MUTATIONS AND MOLECULES INFLUENCING BIOLOGICAL RHYTHMS

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INTRODUCTION

Rhythmically oscillating phenomena in biological systems have been analyzed in many ways: behaviorally (reviews: Moore-Ede et al 1982, Saunders 1982), neurobiologically (reviews: Turek 1985, Jacklet 1985), and biochemically (reviews: Takahashi & Menaker 1984, Johnson & Hastings 1986). Over the last four years, another angle from which these rhythms has been approached—“clock genetics”—appears to have also come to the fore. Yet, this area of rhythm studies has been around for at least 20 years (previous summaries: Feldman et al 1979, Konopka 1981).

In the present review we update several of the recent genetic advances and also discuss how such investigations are moving into the molecular area in studies of two organisms, Drosophila and Neurospora.

The biological clocks underlying rhythmic fluctuations have seemed to be an eternal mystery. Whether “clock molecular genetics” will allow us soon to peer into the core mechanisms of the pertinent oscillators remains to be seen.

Interpretation of the studies of these clocks is not always simple. That is, a fair amount of jargon surrounds rhythm analysis. Consequently we begin by listing some of the properties of true clocks, which are deeper than simply that they control various “parameters” that exhibit some kind of regular fluctuations having a given periodicity, such as a “circadian tau,” or cycle durations in the ultradian realm (ca. $10^{-1}$ to $10^{-3}$ of a day; reviews: Shultz & Lavie 1985). We note these additional attributes of
clocks because most of the "clock mutations" to be discussed have turned out to affect more than overt circadian or ultradian periodicities:

1. Thus, clocks not only produce such taus, but also a clock has a phase, meaning, for instance, that the start of a given cycle usually occurs in a particular relationship to an environmental cycle. For example, peaks of eclosion (pupal-to-adult transition) in Drosophila tend to occur just after dawn (or "subjective dawn," see below).

2. When a clock is started (e.g. by a pulse of light) or entrained (e.g. by alternating cycles of light and darkness, such as 12 hr: 12 hr "LD") it will subsequently free run in constant conditions (whereby the lights do not come on again after the pulse or after the cycles of LD entrainment).

3. Thus, rhythms are endogenous, not mere responses to cyclical environmental cues—meaning that the cycles continue during free-running conditions.

4. A free-running rhythm can be phase-shifted, e.g. reset by application of the same kind of light pulses used to set the phase in the first place.

5. The phase advances and delays that occur under these circumstances define a phase-response curve (PRC) that has quite similar properties in many species of microbial and metazoan eukaryotes; i.e. delays are induced when the resetting signal is delivered during the early subjective night (portion of a free-running cycle that extrapolates back to when it was dark during entrainment), advances occur during late subjective night, and the clock is insensitive to resetting cues during the subjective day.

6. Clocks are temperature-compensated: circadian, and even ultradian (e.g. Lloyd & Edwards 1984), taus are much the same over rather wide temperature ranges. This seems most meaningful in poikilothermic organisms, such as Drosophila (e.g. Zimmerman et al 1968) plus Neurospora (e.g. Gardner & Feldman 1981). Temperature compensation is also meaningful in certain vertebrates (such as hibernating species, e.g. Menaker 1959, Rawson 1960), but it does not occur so well in others (e.g. Richter 1979, Gibbs 1983).

RHYTHM MUTANTS

Mutations that alter or seemingly abolish circadian rhythms have been systematically isolated in two Drosophila species, D. melanogaster and D. pseudoobscura (reviews: Konopka 1979, 1981, Hall 1984), and in the microbes Neurospora crassa (reviews: Feldman & Dunlap 1983, Feldman 1985) plus Chlamydomonas reinhardtii (reviews: Feldman 1982, 1983; also see Mergenhagen 1984). The relevant mutants, defining on the order of a
half-dozen "clock genes" in most of these species (exception: \( N = 2 \) in \textit{D. pseudoobscura}), were recovered in brute force screens, whereby the rather heroic efforts of R. J. Konopka and colleagues plus F. R. Jackson (\textit{Dm}), C. S. Pittendrigh (\textit{Dp}), J. F. Feldman and co-workers (\textit{Nc}), and V. G. Bruce (\textit{Cr}) led to mutants with either longer or shorter than normal taus, or to strains that apparently do not exhibit any circadian rhythmicity. In addition, there are two recently isolated mutants, which were encountered in the course of rhythm experiments as opposed to being the results of deliberate mutagenesis efforts: an arrhythmic mutant in the blowfly \textit{Lucilia cuprina} (Smith 1987) and a short-period mutant in hamster (Ralph & Menaker 1987). Finally, experiments on the "polygenic" control of rhythms have occasionally been performed, not only in \textit{Drosophila} (Pittendrigh 1967) but also in certain mammals (Possidente & Hegmann 1980, Büttner & Wollnik 1984, Hotz et al 1987, Wollnik et al 1987).

Detailed analysis of the phenotypes affected by these kinds of mutations—and, just as important, of the \textit{genes} defined by them—have been extensive and deep only in \textit{D. melanogaster} and \textit{N. crassa}, however. The genic studies \textit{per se} have paved the way for molecular analysis of certain of the most interesting genetic factors that participate in the control of the several clock attributes listed above. We discuss how these genetic studies have specifically led to the molecular work in the latter sections of this review. First, however, we review several recent results concerning the "phenogenetics" of the main clock mutants and mention certain characteristics of the less analyzed variants.

