benzer 1971). This assumes that the presence of *per*⁰¹, including in a heterozygote involving *per*^S, provides no function (as

was verified in subsequent genetically and molecularly based studies; see, e.g., Smith and Konopka 1981; Bargiello and Young 1984; Yu et al. 1987). The allelic interactions and gene-dosage effects implied by these findings are discussed later in terms of their potential forcefulness for inferring how the *period* gene acts as a potential contributor to circadian pacemaking.

If a gene can thus be altered so that such pacemaking is extant but “off,” the idea is (and was) that this factor’s normal function involves clock functioning as opposed to the formation of some piece of rhythm-related anatomy during development. It would follow that the *per⁰¹* mutant is clockless at the functional level and likely to be normal for the pertinent morphology. Furthermore, because two separate circadian phenotypes come under the sway of the gene, one is encouraged to infer a “central” clock function as opposed to a process that operates on behalf of only a particular rhythmic attribute. In other words, if solely eclosion or adult behavior were mutationally altered, it could be supposed that the gene in question functions within a parochial “output pathway” originating at the core pacemaker; this pathway would end at the specific regulation of merely a particular rhythmic character.

Consider again the *per^S* and *per^{L1}* mutants. They were so dramatically altered for their cycle durations (in constant darkness or DD) that the implied *period* gene would not quit. But it is notable in this regard that one mutation at this locus causes only a mild circadian abnormality—*per^{Clk}*, a mutant that exhibits 22.5-hour periodicity (Dushay et al. 1990, 1992). What if *per^{Clk}*, by bad luck, had been the only mutation induced in the gene? The *per* story might have fallen by the wayside. This is what happened with regard to a little-known mutation on another *Drosophila* chromosome—*Toki*, which was induced by chemical mutagenesis and found to cause approximately 25-hour periodicities (Matsumoto et al. 1994). A certain degree of malaise set in, in the sense that an absence of substantial abnormality seemed to stimulate no further studies of the mutant or the gene that it defines. In addition, *Toki*’s genetic etiology was localized approximately to a region within the second chromosome of *D. melanogaster*. (All that could be said is that this mutation is not allelic to much better known rhythm mutations on chromosome 2.) A second mutant allele of *Toki*, causing some sort of striking abnormality, might have held the gene in good stead.

We now turn to mouse chronogenetics, elements of which led to *Toki*-like scenarios. A handful of rhythm variants has been induced in this mammal (there are more and more such mutants as time goes by; see, e.g., Bacon et al. 2004; Siepka et al. 2007). One is the famed *Clock* (*Clk*) mutant, which causes severely abnormal rhythmicity when the mutation is homozygous (Vitaterna et al. 1994). Chemical induction of *Clk* prompted many studies of its mutational effects as well as analysis of the gene and its product’s action (for review, see Lowrey and Takahashi 2004). In contrast, induction of the *Wheels* (*Whl*) mutant led to *Toki*-like melancholy, because this mouse mutant is only slightly abnormal for circadian period, and descendants of the original *Wheels* isolate

were erratic for exhibition of such defects (Pickard et al. 1995). Furthermore, the mutation acts pleiotropically, owing to *Whl*-induced anatomical defects that seem to be unrelated to rhythm control (Pickard et al. 1995; Alavizadeh et al. 2001). An analogous murine case is provided by the *Wocko* rhythm mutant (Sollars et al. 1996), whose overall phenotypes (Crenshaw et al. 1991) were such that the investigators were “quits” in terms of digging deeply into the etiology of the less than exciting chronobiological abnormality.

This brings us back to *Drosophila* and to alterations of neural morphology, which can indeed be associated with rhythm mutants. The case in question involves the *disconnected* (*disco*) gene, which was recognized initially by a mutant exhibiting severely abnormal optic ganglia in the anterior central nervous system (CNS). *disco¹* (the first mutant of this type isolated) was one of several variants in *D. melanogaster* recognized by neuroanatomical screening *per se*; later, several of these mutants were shown to exhibit behavioral anomalies. Thus, taking the collection of brain-damaged variants and “screening through” them for rhythm defects disclosed *disco¹* as the only substantially abnormal type (Dushay et al. 1989; cf. Vossell and Young 1995): Mutant cultures eclosed aperiodically and individual adults behaved arrhythmically. By this time in *Drosophila* chronogenetic history, the *per* gene had been cloned and was being assessed as to where it makes its products in fruit fly tissues (Liu et al. 1988; Saez and Young 1988; Siwicki et al. 1988). Among such locations were certain *laterally* located brain *neurons* (Siwicki et al. 1988), now called LNs (for review, see Hall 2005). Intriguingly, the apparent anatomical problems within a *disco¹* CNS extended a bit more deeply than visual system ganglia, because next to no *per*-expressing LNs were detectable in the adult brains of the mutant (Zerr et al. 1990; for many further data that speak to this point, see Helfrich-Förster 1998). So, it was inferred that functions operating within these CNS neurons underlie circadian phenotypes—plural, given the bidefective *disco¹* defects. This belied the notion that a mutant exhibiting more than one kind of rhythm deviation would necessarily define a circadian pacemaking factor.

What about the etiology of *disco¹*’s neurobiological and behavioral phenotypes (*n* = at least four abnormal ones)? Could it be that the combination of optic-ganglia and LN abnormalities, and their chronobiological consequences, does not have a simple underlying causation? It might be, for example, that *disco¹* itself leads to nonformation of the visual system elements (the most salient anatomical subnormality) but that something else in the “genetic background” causes the more subtle absence of LNs (or at least lack of *per⁺* expression in these approximately ten pairs of brain neurons). Another possibility is that *disco¹* does make both morphological aberrations manifest themselves but that this mutated allele is an odd duck that promotes these problems in some sort of epiphenomenological manner. What might this mean? Well, consider the *no-action-potential/temperature-sensitive* mutant: *Drosophila*, of either sex mind you, exhibit paralysis at high temperature, a condition that causes nerve conduction to fail (Wu et al. 1978). Later, it was shown

that *nap^{TS1}* is mutated within a gene called *male-lethal* (*mle*); most mutations at the locus, by definition, cause only males to die during development (Kernan et al. 1991). The action potential mutation within the gene is apprehended to mediate some weird kind of “gain-of-function” defect that is quite apart from the workhorse role of *mle⁺*.

In this context, it was warranted to test additional *disco* mutations to determine if they would cause dual defects—in visual system formation, on the one hand, and in terms of rhythm subnormalities, on the other hand. Comfortingly, it seemed, the *disco²* and *disco³* mutants, which were induced at a completely different institution and at a separate time compared with the origin of *disco¹* (Steller et al. 1987), were similarly arrhythmic for eclosion and adult locomotion (Dushay et al. 1989). Discomfortingly, it turned out that the effects of independent “hits” within the *disconnected* gene were not being tested: *disco²* and *disco³* are molecularly identical to *disco¹* (M. Freeman, pers. comm.), in that all three mutants are accounted for by the self-same nucleotide substitution (Heilig et al. 1991) within the gene’s open reading frame (ORF).

This kind of quandary has surfaced for other genes as well, some of them rhythm-related: The seminal arrhythmia-inducing *period* mutation (Konopka and Benzer 1971; Baylies et al. 1987; Yu et al. 1987) is molecularly identical to *per⁰²* and *per⁰³* (Hamblen-Coyle et al. 1989), and *per^{L1}* has the same property vis-à-vis *per^{L2}* (Gailey et al. 1991). In *Neurospora crassa*, induction and isolation of the *frequency* (*frq*) mutants were crucial for kick-starting molecular elucidation of the circadian clock mechanism in this fungus (for review, see Dunlap et al. 2004). For a while, it seemed intriguing that period changes caused by *frq* mutations entailed “quantal” ways that such cycle durations could be altered: All three of the *frq²*, *frq⁴*, and *frq⁶* mutants exhibited 19-hour free-running periods, and two other such mutations—with separate allele numbers 7 and 8, signifying that they were nominally isolated independently—each led to 29-hour periodicities. But the first three of these variants were identical to each other molecularly, and the second two had the same property in terms of their intra-ORF changes at the self-same site (Aronson et al. 1994). Incidentally, the notion that *Drosophila*’s *male-lethal* gene could commonly be mutated to cause nerve conduction failure collapsed when it was found that the original *nap^{TS1}* mutation at this locus was identical to the intragenic nucleotide substitution in *nap^{TS}* alleles 2 through 5 (Kernan et al. 1991). For *disco*’s part, the original allele number 1 was found to be identical not only to the aforementioned *disco²* and *disco³*, but also to mutation number 4 (cf. Steller et al. 1987; Heilig et al. 1991; M. Freeman, pers. comm.). To explain these annoying, or at least puzzling, cases, one could invoke intragenic “hot spots” that are susceptible to reinduction of mutations at the same sites in question. Alternatively, mutant screenings performed in part sloppily can lead to strain contaminants, whereby a supposedly new isolate in fact involves pulling aside an extant mutant already in the laboratory. Mercifully, with regard to interpreting the effects of *disco¹* on *Drosophila* rhythms, a bona fide independent mutation was induced at the locus. This is

disco¹⁶⁵⁶, for which an amino-acid-changing nucleotide substitution occurred at an ORF site different from that responsible for the *disco¹* missense mutant (Heilig et al. 1991). And *disco¹⁶⁵⁶* causes the same kind of locomotor arrhythmicity observed originally for *disco¹⁼²⁼³* (Hardin et al. 1992).

This result led to some modest relief, in that the effects of these independently isolated *disconnected* mutations were mutually confirming: Two separate variants are better than one, at least in terms of discounting the possibility that one of them entails an occult mutation that would be difficult to elucidate in terms of connections between altered genotype and aberrant phenotype. The same situation is pointed to by the *Clk* mutation in mouse. It should not necessarily be apprehended as the most conventional type of mutant, many of which suffer from decrements or loss of gene product functions. Thus, the seminal mutation at this murine locus is an “antimorphic” variant (also known as a “dominant negative”). An allelic type with this property is inferred from the fact that a deletion of the *Clk* locus heterozygous with *Clk⁺* leads to no locomotor rhythm anomalies (King et al. 1997a), whereas the mutation over “+” by definition causes altered rhythmicity: The mutant strain was isolated among F₁ mice stemming from chemically mutagenized males (treated with a substance called ethylnitrosourea [ENU]) mated to genetically normal females (Vitaterna et al. 1994). This mutant type spawned a large number of phenogenetic investigations, which uncovered several abnormalities within and without the chronogenetic arena (see, e.g., Naylor et al. 2000; Low-Zeddies and Takahashi 2001; Turek et al. 2005; Oishi et al. 2006). All such studies were rooted in the effects of the one ENU-induced allele, which is accounted for by an intragenic change (call it “altered” or *alt*) that would lead to modified final products (CLK protein isoforms) (King et al. 1997b; cf. Antoch et al. 1997). At last, a second *Clk* mutation was created by knocking out the normal gene (DeBruyne et al. 2006); the rhythm phenotypes associated with homozygosity for this *Clk*-null allele (superscript “minus”) are in a way “too normal.” For example, locomotor periodicities of *Clk^{alt}/Clk⁻* mice are barely if at all different from those of wild-type individuals (DeBruyne et al. 2006), whereas *Clk^{alt}/Clk^{alt}* animals typically exhibit greater than 27-hour periods that often degrade into aperiodic locomotion in constant darkness (see, e.g., Vitaterna et al. 1994; King et al. 1997b). This situation is not a disaster, whereby the *Clk*-null’s phenotypic properties would discount the significance of this gene’s actions on behalf of murine chronobiology. But the dramatically different effects of the second mutant allele compared with the first one isolated have necessitated further inquiry (DeBruyne et al. 2007).

The opposite scenario is in force with regard to a molecular relative in the mouse of one of the two *timeless* (*tim*) genes in *Drosophila*. The mammalian “*mTim*” factor in question was shown to be involved in clockwork function, in conjunction with identifying the pertinent stretch of murine DNA in its normal form by similarity to what turned out to be *Drosophila*’s *tim2* gene (Benna et al.

2000), whose synonymous name is *timeout* (for review, see Gotter 2006). The initial mammalian experiments involved, in the main, transfection of *mTim* into cultured cells to interrogate effects on expression of other clock factors cointroduced into them (for review, see King and Takahashi 2000; Lowrey and Takahashi 2000). But these findings did not speak to the *in vivo* relevance of *mTim* functions. The latter were investigated by, as one might have guessed, knocking out the gene, which led, if you will, to *too much* of an abnormality: mice that die during development when the newly manufactured *mTim*⁻ mutation was homozygous (Gotter et al. 2000). Furthermore, the viable *mTim*^{-/+} type exhibited no locomotor rhythm abnormalities, i.e., no dosage effect of heterozygosity for an absence of the gene (Gotter et al. 2000). (Recall from above that a *per*-null variant does have a dosage effect that causes circadian period lengthening.) These murine findings led to disparagement of *mTim* as a pacemaking “player” (the title of the paper just cited is “A time-less function for mouse *timeless*”). And whether or not subsequent analysis of decrementing mTIM protein levels seemed to resurrect the rhythm-related significance of this gene product (Barnes et al. 2003), the fact that *mTim* encodes a developmentally essential factor should *not* have undermined its potential meaning from chronobiological perspectives.

Here is the reason for this scolding: Several clock genes in *Drosophila* are *vital* ones, signified originally by induction of mutations at a locus called *doubletime* whose gene symbol is *dbt* (Price et al. 1998). The first-blush set of such variants allowed for viability, even when a *dbt* mutation was homozygous. By luck, a transposon (called a “P element”) had inserted very near to this locus; this facilitated molecular identification of *dbt* as encoding a kinase subunit (Kloss et al. 1998). Additionally, homozygosity for *dbt*^P causes developmental lethality, and neither that mutated allele nor a deletion of the gene over *dbt*⁺ leads to abnormal rhythmicity (Price et al. 1998). Other investigators who unwittingly induced mutations at the same locus (which they called *discs overgrown*) did so squarely with regard to their lethal effects (Zilian et al. 1999). Therefore, the actions of *doubletime* are pleiotropic, *not dedicated* to rhythm control. Stated another way, this gene is a *versatile* factor, which is exploited during development for one or more reasons quite different from the manner by which the enzymatic function gets reused much later in the life cycle to support proper circadian pacemaking.

By now, four rhythm-related kinase subunits are known in *Drosophila*, all pointed to initially by the effects of genetic variants involving the genes encoding the relevant polypeptides. The second of these genetic factors to emerge was *shaggy* (*sgg*, also known as *zw3*), which was reidentified in a transgene-based screen for “overexpression” effects on circadian locomotor periods (Martinek et al. 2001). The *sgg* gene had long been known to be able to mutate to lethality (see, e.g., Shannon et al. 1972), to cause interesting defects in developmental “pattern formation” (see, e.g., Simpson and Carteret 1989), and to encode a particular category of a kinase subunit (see, e.g., Peifer et al. 1994).