**Drosophila Clock Mutants**

Most of the rhythm mutations in \textit{D. melanogaster} have been induced, by chemical mutagens, at one genetic locus, the X-chromosomal \textit{period} (\textit{per}) gene (Konopka & Benzer 1971, Konopka 1987). Some of these \textit{per} mutants have circadian taus far outside the normal range: \textit{per}^\textit{\alpha}, 18–20 hr; \textit{per}^{L1} and \textit{per}^{L2}, 28–30 hr; the majority (\textit{per}^{\alpha1} through \textit{per}^{\alpha4}) are arrhythmic in both eclosion and locomotor activity (re "sleep/wake" cycles of the adult flies). Two other clock mutations on the X chromosome turned up in these screens: \textit{Clock} (\textit{Clk}, activity cycles ca. 22–23 hr) and \textit{Andante} (\textit{And}, eclosion and activity taus, ca. 25–26 hr). Jackson (1983) induced three rhythm-altering mutations on the autosomes of this species: \textit{phase-angle-2} (\textit{psi-2}) and \textit{phase-angle-3} (\textit{psi-3}), each of which cause "too-early" (i.e. pre-dawn) emergence during LD cycles. Each of these mutations turned out, as well, to control free-running periods that are, as in the \textit{And} mutant, slightly longer than normal (cf Pittendrigh 1967). The other autosomal mutation, \textit{gate} (\textit{gat}), is closely linked and could be allelic to \textit{psi-2}. \textit{gat}
causes eclosion to be poorly gated, such that the flies do not emerge during the usual narrow windows of time (Jackson 1983).

Further phenotypic studies of the per mutants showed that the per⁰ cases appear to be arrhythmic, in eclosion, even during LD cycling (Konopka 1981; also see Bargiello et al 1984). Yet, these mutants are, in LD, pseudorhythmic in their locomotor activity, i.e. they regularly become active when the lights come on or go off (Hamblen et al 1986). This, though, is a mere response to environmental changes as opposed to the expression of an endogenous oscillator. Thus, per⁰s are, by definition, not rhythmic in free-running conditions. And, more subtly, they have no apparent clock running during entrainment, whereas the rhythm’s phase for wild-type (per⁺) flies is such that they anticipate lights-on by becoming active ca. 2 hr in advance of this environmental change (Hamblen et al 1986). This kind of “phase lead” is a common feature of vertebrate rest/activity cycles (e.g. Fuchs 1983).

The per results noted above have some parallels with effects of arrhythmic mutations in L. cuprina and D. pseudoobscura. In the former dipteran, the ary mutant was found on the basis of arrhythmic eclosion; it also exhibits this phenotype in free-running locomotor activity, although the adults are cyclically active and inactive in LD (Smith 1987). In D. pseudoobscura, there are five of these, defining two X-chromosomal genes by complementation tests. Three of the mutants, only, eclose arrhythmically in LD; the other two, which are not allelic to each other, exhibit “forced” rhythmic eclosion under these conditions (Pittendrigh 1974). It has also been determined that all five of these mutants are arrhythmic in free-running locomotor activity (Konopka 1979), though further details (e.g. LD vs “DD” comparisons) were not reported. It was shown, near the time of these mutants’ isolations, that the genetic variants are “semidominant”; specifically, for these D. pseudoobscura mutants, females “doubly” heterozygous for a given pair of nonallelic mutations are rhythmic but with longer than normal periods and with a substantially altered phase (Pittendrigh 1974). We mention below other examples of this kind of incomplete recessivity, i.e. for clock mutations in general.

Multiply mutant flies have been produced in D. melanogaster as well to determine whether putatively “synergistic” rhythm defects would occur. So far, however, only additive effects on circadian periodicity (Orr 1982; And plus per and And plus Clk combinations) or phase (F. R. Jackson, unpublished: psi-2, psi-3 double mutants) have been found. These phenotypes are analogous to those exhibited by Chlamydomonas when it is doubly or multiply mutant for rhythm variants (Bruce 1974). (See below for somewhat contrasting results from Neurospora.)

The gene dosage of per’s normal allele has been manipulated. Increased
and decreased copies of \( \text{per}^+ \) lead to shortened and lengthened circadian periods, respectively (Smith & Konopka 1981, 1982). Not only do females heterozygous for the normal allele and a deletion show longer than normal periods, but \( \text{per}^0 \) mutations are similarly semidominant (Smith & Konopka 1981, 1982). Nearly the same phenotype results when a \( \text{per}^L \) mutation is made heterozygous with \( \text{per}^+ \) (Smith & Konopka 1981), thus suggesting that this class of variant is a severe "under-expressor" of information encoded within this genetic locus, with \( \text{per}^0 \)'s being hypothetically "null" (i.e. equivalent to a deletion). Additional force to these suggestions has come from recent molecular studies (see below). The short-period \( \text{per}^s \) mutant is a most interesting case. Like \( \text{per}^0 \) and \( \text{per}^L \), \( \text{per}^s \) is semidominant to \( \text{per}^+ \) (Konopka & Benzer 1971). The aforementioned hamster mutation (\( \tau \)) is quite similar, shortening circadian periods by ca. 1.5 hr in \( \tau/\tau^+ \) animals and by ca. 4 hr when \( \tau \) is homozygous (Ralph & Menaker 1987).