MUTATIONS AT CHRONOGENETIC LOCI MORE COMPLICATED THAN ONE WOULD IMAGINE

An analogous enzymatically based substory emerged, when an old *Drosophila* rhythm mutant eventually was discovered at the molecular level. This occurred many years after the *Andante* (*And*) variant had been chemically induced to make the circadian clock run “moderately slow.” (However, and as is so for any chronovariant at the outset, there was no way to infer that *And* is a pacemaker mutant *per se*.) Cycle durations of *And* adults or of flies emerging from late-developing cultures are only at most 2 hours different from the norm (Konopka et al. 1991). This seems to have militated against immediate or even vigorous attempts to take this gene to the molecular level. (Recall *Toki* and *Wheels* in this regard.) Furthermore, *And* came with a wing anatomical abnormality whose appearance was like that of the classical *dusky* (*dy*) mutants (Konopka et al. 1991). Sure enough, the mutational etiology of the *And*- and *dy*-like defect mapped to one genetic locus on the X chromosome—to the narrowly defined region where *dy* mutations (*per se*) were long known to be located (Konopka et al. 1991). Did some dissatisfaction set in thereby, owing to pleiotropy of the *And* mutant strain? Perhaps, but it nevertheless seemed necessary to test one of the original *dy* mutants for abnormal rhythmicity. There was none (Konopka et al. 1991). However, a contemporary analysis of newly induced *dy* mutants (which involves easier screening compared with rhythm monitoring) turned up a handful of them; about half of these turned out to be *And*-like in terms of longer than normal circadian periods (Newby et al. 1991). It was as if the “*And-dy*” locus were a complex genetic factor, whereby some of its mutations would be bidetfective and others would cause both kinds of phenotypic changes. Or, maybe one was unknowingly dealing with physical overlap of otherwise unrelated stretches of chromosomal DNA, so that only some mutations at such a locus would damage both encoded functions.

These complications were unraveled in conjunction with molecular identification of the pertinent transcription units. It turned out that they are right next door to each other on the X chromosome *and* that the seminal *And* mutant had been doubly hit: One nucleotide substitution is in the *And* gene *per se* (Akten et al. 2003) and the other is in the *dy* gene (DiBartolomeis et al. 2002). The latter encodes a function that need not concern us here (cf. Roch et al. 2003), and the former encodes a kinase subunit. Moreover, the enzyme regulatory factor specified by *And* is developmentally vital, because certain recently identified mutations at this “unitary” (noncomplex) locus are lethal (Jauch et al. 2002). So *Andante* is a pleiotopically acting gene after all but not because of the *dusky*-ness exhibited by the original mutant. The rhythm and the wing characters have nothing to do with each other biologically. Yet, the newer *And*-with-*dy* variants are puzzling in this regard (Newby et al. 1991). Could each of them be a double mutant too? They have not been assessed in this (molecular) respect. Provisionally, one would guess that these mutant types, isolated as wing

abnormals, were errant reisolations of the original doubly mutated *And* variant. This would be a further case of the “no hot spots” supposition invoked above to explain the bugaboos of multiple *discos*, *per*⁰s, and so forth.

In any case, it might be interesting to muse about why induction of *And* involved a double mutation at two extremely nearby chromosomal sites. Could a mutagenesis event involve stochastic accessibility of the chemical agent to a local region of the chromosome? Opening up the chromatin in this manner, within the gamete in question, could lead to nearby double hits more than Poisson would predict. This speculation cannot be dismissed out of hand because of the following: The third “clock kinase” identified molecularly in *Drosophila* involved a chemically induced mutation called *Timekeeper*, symbol *Tik* (Lin et al. 2002). It has dominant effects on behavioral rhythmicity, but *Tik* is a recessive-lethal mutation at a locus encoding a kinase catalytic subunit different from those specified by *dbt* or *sgg*. (TIK is a companion polypeptide to the AND polypeptide; for review, see Blau 2003.) The *Tik* mutant is accounted for by an *intragenic double mutation* that leads to separate amino acid substitutions (Lin et al. 2002).

This kind of mutational event has happened at least one more time in the *Drosophila* chronogenetic business: The most recently reported *timeless* mutant in this insect (at a locus different from the enigmatic *timeout* locus) also turned out to be doubly mutated within the ORF (Wulbeck et al. 2005). Another intriguing feature of this *tim*^{bl} mutant is that it exhibits longer than normal locomotor periods, but eclosion rhythmicity is like that of wild type. In contrast, the original *tim*⁰¹ mutant is aperiodic for eclosion, by definition of how it was isolated, and is similarly arrhythmic for adult behavior (Sehgal et al. 1994). In addition, subsequently induced *tim* mutants that were tested for both characters exhibit parallel abnormalities (Rothenfluh et al. 2000). What if *tim*^{bl} had been the only mutation induced in this gene? The unidirectional phenotype might have caused it to be subconsciously disparaged, such that the gene might have been viewed as a “mere output factor,” able to mutate so that only one particular kind of circadian rhythmicity is changed. As was introduced above, a bidefective mutant seems easier to view as defining a central pacemaker factor. Thus, what flows outward from the effects of a mutation within such a gene (such as *per*, *And*, and *dbt*) would be multiple circadian abnormalities (at least more than one of them). This supposition is of course belied by the case of *disco*, mutation of which leads to both eclosion and locomotor defects. Changes of this gene do not involve clock defects, but instead involve neuroanatomical mutants that suffer damage to the neural substrates of both rhythmic characters.

INPUTS TO THE *DROSOPHILA* PACEMAKER: MORE ABOUT DIFFERENTIALLY VARYING ALLELES AND CONSIDERATIONS OF FURTHER GENETIC COMPLEXITIES

I stretched the truth a little by suggesting that the above-mentioned *tim*^{bl} mutant is defective for only one rhythm-related character. This variant was noticed origi-

nally by virtue of a “phase change” readout by luciferase reporting of clock gene cycling. Live flies can be monitored automatically for this property when they harbor a transgene in which a fair amount of “5” DNA from a clock gene (such as *per*) is fused to *luc* (Brandes et al. 1996), the latter being luciferase-encoding cDNA derived from a beetle (also known as firefly). After *tim*^{bl} was recognized by virtue of this molecular anomaly, it was found that the mutated form of TIM is relatively “unresponsive” to light. Such stimulation normally causes the concentration of this protein to crash, a hallmark of photically effected clock resetting in this organism (Dunlap et al. 2004). How “light gets to TIM” is crucially mediated, it seems, by the direct absorption of such stimulation by *Drosophila*’s version of cryptochrome (CRY), a substance that is activated mainly by blue light (see, e.g., Sancar 2000).

One way that this process began to be elucidated stemmed from the first mutation induced in the CRY-encoding gene (*n* = 1 in this insect, compared with the two or more harbored by various vertebrate species). Thus, the *cry*^b mutant allele was induced and then recognized because it eliminated *per-luc* cycling (Stanewsky et al. 1998). Second- and third-stage testing of the mutation’s effects showed that *tim-luc* cycling was similarly flattened. In addition, the normal daily cycle of TIM protein abundance was eliminated as well, whereby the apparent level of the protein stayed high during the daytime (Stanewsky et al. 1998). In other words, light-induced TIM disappearance was disallowed in *cry*^b flies, as if a normal level of CRY is necessary to interact with TIM and cause it to be targeted for degradation. This supposition was contemporaneously supported by several additional kinds of experiments (whose details need not concern us, although for a review, see Hall [2003]).

The mutant-based side of this particular input substory involved an eventual array of ambiguities. First, note that *cry*^b is a missense mutant, which would not necessarily be a null variant. Whereas little or no CRY^b polypeptide was recognizable in the initial immunohistochemical tests (Stanewsky et al. 1998), subsequent tests have been detecting more and more leaking out of residual CRY levels that emanate from this mutated form of the gene (Busza et al. 2004). A subsequently induced *cry* mutant also was not demonstrable as a loss-of-function variant at the molecular level (Busza et al. 2004). The second and related point concerns the fact that the original *cry*^b mutant has been subjected to most of the requisite *chronobiological* testing. Here, too, light responsiveness of the mutated flies has suggested that they retain “some” CRY-mediated functions. These phenotypes involve, for example, the solid photic resettability of singly mutant *cry*^b adults. However, when this mutation was combined with “externally blinding” variants involving other fruit fly genes, the ability of doubly mutant individuals to resynchronize their locomotor rhythms to altered light/dark (LD) cycles was much more severely attenuated (see, e.g., Stanewsky et al. 1998; Veleri et al. 2007). Nevertheless, these CRY-depleted plus otherwise blind animals could be reentrained to an appreciable degree. One of the issues at hand, which now will be summarized

all too briefly, is that there are “multiple input routes to the clock” in terms of the heads of such pathways being light-responsive. The separate routes are supported by actions of different kinds of “visual genes,” on the one hand, and by different anatomical structures, on the other hand (for review, see Helfrich-Förster 2002). One of the latter such entities involves “deep-brain” photoreception (Emery et al. 2000b). It is mediated by CRY’s presence within several of the aforementioned pacemaker neurons, those that contain clock gene products, the PDF neuropeptide, or both (for review, see Hall 2003).

To make a fly “circadian blind” therefore necessitates at least double damage to the photic input system, which is usually achieved genetically (although see also Ohata et al. 1998). But what if whatever genetic combination involved includes a residually functioning *cry* gene product? Interpretation of modest resettability is confounded by the non-null state of CRY functioning, juxtaposed with the possibility that some additional unknown input route is in play. In any case, it seemed warranted to expand the allelic series for *cry* by one more step. Therefore, the gene was “targeted” for knocking out, as is nicely achievable in *Drosophila* these days by transgene-based tactics (Bi and Rong 2003; Venken and Bellen 2005). True null variants (allelic designations $\theta = \text{zero}$) were thereby created (Dolezelova et al. 2007). But light responsiveness of the clock input system was found not to be thoroughly blinded, even when a *cry*⁰ mutation was combined with another mutation that does (or should) render all peripherally located photoreceptive structures unresponsive (Dolezelova et al. 2007). It was concluded that, indeed, an “extra” input route functions within the system—one that could not be dependent on CRY as it cooperates with phototransduction processes believed to subserve light inputs to all of the relatively peripheral receptor cells known so far. (For the record, those entities are located in the compound eyes, the ocelli, an “extraocular” photoreceptor found at the periphery of the optic ganglia, and possibly some nonLN cells located near the dorsal extremity of the brain.)

Eclosion rhythmicity in *Drosophila* keeps being mentioned, even though this character has largely been replaced by locomotor monitoring as the workhorse bioassay of periodic phenomena operating at the whole-organismal level (for review, see Hall 2003; Price 2005). In this regard (sort of) studies performed long ago on late-developing cultures indicated that synchronization of them involves a relatively simple photic input. First, genetically normal *Drosophila* (of the *pseudoobscura* species) dietarily depleted of rhodopsin could be “entrained” or reentrained to eclose in an appropriately periodic manner, and there was no reduction in photic sensitivity (Zimmerman and Goldsmith 1971). In contrast, adult flies whose opsin-based photoreception is compromised exhibit subnormal sensitivity, even when they are CRY-enabled (see, e.g., Ohata et al. 1998; Stanewsky et al. 1998). The second point is that spectral sensitivity for light-induced eclosion entrainment was shown many years ago to exhibit a plateau “in the blue” (Frank and Zimmerman 1969; Klemm and Ninnemann 1976). A 21st century prediction, therefore, was that *cry*

mutant cultures should not be synchronizable for periodic eclosion by exposure of late-developing animals to LD cycles or that populations of mutated flies striving to emerge rhythmically (with near-dawn peaks) in that photic condition could not exhibit such periodicity. Defective exhibition of the latter characteristic was reported as a result of one study: LD arrhythmicity for *cry*^b eclosion (Myers et al. 2003). But another study showed that this mutant type exhibits strongly periodic adult emergence, after being developmentally entrained via LD cycles (including through the metamorphic period) and then left to free-run in constant darkness (DD) during the times of actual eclosion events (Mealey-Ferarra et al. 2003). Even adding a blinding mutation to a *cry*^b-containing genotype (which disallows LD synchronization of clock neurons within the *larval* brain) left the doubly mutant animals in a rhythmically eclosing state (Mealey-Ferarra et al. 2003).

Could it have been that the differing experimental protocols in these conflicting studies were also “off” in some unknown manner with regard to the photic and other (local) conditions? If so, and if the non-null *cry*^b type hovers at a sharp margin of entrainability for eclosion rhythmicity, separate sets of results could be at variance. Thus, it was thought that analysis of *cry*⁰ for periodic eclosion might resolve the conundrum. The outcome was that metamorphosing CRY-less flies exposed to LD cycles emerged in solidly rhythmic ways, when the subsequent conditions remained (environmentally) periodic or were shifted to DD (Dolezelova et al. 2007). A new finding was that LD-entrained cultures of the *cry*⁰ mutants did not go arrhythmic when shifted to constant light (LL) during eclosion monitoring (Dolezelova et al. 2007). In contrast, wild-type *Drosophila* emerge aperiodically under this condition (see, e.g., Chandrasekaran and Loher 1969). Therefore, CRY was concluded to be utterly *dispensable* in terms of functioning on the input side of this process, which operates at the transition between late metamorphosis and early adult behavior. However, this blue-absorbing protein is somehow *involved*, or else its elimination would have left *cry*⁰ cultures-slash-flies in a responsive state insofar as the arrhythmia-inducing effects of constant light are concerned. Furthermore, the periodic eclosion of this null mutant type *in* LD was slightly aberrant: Adult-emergence peaks occurred slightly before dawn, whereas those defining rhythmic wild-type eclosion and that exhibited by *cry*^b flies occurred slightly after the dark/light (DL) transition (Dolezelova et al. 2007).

At all events, dedicated generation of null alleles for one of the key genes acting on the input side—and combining a given such *cry*⁰ mutation with those known to be null with respect to any of the companion visual genes—did not crack the cases in question. But it was arguably important to try, as opposed to endlessly milking the relatively dull tools provided by *cry* missense mutants.

There are additional genetically defined functions operating on behalf of photically mediated chrono-inputs in *Drosophila*. One of them is an “F-box” protein (cf. Siepka et al. 2007) encoded by the fly’s *jetlag* gene, which specifies a factor that contributes to light-induced TIM degradation (Koh et al. 2006). Mutations at the *jet* locus were

encountered somewhat serendipitously, at least in part because they were naturally occurring variants segregating in laboratory strains of *D. melanogaster*. A key effect of such a mutation was to render behaving flies largely unresponsive to the normally arrhythmia-inducing effects of LL. (By the way, anomalous retention of locomotor rhythmicity under this photic condition is also a property of *cry* mutants, as was first shown by Emery et al. [2000a].)