The analogous mutation in \( \text{Drosophila} \) is formally an "over-producer" of some kind, in the sense that \( \text{per}^r \) leads to the same kind of shortened circadian period as do genotypes involving extra copies of the normal allele (Smith & Konopka 1982). Further analysis of the relevant behavioral data indicated that circadian periodicity is a logarithmic function of \( \text{per} \) activity, and led to the inference that \( \text{per}^r \)'s activity level is ca. 35 times that of \( \text{per}^+ \) (Côté & Brody 1986). The guess, then, was that molecules produced by this particular mutated form of the gene are intrinsically abnormal, as opposed to a hypothesis whereby transcription would occur at a vastly increased rate. This, too, has been confirmed molecularly (see below).

This \( \text{per}^r \) mutant is also interesting in its responses to phase-shifting light stimuli: The PRC has a normal (12 hr) subjective night, but the subjective day is shortened to ca. 7 hr (Konopka 1979). Also, phase advances and delays of eclosion peaks, induced during subjective night, are larger than in wild-type (Konopka 1979). These data led to a "membrane model" of circadian rhythm control by the product of this \( \text{Drosophila} \) gene (Konopka & Orr 1980); the ideas here are similar to the "ionic flux" formalisms developed more generally to model circadian clocks (Engelmann & Schrempf 1980). However, the molecular data extant on the \( \text{per} \) gene product (see below) do not yet give further force to this kind of clock model.

Temperature compensation is somewhat abnormal in the rhythmic \( \text{per} \) mutants: Whereas the wild-type was always 24 hr, taus were a bit shorter or longer than normal, respectively, in \( \text{per}^s \) and \( \text{per}^L \) adults tested for activity rhythms at relatively high temperatures. Both mutants gave closer-to-normal periodicities in low-temperature experiments (Konopka 1979). In contrast, the \( \text{And} \) and \( \text{Clk} \) mutants (Orr 1982) and the \( \text{Chlamydomonas} \)
rhythm mutants that were tested appropriately yielded flat (compensated) plots of period vs temperature.

The cases of arrhythmicity induced by \( \text{per}^a \) mutations or deletions of the locus have been reanalyzed. It turns out that these genotypic changes do not really lead to all-out aperiodic behavior in locomotor activity tests. Instead, ultradian rhythms are routinely found lurking in the data. These periodicities (mean values, ca. 10–15 hr) are extracted from 50–70% of the \( \text{per}^a \) or per-minus (\( \text{per}^- \)) flies whose activity was monitored, followed by application of rather special algorithms designed to "pull rhythms out" of noisy data (Dowse et al 1987, Dowse & Ringo 1987). The \( \text{per}^- \) type used here could be only suggested to be really deleted of the locus (Smith & Konopka 1981); that this genotype is a true deletion of the per gene turned out to be true at a higher level of resolution (see below).

The significance of per's action has entered the ultradian realm. Genetic variants involving this gene affect rhythms whose normal taus are markedly shorter than the cyclic durations with respect to which the original per mutants were isolated. First, there are ca. one-minute periodicities in the courtship song of \( D. \) melanogaster males which are shortened by at least 25% in \( \text{per}^a \) and lengthened by ca. 50% in \( \text{per}^{L1} \); \( \text{per}^{a1} \) seems to eliminate the song rhythms (Kyriacou & Hall 1980, 1986). These oscillations are temperature-compensated over a ca. 20°C range (Kyriacou & Hall 1980). Second, the heart in Drosophila larvae beats regularly, as if this phenotype is an ultradian rhythm (frequency, ca. 3 Hz), and Livingstone (1981) observed that heartbeating is erratic in late-stage larvae expressing \( \text{per}^a \). This finding has recently been confirmed and quantified by C. P. Kyriacou (unpublished) and independently by H. D. Dowse (unpublished).

The high-frequency courtship song rhythms are behaviorally significant in terms of their effects on female responses to a male's eventual mating attempts (Kyriacou & Hall 1982, 1984, 1986). These results might have been expected, given that the males from several closely related Drosophila species sing with different taus [ranging from 30–40 s in \( D. \) simulans (Kyriacou & Hall 1980, 1986) to 70–80 s in \( D. \) yakuba (Kyriacou & Hall 1987)].

Fly rhythmicity, including that involving courtship, has been examined in some neurogenetic experiments. Action potential mutations (that cause paralysis and turn off nerve conduction at high temperatures; review: Tanouye et al 1986) were shown to stop the "song clock" that is inferred to underlie this behavioral rhythm (Kyriacou & Hall 1985). In contrast, TTX treatments applied to known neural oscillators in molluscs (Eskin 1977) and mammals (Schwartz et al 1987) do not arrest the relevant circadian clock: The physiological or behavioral oscillations are shut down
during the treatment; but when rhythmicity returns after washing the toxin away, the phase is the same as it was before the treatments. Thus, the song clock in *Drosophila* and circadian clocks—at least in these other species—seem to have a different neural basis (though this notion is admittedly rather vague). Other quasineural studies of the fly’s rhythms involved genetic mosaics (Hall 1984), i.e. flies that were part per-mutant and part-normal (within each animal). The effects of *per* on the oscillator controlling the song rhythm was thus localized to the thorax, probably within the ventral ganglia, whereas the effects of this mutation of circadian periods “mapped” to the brain (cf Konopka et al 1983).