In a companion study to the *jetlag* study just cited, mapping the etiology of “LL rhythms” indicated, however, that the genetic difference underlying the phenotypic difference (vis-à-vis arrhythmicity of wild type in LL) was *not* located in the chromosomal interval that harbors *jet* (Peschel et al. 2006). The requisite meiotic recombinants involved the following crucially considered genotypes, any of which included a *jet* mutation: one allelic type at the *tim* locus versus an alternative allele. (The *tim* gene is located on the same chromosome as *jet*, nearby.) These *tim* “isoalleles” (Rosato et al. 1997) are defined by one form that produces two TIM proteins (both encoded by the so-called *LS-tim* allele), whereas the other one makes only one form of the polypeptide (the short one called S-TIM). Only when a *jet* mutation was combined with *LS-tim* did the abnormally rhythmic behavior in LL occur (Peschel et al. 2006). Meanwhile, it has been revealed that LS-TIM is relatively unresponsive to light, degradation-wise, at least in part because this form of the protein seems physically to interact with light-stimulated CRY in a relatively weak manner (Sandrelli et al. 2007).

The upshot of these studies is that one needs to mind the genetic background! A given singly mutant type might or might not lead to the expected defect, unless it is conjoined with a separate player, one that, by itself, might cause a minimally appreciable abnormality for the chronobiological character in question. (Indeed, in the case at hand, neither the *LS-tim* nor the *S-tim* isoallelic types retains anomalous rhythmicity in constant light.) The “background warning” should be especially in force if neither of the other variants is rarely met. Indeed, both of the *tim* isoalleles are commonly encountered, including and especially in natural populations, where they vary in a geographically systematic manner: The *LS-tim* form is found at relatively higher frequencies in northern latitudes (Tauber et al. 2007). This harks back to the “*per* clines,” previously established to exist in natural populations analyzed north-to-south with regard to the intragenic length of a coding sequence that specifies a series of threonine-glycine amino acid repeats, located approximately in the middle of a given PER polypeptide (Costa et al. 1992; Sawyer et al. 2006).

MIND YOUR GENETIC BACKGROUND ALSO WHEN STUDYING OUTPUTS FROM THE CLOCK

The matter of pathways that connect central pacemaking to the distal regulation of revealed rhythmicity was introduced earlier. Increasing numbers of output-pathway endpoints are being recognized in *Drosophila* (for review, see Hall 2005). One to arrive on the scene in relatively

recent years is the control of sleep versus wakefulness. This phenomenon goes deeper into a matter of rest versus activity, in part because of the regulatory factors contributing to sleeping and to recovery from sleep deprivation (for review, see Greenspan et al. 2001; Hendricks 2003). Nevertheless, sleep/wake cycles come under the sway of circadian clock control as well. It would follow that isolation of sleep mutants might define genes that function on an output pathway operating downstream from the clock; such mutants might thereby be “specifically” defective and would not exhibit, for example, abnormalities of eclosion rhythmicity or that which underlies varying sensitivity of an appendage to odor stimuli (see, e.g., Krishnan et al. 2001). So, a screen for newly induced sleep-defective mutants turned up one; mapping the etiology of the “mini-sleep” character suggested that what became mutated was the famed *Shaker* (*Sh*) gene, which encodes a particular category of potassium channel subunits (Cirelli et al. 2005). Indeed, the *mini-sleep* mutation involved a novel site change within the *Sh* locus. These investigators wondered whether only this particular amino acid substitution within a SH polypeptide would cause the sleep anomaly, imagining in turn that any “hyperexcitable” mutant involving this gene would not necessarily be so fidgety that it would not sleep as much as normal flies do during a given daily cycle. Thus, they tested some of the classical *Sh* mutants for sleeplessness, but most of these variants tested as normal. What, however, if these long-maintained mutant strains had accumulated *genetic modifiers* that act to ameliorate would-be effects of the main (*Sh*) mutation? This is a frequently invoked shibboleth in the biogenetic business. But the supposition had real meaning here, because outcrossing the old *Sh* mutants to genetically normal *Drosophila* and reextracting shakerness from among the segregants led to substrains that exhibited the mini-sleep character. Apparently, the kind of change within the gene that leads to generic hyperexcitability will also cause restlessness. A further question was whether the connection between being hyper and sleeping minimally is so non-specific that mutations in other genes that encode different kinds of potassium channel subunits would cause the same abnormality. The answer seemed to be no, for neither *Hyperkinetic* (*Hk*) nor *seizure* mutants slept abnormally (Cirelli et al. 2005). Elements of this state of affairs were dubious when this study was reported, because the “non-*Shaker*” potassium channel variants were not outcrossed to ask whether the normal sleep phenotypes determined for some these “other” potassium channel mutants were influenced by factors lurking in the genetic backgrounds. Sure enough, a subsequent study undermined the hope for a *Shaker*-o-centric view of the connection between hyperexcitability and sleep anomalies, because *Hk* mutations were said to cause sleep-duration decrements, and the *Hyperkinetic* variants in question had been outcrossed to regularize their genetic backgrounds with the requisite control strains (Bushey et al. 2007).

An opposite kind of genetic modifier problem turned out to undermine the interpretability of another kind of output mutant in *Drosophila*. The question eventually asked was whether the *abnormality* is contributed to by

factors other than the “main” mutations. Those gene changes had occurred long ago in a gene called *ebony* (*e*), because the flies exhibit dark body color. Various pigment mutants of this sort turned out also to be neurotransmitter variants (not surprising, because the biochemical pathways subserving melanin production overlap with those that manufacture amine-containing transmitter substances). Several different *e*-mutated alleles were found to make flies largely arrhythmic for adult locomotion, but all such mutants exhibited normally periodic eclosion (Newby and Jackson 1991). Meanwhile, the *e* gene was becoming ever better characterized as to how its product, β -alanine synthase, regulates levels of dopamine and histamine (see, e.g., Borycz et al. 2002). It was as if one or both such substances were involved in intercellular communication along an anatomical pathway that links the proximal outputs of the clock to downstream entities that modulate walking behavior of the mature animal. An odd twist to this substory is that *e*⁺ seems to function on behalf of some kind of neurochemical processes operating in *Drosophila* glia (see, e.g., Richardt et al. 2002). This prompts mention of the fact that at least some of the clock genes in this insect are expressed in hosts of glial cells distributed throughout most CNS ganglia (Siwicki et al. 1988; Ewer et al. 1992; Helfrich-Förster 1995; Kaneko and Hall 2000). Why? Unknown—although it is worth mentioning further that the chronobiological meaning of glial cells harbored within a pacemaker structure in mammalian brains (Weaver 1998) was signified by at least one study (Prosser et al. 1994). In context of the current article being largely historical, it must be noted that the matter of “clock glia” potentially contributing to the regulation of actual *Drosophila* chronobiology (see especially Ewer et al. 1992) is one of many matters that are not properly a part of current concerns.

That aside, we plunge ahead to continue considering *ebony*-related neurochemistry. Thus, a companion set of *Drosophila* mutants to the *e* mutants are those altered in the *tan* (*t*) gene. These body color variants are subnormal for β -alanine hydrolase; once again, dopamine and histamine levels are compromised compared with concentrations of these substances in wild-type flies (Borycz et al. 2002). We therefore surmised that testing *t* mutants for rhythmic characters was in order. Putative effects of the *t*¹ and *t*⁵ mutations on eclosion and locomotor rhythmicity were assessed. Both mutant alleles supported normality for both characters (Fig. 1), unlike the reported effects of *e* mutations on the latter phenotype (alone). This outcome must be regarded as provisional, however: The *t* mutants (Fig. 1) will have to be outcrossed to wild type, followed by re-extraction of individuals with anomalous body color, and then retesting the *t*¹- and *t*⁵-mutated substrains for both rhythm phenotypes.