There are further, albeit now somewhat old and not-followed-up, findings in the area of *per* neurobiology: Transplantation of an adult *per* brain to the abdomen of a *per* fly can in some cases “correct” the defect associated with the host genotype, whereby rhythmic and short-period locomotor activity cycles are manifested (Handler & Konopka 1979). However, this finding does not necessarily implicate some sort of circulating factor—perhaps under the control of this gene’s action—as a component of the clock system of intact flies (i.e. including the brain in its usual location). Still, other “neuro-humoral” findings should be considered: Cells of a neurosecretory cluster are in somewhat ectopic locations in the adult brains of *per* in *D. melanogaster* and in the two types of arrhythmic mutants in *D. pseudoobscura* (Konopka & Wells 1980). Could these brain abnormalities be related to the deficit in octopamine synthesis in the *melanogaster* *per* mutants (Livingstone & Tempel 1983)?

**Neurospora Clock Mutants**

The clock variants in this lower eukaryote have, as for the *Drosophila* mutants, been analyzed in many ways since they began to be isolated at about the same time that studies of the fly’s clock genetics began. Moreover, the results gathered from studies of these fungal mutants in several ways parallel the findings from *Drosophila*, at least in the sense that the multiplicity of clock-functional abnormalities caused by certain mutations in the two organisms are interestingly similar.

Mutations at six genetic loci in *N. crassa* have been induced (by chemical mutagenesis or UV treatment) and isolated with respect to defective circadian rhythms (Feldman & Dunlap 1983). The *frequency* (*frq*) gene has been “hit” the most times, in that there are eight independently isolated alleles. Only one mutation has been found for the other five loci, called *period* (*prd-1* through *prd-4*) or *chrono*. Most of these mutations cause periodicities of conidial banding patterns (associated with growth) to be shorter or longer than the normal 21.5 hr.

In addition, a number of metabolic and biochemical mutants have been
found—subsequent to their isolation on "nonclock" criteria—to have altered tans. The effects can be mild when such variants are tested under standard conditions (review: Feldman & Dunlap 1983) or quite dramatic under certain nutritional conditions. For example, the partial fatty acid auxotrophic cel– strain exhibits normal cycle durations at ca 22°C, but supplementing the medium with certain unsaturated fatty acids lengthened periods by 5–19 hr (Brody & Martins 1979).

The most intensively studied clock gene in N. crassa is the frequency (frq) locus on chromosome VII. This gene, like per, has been mutated to yield short-period and long-period variants (Feldman 1985). In addition, what amounts to an arrhythmic type has recently been induced. Thus, the frq9 allele causes growing cultures to exhibit essentially continuous conidiation under standard growth conditions (Loros & Feldman 1986). In other media, rhythmicity can be seen, though it can require a week of growth to be initiated and then has highly variable periods and phases among different cultures (Loros & Feldman 1986). Thus, frq9 can be regarded as analogous to D. melanogaster's per's, which are only quasi-arrhythmic (see above).

Another important property of frq9 is that tau is strikingly temperature dependent, to the extent that this mutant seems to have lost temperature compensation (Loros et al 1986). More generally, it turns out that all the long-period frq mutants give periods that are inversely related to temperature (though with Q10s less than the values of 2 found for frq9), whereas short-period mutations at this locus leave temperature compensation intact (Gardner & Feldman 1981). Somewhat of a parallel is found in the aforementioned cel– mutant, which loses its temperature compensation when grown below 22°C on medium unsupplemented with fatty acids (Mattern et al 1982).

Included in the temperature-compensation-defective frequency mutants is the long-period frq7 allele (Dunlap & Feldman 1987). In addition, this mutation leads to an abnormality in phase-shifting—not by light, which can of course strongly reset Neurospora's clock, but in regard to advances and delays that are caused by protein synthesis inhibitors (cf Nakashima et al 1981). Indeed, such treatments generate PRCs, which are nonsuperimposable on the standard ones elicited by light-pulses, in a variety of systems (e.g. Dunlap et al 1980, Lotshaw & Jacklet 1986). This might mean that protein synthesis is important for the clock's operation only at certain times within a cycle (i.e. these PRCs almost by definition include insensitive phases). In experiments on frq7, then, pulsatile cycloheximide applications essentially eliminated this PRC, although the mutant is by all means still rhythmic when treated in this manner (Feldman & Dunlap 1987). The strong implication here is that protein synthesis per se is not a
part of the "oscillatory feedback loop" that underlies this organism's rhythmicity.

Other experiments involving resetting *Neurospora*'s clock (in these cases, by light stimuli) have shown that the subjective day is the portion of a cycle nonrandomly altered by *frq* mutations. For example, essentially the entirety of the tau-shortening effects of the *frq*\(^2\), *frq*\(^4\), and *frq*\(^6\) mutations can be accounted for by the fact that the middle-to-late subset of the light-insensitive half of a free-running cycle is but 65% of normal (Feldman 1985). A similarity to *Drosophila* 's *per* mutant is evident (cf Konopka & Orr 1980).

Most of the clock mutations in *Neurospora* are semidominant in their effects on tau, as determined in "heterokaryon" experiments involving the production of "forced" diploids with syncitia containing nuclei of different genotypes (Feldman & Dunlap 1983). Recall that the rhythm variants in two *Drosophila* species and in a hamster are superficially similar in this genetic property (see above), as are the mutants of *Chlamydomonas* (Bruce & Bruce 1978). An exception, in *N. crassa*, is *frq*\(^9\); this mutation is recessive to the wild-type allele (Loros & Feldman 1986). Such a result has been interpreted to mean that *frq*\(^9\) is a null mutation that may completely inactivate the locus. Whether or not this is so, the recessivity of this allele is extremely useful in designing "molecular gene rescue" experiments aimed at identifying cloned material that must include the entirety of a genetic locus like this one (see below).

Further usage of heterokaryons has shown that the relative proportions of nuclei—i.e. carrying alleles associated with shortened, lengthened, or normal periods—allow good predictions of the tau that will be exhibited by a given genetically mixed type (Feldman & Dunlap 1983). Again, dosages of clock genes matter (cf Smith & Konopka 1981, 1982).

Many multiply mutant strains of *N. crassa*, involving variants at the various rhythm-affecting loci, have been tested for tau (Lakin-Thomas & Brody 1985). In many such genetic combinations, it appeared as though the resultant periods may best be explainable by "multiplicative" predictions, as opposed to merely additive effects on tau of the relevant single mutations. Thus, one might further infer that the products of these genes interact in some manner (perhaps even in terms of physical associations of the hypothetical proteins). In other multiple mutants, a given factor was sometimes epistatic to another; for example, the effects of *cel*\(^-\) on period and temperature-compensation are not seen in a *prd-I* genetic background (Lakin-Thomas & Brody 1985). Hence, the influences of these two genes on the clock could be viewed in terms of sequentially acting products.

Whether or not these kinds of genetical formalisms are themselves
forceful, or have specific heuristic value, it is clear that well more than one putative clock gene must be studied, in a given species, before one can begin to understand how the organism constructs and operates its biological clocks. In this regard, the experiments involving "only certain" of the relevant genes, which are now being studied at more concrete levels and are discussed below, must be viewed as inadequate. But they are a start—perhaps toward understanding the molecular mechanisms of living oscillators.

MOLECULAR BIOLOGY OF CLOCK GENES

The per Locus of Drosophila melanogaster

Isolation of DNA sequences that were eventually identified as encompassing all of this clock gene required an extensive array of background "cytogenetic" information on the locus (e.g. a narrow localization of the per mutations' site, by mapping them with chromosomal aberrations such as deletions; Young & Judd 1978, Smith & Konopka 1981, 1982).

Thus, per was cloned by chromosomal "walking and jumping" procedures (see Bender et al 1983 for a general description of this method) by Bargiello & Young (1984) and by microexcision experiments (Reddy et al 1984). These approaches worked because per had been pinned down to a tiny region, probably an interval between two small chromosome "bands" on the fly's X chromosome (see above). What could not work, of course, is the commonly applied cloning strategy of working backwards from a known protein product to the relevant genomic sequences.

The boundaries of per were rather precisely delimited by molecular mapping of nearby chromosomal "breakpoints" (e.g. ends of deletions). These data were in fact obtained in conjunction with the walking/jumping efforts and the experiments immediately proceeding from the microexcision of DNA that included the gene. An important bonus in the results was proof that the aforementioned per− genotype is indeed homozygously deleted of ca. 10 kb of DNA (Bargiello & Young 1984, Reddy et al 1984). Subsequent work (see below) showed that this deletion is devoid of the entirety of the gene, i.e. the critical component of the locus's molecular expression.

RNA transcripts complementary to per-locus clones were detected in the initial molecular experiments (Bargiello & Young 1984, Reddy et al 1984). Attention was focused primarily on two of them: a 4.5 kb RNA and a ca. 1 kb species, both of which are missing in the aforementioned per− flies. The smaller of these two RNAs was immediately intriguing, because it oscillates in its abundance over the course of a given day, in wild-type flies that are in LD entrainment or in free-running conditions.
BIOLOGICAL RHYTHMS

This molecular circadian rhythm (there is one peak per day, in the middle of the day or subjective day) seems tied into clock functions, because not only does the 1 kb RNA originate from at least the vicinity of the per locus, but the fluctuation is also dampened in the per° mutants (this transcript’s abundance stays at the low, night-time level; Reddy et al 1984, Hamblen et al 1986). Loosely analogous findings have come from other systems: Gross RNA levels can fluctuate in microbial organisms (either in circadian or ultradian domains, respectively; Walz et al 1983, Lloyd & Edwards 1984). Higher up the evolutionary scale, a transcript encoding vasopressin in rat oscillates in its abundance, as determined by in situ hybridization to brain regions. Moreover, the fluctuations can be detected only in the key clock center in this mammal, the suprachiasmatic nucleus (SCN; Uhl & Reppert 1986, cf Turek 1985).

Subsequent experiments on the fly’s fluctuating RNA have indicated, perhaps ironically, that this transcript is not the critical component of the per locus’s expression. The relevant experiments involved construction of germ-line transformants (or “transgenics”), whereby various DNA fragments from the environs of the gene were transduced into per° or per− “hosts.” The aggregate pattern of “arrhythmicity rescue” showed that clones homologous only to the 4.5 kb transcript (see above), but not those that could encode the 1 kb species, are able to restore rhythms to these mutants (Bargiello et al 1984, Zehring et al 1984, Hamblen et al 1986). One important inference from these results is that the effects of per° mutations on the smaller RNA’s abundance oscillation are indirect (e.g. a given per° is not directly mutated in the genomic source of the 1 kb transcript). Also note that, whereas “4.5-covering” clones rescue both circadian and courtship song arrhythmicity, those which cover the fluctuating RNA have no influence on either kind of mutant phenotype (Hamblen et al 1986).