Speaking of outcrossing and now focusing on the mutant type that created the clock-output scenario in question: First, we found that both *e*¹ and *e*¹¹ mutants exhibited normal eclosion profiles (Fig. 2A), as originally reported (Newby and Jackson 1991). However, the first of these mutant alleles allowed for normal locomotor rhythmicity (Fig. 2A,D), quite at variance with the earlier study. For *e*¹¹, the rest/activity cycles of adults were

“weak” overall (Fig. 2C,D), allowing one to cling to the notion that the gene has a chronobiological role. However, outcrossing *e*¹ and *e*¹¹ to wild type (seven times) and reextracting the requisite darkly pigmented substrains showed that adults taken from them were “strong” and otherwise normal for locomotor rhythms (Fig. 2E,F).

In our hands, therefore, the connection between pigmentation, *these* neurotransmitters, and regulation of rhythmic behavior is solely a matter of one or more factors harbored in the genetic background of a particular *ebony* mutant. One soft feature of this analysis is that whatever these factors may be is unknown—as to whether few or many rhythm-undermining variants are harbored in the *e*¹¹ strain, let alone where within the genome these putative output factors would be located.

A different tack is taken in this arena nowadays. The genetic tactic in question involves noticing that strains which vary among each other for “some” phenotypic character, followed by performing crosses between strains, which can result in specifiable genetic segregants and recombinants. The specificity at hand means that quantitative trait loci (QTL) can be chromosomally mapped: How many such factors are pointed to, and where is each located within genome (at least roughly)? To which features of the overall phenotype do they contribute (speaking to the fact that locomotor rhythmicity involves a variety of quantifiable attributes, such as the “free-running circadian period” and “phase angle of entrainment” metrics dealt with in the study cited shortly below)? What is the magnitude of a given QTL’s contribution to the character difference observed by monitoring behavior of the starting strains? A few chronogenetic investigations of this sort have been

Figure 1. Adult emergence and locomotor rhythms of *tan* mutants. (A) Emergence of flies from metamorphosis, monitored in constant darkness (DD) as described by Mealey-Ferrara et al. (2003), led to the eclosion profiles in the left-hand two columns; high-frequency components were filtered in the second column (cf. Levine et al. 2002). These time courses were analyzed as plotted in the right-hand three columns via maximum entropy spectral analysis (MESA) and the two other formal treatments of the data plotted rightward (for principles and practices for these methods, see Levine et al. 2002). The two *tan* mutants tested are indicated by their superscripted allele designations. The *+/+* genotype refers to a *tan*⁺ wild-type control, which was not isogenic with the mutants. (B) Locomotor activity of the *tan*¹ mutant, monitored in DD as described by Dolezelova et al. (2007), for example. The second row gives an example of behavior performed by a female heterozygous for the X-chromosomal *t*¹ mutation and a deletion (*Df*) of the genetic locus. The third row exemplifies behavior of a *t*¹/*t*⁺ heterozygote. The left-hand column displays double-plotted actograms (days 1–2 of locomotor movements on the top line of a given plot, days 2–3 on the second line, etc.) Formal analyses of these behavioral records (right-hand three columns) were again performed as described by Levine et al. (2002). (C) Locomotor activity of the *tan*⁵ mutant; labels and plot types are the same as those in B. (D) Relative robustness of locomotor rhythms exhibited by “average flies” expressing various *tan* alleles (the *n* indicates numbers of individual flies monitored). The homozygous and heterozygous such genotypes (see B and C) are displayed across the abscissa. The ordinate values are a measure of “rhythm strength,” as described by Levine et al. (2002), including a cutoff line horizontally coursing across the plot; RS values above that line imply “significantly” periodic behavior.

Figure 1. (See facing page for legend.)

performed, notably in mouse. A quite extensive one uncovered 14 QTLs, scattered among most of the rodent's 20 chromosomes (Shimomura et al. 2001). By this time, chromosomal sites that harbor several of the "main" clock genes in this mammal were known; additional such loci were pinned down as an accompaniment to this QTL analyses, i.e., the intrachromosomal sites corresponding to clock genes originally identified molecularly in their normal forms (Shimomura et al. 2001). Three of these genes that are among the most conspicuous chronogenetic factors in mammals are murine (*m*) relatives of *Drosophila*'s *period* gene (the former being known as *mPer1* through 3, i.e., three separate genes). It could be regarded as disappointing that no QTL (in the study just cited) apparently corresponded to a previously known rhythm-related locus. Several of the latter have been subjected to gene knock-outs, and such null mutations were frequently found to cause subnormalities or anomalies associated with murine locomotor rhythmicity (as nicely summarized by the list contained within Stanewsky 2003). For example, knocking out *mPer1* and *mPer2* and then combining the two nulls lead to severely subnormal rhythms of wheel-running behavior (for review, see Stanewsky 2003).

One idea underlying the QTL approach was that homing in on certain rhythm-related genetic loci would sug-

gest that they entail milder genotypic changes within some of the "major" clock genes such as *mPer* genes. Such *gedanken* outcomes could independently confirm the chronobiological meaning of such genes, along with enhancing one's appreciation of what a given such factor is "about" in terms of how it contributes to a particular feature of circadian rhythmicity. Inasmuch as this kind of picture could not be painted by the results of the Shimomura et al. (2001) study, a different and just as good inference suggests itself: Tapping into naturally occurring variation can lead to identification of genetic factors *heretofore unknown* to be involved in the process. Bad luck could have militated against mutating these loci to isolate mutants exhibiting whatever rhythm irregularity was being screened for. That said, newly induced mutants that are saved *and* followed up tend to show rather substantial differences from the norm (prompting rereferrals to Vitaterna et al. 1994; Bacon et al. 2004; Siepka et al. 2007). It is therefore arguable that a QTL associated with modest variation for the character in question (Shimomura et al. 2001) might not get delved into deeply, unless more severely mutated allelic changes at the locus will be induced or otherwise encountered later on.

BACK TO THE BEGINNING: MULTIPLE GENOTYPIC VARIANTS INVOLVING *DROSOPHILA*'S *PERIOD* GENE AND THEIR IMPLICATIONS FOR FUNCTIONING OF ITS PRODUCT

Mentioning the mouse *Period* genes (shortly above) and their engineered mutations was done judiciously, for it brings us back to the seminal mutants of this kind and to the first clock factor that was identified in concrete form. In this light, the *period* mutants in *Drosophila* and the locus it defined were subjected to rather extensive phenogenetic analyses. The plural noun just invoked refers to more than extensive chronobiological testing of a given individual mutation's effects. In addition, many *per*-related genetic *combinations* were produced to ask whether the effects of such genotypes might lead to inferences about how the gene functions. For example, *per* mutations that allow for rhythmic behavior were made heterozygous for the normal allele; all such combinations indicated the aforementioned semidominance of these mutant alleles. Furthermore, the period alterations—observed for instance in *per^S/+* or *per^L/+* heterozygotes—involved departures from the norm greater than those caused by *per⁰¹/+* (Konopka and Benzer 1971; Smith and Konopka 1982). It follows that the effects of the period-shortening and -lengthening alleles are worse than that of the "zero" mutation. That the latter might indeed be null was suggested, early on, by the fact that a deletion of the gene, over +, leads to the same modest period lengthening as observed for *per⁰¹/+* *Drosophila* (see, e.g., Smith and Konopka 1982).

This particular result points to "cytogenetic" studies of *period* that formed a part of this analytical process. A further feature of such manipulations was to assess the effect of a duplication of the chromosomal region con-

Figure 2. Adult emergence and locomotor activity rhythms of *ebony* mutants. (A) Periodic eclosion of two *e* mutants (distinguished by the different superscripts), compared with a wild-type control (+/+), the flies of which were not isogenic with the *e* mutants. Plotting and analytical tactics are the same as those in Fig. 1A. (B) Free-running locomotor activity rhythms of flies (monitored in DD), which were homozygous for *e¹*, heterozygous for that mutation and a deletion (*Df*) of the locus, or heterozygous for *e¹* and the normal (+) allele. Plotting and analytical tactics are the same as those in Fig. 1B. (C) Locomotor rhythmicity influenced by the *e¹¹* mutant allele harbored in a long-inbred strain. Columns, rows, and genotypic labels are the same as those in B (cf. Fig. 1B,C). (D) Robustness of locomotor rhythmicity influenced by the *e¹¹* allele. The metric and the plotting conventions are the same as those in Fig. 1D. The asterisks designate significantly reduced rhythm strength caused by homozygosity for *e¹¹* or "uncoverage" of that mutation's effect by a deletion (*Df*). (E) Locomotor rhythmicity influenced by *e¹* or *e¹¹* after flies carrying a given such mutation were outcrossed to a wild-type strain, followed by crossing the resulting heterozygotes ("over +") to each other and selection of homozygous mutant individuals among the offspring; those *e/e* flies were outcrossed again to +/+ to create round-2 worth of heterozygotes, further enriched for the wild-type genetic background. This crossing procedure was repeated such that seven rounds of *e/+* heterozygosity were in force; after the last such round, mutant homozygotes were selected (as usual), and it was these *e¹/e¹* or *e¹¹/e¹¹* flies and their true-breeding homozygous descendants that were tested for locomotor rhythmicity. Examples of such behavior are displayed (along with the analytical plots) as in B and C. (F) Strong locomotor rhythms associated with flies carrying *e¹* or *e¹¹* in, or derived from, the outcrossed (outX) strains. The one "derived" type is the *e¹/+* heterozygote generated by crossing (outX) homozygotes to the wild-type strain used in the outcrossing (= "isogenization") procedure. The +/+ control (average rhythm strength) bar resulted from testing flies from that wild-type strain. The four ordinate values (cf. Fig. 1D and Levine et al. 2002) were statistically indistinguishable from one another.

taining per^+ , whereby increased dosage of the normal form of this gene led to shorter than normal periodicity (Smith and Konopka 1982), a finding that prompted the notion that per^S might be a “hypermorphic” mutation, leading to an enhanced level of the encoded function. But this was belied by a combination of allele effect and cytogenetic analysis: If the $per^S/+$ genotype is making “lots more product,” expression of the + allele in this heterozygote would contribute to the overall (heightened) concentration of whatever the gene encodes. If this supposition has meaning, a per^S overdeletion (per^-) heterozygote would produce slightly less product and result in a somewhat longer cycle duration compared with $per^S/+$. But the latter genotype leads to about 21.5-hour periodicity, whereas per^S/per^- flies run at about 20 hours (as cited and interpreted by Côté and Brody 1986). Thus, a better way to view this subscenario is to assume that the effect of per^S is not to “add” to the effect of the + allele but instead to *interfere* with “normal function” (cf. Côté and Brody 1986). The hedge just quoted is that this might have to do with the product of the normal per gene or with factors specified by other rhythm-related genes, or both.

The upshot of all these phenogenetics was the following: (1) One job of the *period* gene product is to *interact* with other chronoactive factors (suggested by the dominant negativity of period-altering mutations, again as exemplified by the phenotype of $per^S/+$ flies), and (2) the level of per ’s product is *rate-limiting* for period control (implied by the gene-dosage effects, which by the way do not obtain for some of the other clock genes in *Drosophila*, such as *timeless* or *doubletime*).

To what extent did any of these phenogenetic phenomena suggest what the *period* gene product actually is? Intriguingly, nothing whatsoever suggested itself in this regard. Any and all of the analytical outcomes just summarized could have allowed one to speculate that the presumed PER polypeptide possesses some sort of enzymatic function or that it could be inserted into cellular membranes, or it could be “anything.”

Distinctly different kinds of phenogenetic stories have been told in biogenetic history (referring to genetically based investigations that have been aimed at understanding physiological, developmental, and neurobiological processes as opposed to hard-core genetic processes). Take the *lactose* operon in *Escherichia coli*. The early days (years, really) of studying it were rooted in recognition of many different kinds of mutations at this complex bacterial locus. Furthermore, different allelic types that mapped to a given subsite within the locus were recovered. Pitting a given mutant phenotype against the normal inducibility of *lac*-encoded protein products led, in time, to a magnificently conceived scheme as to how the regulatory factors comprising portions of the locus operate, i.e., what these “*cis*”- and “*trans*”-acting entities would consist of at the concrete level. In this regard, when examinations of *lac*-contained or -encoded regulatory items became molecular, elucidation of those factors was arguably anticlimactic, thanks to the broad and deep analyses of genotype with phenotype connections that had been previously per-

formed (Jacob 1997). An analogous metazoan case is provided by a famous subset of the many developmental mutants known in *Drosophila*. Again, “pure” phenogenetics of the manner by which key mutations alter pattern formation of the developing animal indicated that the corresponding genes were “selector” factors (see Chapter 5 in Lawrence 1992). It was just a matter of time for these genes to be identified at the DNA level, and the chance that “clone and sequence” would *not* have revealed these sequences to encode gene-regulatory factors (transcription ones) was nil (Lawrence 1992).

In marked contrast, the *period* clock gene in *Drosophila* had to be cloned, absent anyone’s wherewithal to deduce the nature and functionality of its product. Furthermore, sequencing the genes ORF led to no clues—or worse—as to what the encoded polypeptide is about (for an early review of what happened over the course of about 10 years postcloning, see Hall 1995). This initially featureless protein had to be elucidated for the circadian-pacemaking role it plays, largely by analyzing the manner by which *per* gene products are expressed. This means, for instance, that PER is found within the nuclei of neural cells, naturally (although in many other tissue types as well), and, from a temporal perspective, the levels of *per* mRNA and of PER protein were found to exhibit systematic daily oscillations (again see Hall 1995). These facts stimulated further inquiries that began to crack the case (as surveyed by Dunlap et al. 2004), a case so filled with cracks that perhaps one more would not hurt. The “one more” in question entails, in a nutshell, a scheme in which cyclically fluctuating PER feeds back to impinge on the transcribability of the first-stage gene product. Therefore, such mRNA cycling, as controlled in part by PER cycling, became viewed as a core component of the oscillator mechanism (Hall 1995; Dunlap et al. 2004).

The meaningfulness of *per* product cycling as it gets controlled transcriptionally has been called into question, however. First, an empirical study was designed to “drive” *per* expression or that of a *timeless*-coding sequence, or both, in temporally constitutive ways. The relevant transgenic strains were set up to activate these clock gene product generations via a heterologous transcription factor whose own production was controlled by a gene-regulating stretch of DNA that is “always on” (Yang and Sehgal 2001). Therefore, *per* and *tim* mRNA levels would be temporally flat, at least in terms of how the primary gene products are transcribed. The transgene combinations were introduced into genetic backgrounds that harbored *per*- or *tim*-null mutations (or again, both, as the case may be). Even when the only DNA sequences that could generate functional PER and TIM were encoded by constitutively generated mRNAs, varying proportions of the adult flies exhibited locomotor rhythmicity (Yang and Sehgal 2001). Therefore, transcriptionally supported rhythmicities of these coding sequences were deemed not necessary for periodic biological readout.