Perhaps the most important result of these transformation experiments has been to allow investigators to focus on the 4.5 kb, per-derived transcript. Thus, from nucleotide sequencing of the relevant genomic clones, and cDNA clones complementary to the genomics, the entire informational content of this portion of the locus has been determined (Jackson et al 1986, Yu et al 1987b, Citri et al 1987). Before considering the nucleotide sequencing details (and, for example, the amino acid sequence that was inferred; see below), one first sees that the 4.5 kb RNA’s gross structure consists of eight specific exons. Yet, this refers to the major transcript, because it turns out that there is heterogeneity in the primary expression of this clock gene: At least two additional (and less abundant) RNA species are detected by cDNA analysis (Citri et al 1987), and they would encode three different proteins (differing in amino acid composition
and sequence in regions relatively near the C-terminal end of the conceptual proteins). These different, but of course related, RNA species are produced by “alternative RNA splicing” events. Their biological significance is not yet known, but it is possible that the different per proteins (each?) play separate roles, i.e. in terms of the different kinds of rhythmic phenotypes reported to be influenced by this gene (Konopka & Benzer 1971, Kyriacou & Hall 1980, Livingstone 1981, Weitzel & Rensing 1981). This idea has begun to be examined via creation of “mini-gene transformants” involving transduction of two separate types of intron-less cDNA “cassettes” transduced into the genome (Citri et al 1987). Either cassette allows for circadian locomotor rhythmicity, but the phenotypes are not entirely normal, i.e. in comparison to the transformants involving the gene in its entirety (introns plus exons). Other per-influenced phenotypes are now being examined in these transformants. It may be necessary for all three of the different types of cDNA cassettes to be combined in a given fly for fully normal circadian rhythms, and the other cycling phenomena just listed, to take place.

Looking at the conceptual per protein(s) (Shin et al 1985, Jackson et al 1986, Reddy et al 1986) led immediately to scrutiny of a certain small part of the amino acid sequence: A perfectly alternating series of threonine-glycine repeats (summing to about 40 residues). This rather striking region, which would be in common to all the known alternatively spliced forms (Citri et al 1987), is qualitatively and quantitatively similar to a series of serine-glycine pairs in the core protein of a chondroitin sulfate proteoglycan from rat (Bourdon et al 1985, 1987). Indeed, per’s product does have proteoglycan-like characteristics, as determined by application of antibodies used to “track” the material in test-tube assays (Reddy et al 1986, Bargiello et al 1987). A word of caution is that these preliminary results do not imply that the clock gene here produces a protein that is solely some kind of “extracellular matrix” material—which one might, in turn, believe to be significant only for the structure of some kind of fly tissues. Whereas vertebrate proteoglycans often have such attributes, they can in certain cases be found inside cells where they may play “physiological” roles (Evered & Whelan 1986).

Where in fact are per products expressed (cf discussion of mosaic experiments, above, that also asked this question)? First, there are results from in situ localization of the 4.5 kb transcript in the embryonic CNS (James et al 1986), at a stage when this RNA can be detected, as well, in “Northern blotting” experiments (Young et al 1985, James et al 1986). This expression, which was seen in all of the segmental brain plus ventral ganglia, was found to wane during late embryogenesis (James et al 1986). Other embryonic tissues in which the per gene product is present are the
paired salivary glands (Bargiello et al 1987). In larvae, as well, per-produced protein is detectable in the salivary glands (and the material indeed appears to be at cell boundaries; see above)—although mRNA transcribed from this gene is at very low abundance during these intermediate developmental stages (Young et al 1985, James et al 1986). During metamorphosis, more robust per expression reappears and reaches even higher levels in adults (Young et al 1985, James et al 1986). At the latter stage, “head vs body Northern” revealed an enrichment in the anterior tissues (vs what obtains for expression of many other mRNAs, including a transcript which “neighbors” the 4.5 kb species; James et al 1986). A further series of experiments, using an antibody against a portion of the per protein, has revealed this gene’s expression in the central brain of adults and also in the eye plus optic ganglia of the visual system (Siwicki et al 1987).

These results on the clock gene’s spatial expression are intriguing from two angles: First, one cannot assert an “exquisitely local” significance to per’s action, which would have been possible if the transcript had been detectable only in a narrowly defined portion of the brain (a hypothetical analog, in Drosophila, of the vertebrate SCN?). Second, per expression early in the life cycle could mean that the gene has developmental significance per se. That is, might it contribute to cell differentiation and pattern formation in the CNS that, at least in part, has to do with the construction of neural oscillators? Furthermore, might there be no influence of the gene product(s) on the actual “ticking” (ongoing physiological operation) of the fly’s clocks? These questions are raised, because couplings among “high-frequency” oscillators have long been suggested (e.g. Klevecz et al 1984) to be involved in the formation of lower-frequency, i.e. circadian, clocks. Such a possibility can be viewed in the context of the fact that ultradian oscillators are still “running” in flies whose per gene is gone or hypothetically nonfunctional (Dowse et al 1987, Dowse & Ringo 1987). Therefore, such genetic variants may not really have stopped the clock but instead could result in a formation failure of the relevant interneuronal connections—the literal correlates of formalistic couplings. The involvement of per in communication among cells, albeit non-neural ones, has now been addressed in less abstract terms: Electrical couplings, as well as dye movements, between larval salivary gland cells (see above) are strikingly affected by per mutations, such that coupling strengths vary directly with the known or inferred levels of the gene product’s function (Bargiello et al 1987).

The preceding discussion tacitly asks some questions about mutated forms of per (also see section on the phenogenetics of Drosophila clock mutants). Specifically, are per’s really null at the level of gene action? And
what about the long- or short-period alleles? These matters have been approached by DNA sequencing of per genes cloned from the mutants. In one set of studies (Yu et al 1987b), the portions of the mutant-derived clones that could be said to account completely for the aberrant rhythms were identified, so that "brute force" sequencing of the entire mutated genes was not necessary. This was done by transforming per" hosts with "chimeric" fragments, each derived partly from mutant clones, with the remainder of each cassette coming from per+. In this way, the locations of the mutated sites in per" and per were narrowed down to a specific 1.7 kb subset of the locus (Yu et al 1987b). A "modest" amount of sequencing then revealed that per" is caused by a nonsense, translation chain-terminating codon, which is rather centrally located in the gene and "upstream" of the Thr-Gly repeat; per"s difference from wild-type was found to be a serine-to-asparagine missense mutation, located between the mutated site in per" and the repeat region.

This strategy for molecularly pinpointing a given mutation is of some general interest, because it is more than a labor-saving device. In addition, it can show that a given alteration is (or is not, as the case may be) the only genetic etiology of the phenotypic abnormality. In other words, what if, say, three "site differences" were found in per vs per"? Two of them might have been outside the confines of the aforementioned 1.7 kb subset of the locus. Yet, these two would, in light of transformation results, be concluded as irrelevant to the "fast clocks" associated with this mutant. By the way, it was speculated that the per"-defined serine, given the specific amino acids neighboring it (see Yu et al 1987b, for background literature), might be a site for phosphorylation of per proteins in the wild-type. [Note: This site would be included in all the known per products derived by alternative splicing (cf Citri et al 1987).] Such a covalent modification could "down regulate" the products' activities (cf Cochet et al 1984), thus modulating the normal timing functions such that they contribute to proper circadian and ultradian taus; in contrast, the nonphosphorylatable asparagine residue at this site in per" could cause the timers to run out of control.

The intragenic sites that are altered in per" and per have been confirmed by the sequencing data of Baylies et al (1987). In addition, these workers determined a nucleotide substitution in perL1 that results in a valine-to-aspartate change, upstream of the other two mutated sites. One would hypothesize, then, that this nonconservative substitution in the long-period mutant causes hypoactivity or perhaps relative instability of the protein molecules (see above).

There is additional force to the notion that abnormally low expression of per—perhaps at any level of gene action—causes clocks to run more
slowly. Baylies et al (1987) determined circadian periodicities for a series of transformant strains, each carrying a \( per^+ \) fragment transduced to a different genomic location. The taus ranged from a bit longer than normal to more than 35 hr, and these phenotypes were inversely correlated with abundance levels of the 4.5 kb RNA transcribed from the transduced DNA insert. [Note: The genetic background of these transformants had to be \( per^- \), because \( per^- \)'s do not cause a change in this transcript's level (Bargiello & Young 1984, Hamblen et al 1986). For that matter, \( per^{L^{-1}} \), as well, has a normal abundance of the 4.5 kb RNA (Bargiello & Young 1984), so one cannot conclude at this level of analysis that it is hypoactive.]

Thus, much is known about \( per \)'s molecular biology. Yet, and even though the extant data are obviously not enough, it will be important to expand these kinds of investigations to other clock genes in the two \( Drosophila \) species for which the rhythm mutants exist. But not many of the five to six clock genes in \( D. melanogaster \), and none of the two in \( D. pseudoobscura \), are readily clonable: The only other locus for which the necessary, fine-level cytogenetic mapping data exist is \( Andante \) (Smith 1982). This "moderately slow" clock mutant is, by the way, interesting in that it lengthens song-rhythm periods as well as circadian taus (C. P. Kyriacou, unpublished; cf Jackson 1983). One important exception is that males expressing the X chromosomal \( Clk \) mutation sing with normal, not shortened, periodicities (C. P. Kyriacou, unpublished; cf Konopka 1987). This is perhaps as it should be: The circadian and song clocks could—and, given these genetic results, do—share components. But they are not the self-same thing, so one would expect them to be independently changeable in certain instances.

A further example of this kind comes from in vitro mutagenesis experiments on \( per \). The Thr-Gly repeat region, whose discovery was of heuristic value for biochemical reasons, has also proved to be most interesting from the behavioral standpoint. When the repeat is removed from transformation cassettes that are then introduced into the fly's genome (\( per^o \) genetic background), circadian rhythms were almost entirely normal (Yu et al 1987a). This somewhat unexpected result is not miraculous: A similar kind of gene deletion, created by recombinant DNA methods in the low density lipoprotein (LDL) receptor, led to no detectable abnormalities in LDL receptor function, when the mutated factor was transduced into cultured cells (Davis et al 1986). The deletion in question was in fact rather analogous to that produced for \( per \): Each was about the same size, and that for the LDL receptor gene encodes a domain of the protein that is a substrate for "\( O \)-linked" glycosylations (Davis et al 1986), as is also inferred to occur for the threonine residues in \( per \)'s Thr-Gly repeat (Reddy et al 1986, Bargiello et al 1987).
When the courtship song rhythms were examined, in the transformants carrying repeat-deleted DNA inserts, striking changes were found: The cycle durations were 20–25 s shorter than in the control transformants (Yu et al. 1987a), thus implying that this domain of the *per* gene is somehow much more concerned with this ultradian rhythm than with the “main” (i.e. circadian) clock function.