These results have inspired some who hover round the clock molecular stories that have been told for various organisms to disclaim as follows: “Transcriptional feedback oscillators: Maybe, maybe not...” (Lakin-Thomas 2006). An appreciable portion of what this author argued

(under the title just quoted) involved denigration of the standard view of rhythm regulation in *Drosophila*, which assumes (to quote this author further) that it is “‘important’ for function” for the final gene products in question to feed back in order that they can modulate cyclically varying generation of the molecules that encode them (that specify, in this case, PER and TIM proteins). Lakin-Thomas (2006) did give a nod to one feature of the Yang and Sehgal (2001) results, which was that “rhythms in the double constitutive flies [see Fig. 3] are somewhat weak.” Nevertheless, this iconoclast went on to proclaim that “I take the glass half-full attitude: as many as half [sic] were rhythmic” (Lakin-Thomas 2006). With around “half” the animals in question performing rhythmically at the behavioral level (see Fig. 3) (referring to some of Yang and Sehgal’s transgenic strains), such a result was surmised to mean: that’s it! Any rhythmicity eked out of a situation in which transcriptional control of molecular cycling cannot be operating was viewed as undermining the original “case-cracking” discoveries of the transcriptional feedback oscillators (which, again, are laid out in their simplest form within the review by Hall 1995).

What are we to make of these sour notes? First, some further empiricism: Wondering how “full” is the “glass,” we retested the triply transgenic strain designed to make *per*- and *tim*-coding sequences simultaneously transcribed in a constitutive manner. The “rescue” of *per*⁰¹/*tim*⁰¹ arrhythmicity (whereby either such null mutation alone is sufficient to cause that property) was such that a mere 19% of the locomotor-monitored flies exhibited significantly periodic behavior (Fig. 3A). This less-than-half outcome is worse than what was reported from the results of several analogous locomotor tests (Yang and Sehgal 2001). Furthermore, about one third of the rhythmic “doubly constitutive” individuals that behaved rhythmically did so in an ambiguous manner, notably by exhibiting more than one periodic component in conjunction with their free-running cyclical behavior (see examples in Fig. 3B–E). Even worse, in a way, the unambiguously rhythmic cases involved cycle durations distributed over an approximately 6-hour range (Fig. 3F), even though the numerical boundaries were in the circadian ballpark. In marked contrast, genetically normal *Drosophila* yield locomotor periods that are tightly clustered near 24 hours (see, e.g., Hall 2003, 2005; Price 2005).

This calls into question whether a given abnormal rhythm-related genotype and its attendant phenotype are what we are all about investigatively. In other words, are we not attempting to deduce from such genetic experiments what is necessary for *rhythm normality*? Should we not at least consider that certain genetically effected abnormalities are rather uninformative, especially when the outcome is a crude caricature of the normal process? Minimally, the rhythmic behavioral glass seems so far from “half-full” in the current case (Fig. 3) that one can surmise a prime role for transcriptional control of *molecular* cyclings if anything in hailing distance of normally periodic *biology* is to occur.

Furthermore, or more specifically, it appears that *all*

levels at which clock gene products are regulated, such that they exhibit daily oscillations, are correlated with wild-type rhythmic phenotypes. The following are some experimental results that speak to this issue: (1) When a *per* transgene lacks its 5′ regulatory sequence, the *transcription rate* at which the encoded RNA is produced is temporally flat (So and Rosbash 1997), but (2) the *steady-state abundance* of *per* mRNA cycles nonetheless, as do the apparent levels of PER protein (Frisch et al. 1994, pointing to many subsequent studies that revealed PER cycling to be controlled in part posttranslationally, as shown seminally by Cheng and Hardin 1998), (3) the transgenic situation in question (Frisch et al. 1994) is enough to support routine rhythmicity of the adult flies, but their period and phase values are appreciably “off” the norms, and (4) additional kinds of *per*-manipulated transgenic types showed that control of RNA production at the transcriptional level alone is sufficient for this “level” of gene product to cycle rather robustly (referring to reporter DNA sequences that had been fused to *per* regulatory sequences), but the temporal dynamics were distinctly aberrant unless the pertinent heterologous DNA sequence (*luc*) was fused to well more than half of the *per*-coding sequence (Stanewsky et al. 1997). The latter set of results means that—fair enough—transcriptionally controlled cycling of clock gene expression does not tell the entire tale. But Points 1 and 2 indicate that the level of control, at the primary stage of gene expression, is far from unimportant.

All of this said, the issues remain up in the air. At least it is the case that mediating production of factors encoded by certain key clock genes—in a systematically varying manner, along with tweaking the temporal dynamics of these molecules after they are primarily generated—will remain objects of much further study. A nice recent example shows how these issues can and must be analyzed, in part from the perspective of transcriptional control of circadian oscillators (Kadener et al. 2007). This alludes to the fact that retesting whatever set of naysaying results that remain in the wind (Yang and Sehgal 2001) is insufficient (Fig. 3 notwithstanding).

At all events, it is time to wind things up because it appears as if the verbal wranglings with which the foregoing passages are replete has strayed rather far from the chronogenetic theme of the piece.

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Figure 3. (See facing page for legend.)

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Figure 3. Behavior of *Drosophila* whose transcription of the *period* and *timeless* genes was engineered to be temporally constitutive. (A) Flies of the triply transgenic genotype indicated at the top were monitored for DD behavior (cf. Figs. 1 and 2). This transgenic type was created by Yang and Sehgal (2001) and supplied by A. Sehgal. The genetic background of that strain included a loss-of-function *period* mutation (*per⁰¹*), but the *timeless* allele was genetically normal; thus, crosses were performed to generate a substrain in which a *tim*-null allele (*tim⁰¹*) was homozygous on the second chromosome. This derivative strain also retained the “driver” transgene (containing a DNA sequence derived from the “pan-neuronally” expressed *elav* gene), along with the two “drivee” transgenes (containing, respectively, a *tim⁺*-derived cDNA and a *per⁺*-derived one). This multiply variant type of fly is designed to transcribe the TIM- and PER-protein-coding drivee transgenes in a manner that does not systematically vary across a given day (owing to *elav* being expressed in such a “constant” manner). However, immunoreactivity for TIM and PER do “cycle” to define about 24-hour periodicities (Yang and Sehgal 2001), owing to posttranslational effects on the levels of these polypeptides (see, e.g., Cheng and Hardin 1998). The proportion of rhythmic individuals was determined (after DD monitoring), according to the metrics used to create Figs. 1 and 2. (B–E) Examples of locomotor activity plots (as in Figs. 1 and 2), including cases involving a singly periodic component (as indicated by the circadian “h” values) and others that were ambiguous (ambig.), because more than one such component was analytically extracted (shown here via periodograms, again cf. Figs. 1 and 2). With reference to A, 6 of the 20 “significantly rhythmic” individuals gave ambiguous outcomes. (F) Distribution of free-running cycle durations, resulting from analysis of the 14 behavioral records (cf. A) that yielded single-period values. The rather wide spread of these periods for the “*per⁺/tim⁺*”-constitutive” transgenics (among the low proportion of such flies that behaved rhythmically) is distinctly different from normal circadian behavior: Nearly all wild-type flies locomotor-monitored in this manner are not only significantly periodic, but their free-running periods are nearly always in the range of 23.5 to 24.5 hours (see, e.g., Hall 2003, 2005).

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