**Clocks and Molecules in Neurospora crassa**

Studies of clocks and molecules in *Neurospora crassa* are not yet as mature as those of *Drosophila’s* per. Interestingly, some of the early data in this area, from the fungal system, started with *per* clones: There is preliminary evidence for *per*-homologous factors in *N. crassa*; two clones from a genomic library, produced from this fungal genome, were found to hybridize to sequences from separate subsegments of *per* (Feldman et al. 1986). Neither *per* region included the Thr-Gly repeat. This is mentioned because interspecific homologies between *per* and cloned material from several other organisms have been detected; but the only cases analyzed in detail—whereby the isolated *per* homologs from mouse and from *Acetabularia* were sequenced—indicated that the only regions in common between the clones referred to *per*’s Thr-Gly repeat (Shin et al. 1985, Li-Weber et al. 1987). Therefore, it could be that families of “generalized” proteoglycan-encoding mouse genes were identified in these experiments. Yet, perhaps one or more of these factors is somehow involved in murine and/or algal clocks.

The *per*-homologous *Neurospora* clones have not yet been sequenced, but it is known that one of them maps, via application of Restriction Fragment Length Polymorphism principles and techniques, tantalizingly near a clock locus (J. F. Feldman, unpublished). It remains to be seen if connections between both formalistic and concrete genetics in these two very different organisms is to occur, i.e. after the necessary further experiments on the *N. crassa per* homologs are performed.

In the meantime, a genuinely exciting advance has occurred in the *Neurospora* system. It began when investigators noticed that the *frequency* gene is genetically quite near a locus for which there is a molecular “handle”: The *oli* gene on chromosome VII. This locus is defined by oligomycin-resistant mutants, which actually are abnormal in their circadian taus (review: Feldman & Dunlap 1983). In fact, *frq* and *oli* were once thought to be the same gene (Dieckmann & Brody 1980). Therefore, the mitochondrial ATP synthetase known to be encoded by *oli* was suggested to have an important relationship to rhythm control in this organism, which it indeed might (Brody et al. 1985). But it is now known...
that \textit{frq} and \textit{oli} are separate genetic loci, located approximately two map units apart (Loros et al 1986). Nevertheless—and this is the critical factor—\textit{oli} has been cloned (Viebrock et al 1982), and such material can be used as the starting point for a chromosomal walk.

This search for \textit{frq} clones has been performed by J. C. Dunlap and R. McClung (unpublished), by deliberately walking in both directions from \textit{oli} (because there are no physical landmarks in \textit{Neurospora}, such as the chromosome breaks near the fly's \textit{per} locus, to orient the walk). After ca. 70–90 kb of walking in the two directions—and knowing that one genetic map unit is about 25 kb in \textit{N. crassa}—transformation techniques were applied (see Dhawale et al 1984 for an example of the method). The results were that a series of overlapping clones, transduced into the \textit{frq}\textsuperscript{9} strain (see above), succeeded in rescuing the effects of this arrhythmic mutant and ultimately pinned down the \textit{frq} locus to an 8 kb subset of chromosome VII. This, then, will permit a host of clone and gene product characterizations, similar to those performed for \textit{per}. The data that should be rapidly forthcoming may have greater force than those from \textit{Drosophila}. For example, \textit{Neurospora} does not develop any kind of intercellular "oscillatory circuitry" to make its circadian clock. Therefore, the information encoded within this fungus's \textit{frq} gene is likely to be right at the core of its oscillator, whereas what \textit{per} is and does may or may not be (see above).

A final fillip to the emerging \textit{Neurospora} story is that two oscillating RNAs have been recently discovered, which, as for the transcript encoded near \textit{per}, show peaks of abundance during the day or subjective day (J. J. Loros, unpublished). The DNA sequences encoding these RNAs have been cloned, and the strategy for their isolation (which was intimately connected to the discovery of the abundance fluctuations) has indicated that the kind of periodic gene expression that can be inferred is relatively rare in this fungus. Additionally, these molecular rhythms are tied in to the circadian clock of \textit{Neurospora}, because the peaks of RNA abundance were farther apart in a long-period \textit{frq} mutant than in the wild-type (J. J. Loros, unpublished). It remains to be seen (a) whether the functions encoded by these genes will be tractable and in some way connectable to the clock mechanism (the same sort of question that remains for the oscillating "near-\textit{per}" RNA from \textit{D. melanogaster}), and (b) whether one or more of these \textit{N. crassa} clones in fact defines a locus that, when mutated, leads to an abnormal rhythm.

Many questions—those posed here and others—are going to suggest themselves, as geneticists and chrono-biologists delve deeper into the appropriate genes and the normal vs abnormal rhythmic phenotypes in the bread mold and the fruit fly. As these investigators continue to make progress in their molecular studies, it may well be that the working of
biological clocks, at least in these two systems, will become less and less mysterious.

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