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A Mutational Analysis of the Period Locus of *Drosophila Melanogaster*

Mary K. Baylies

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A Mutational Analysis of the *period* Locus
of *Drosophila melanogaster*

A thesis submitted to the faculty of
The Rockefeller University
in partial fulfillment of the requirements of
the degree of Doctor of Philosophy

By

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ABSTRACT

The *period* (*per*) gene of *Drosophila melanogaster* is fundamentally involved in the generation of biological rhythms. Three classes of *per* mutations which alter circadian periodicity have been identified: *per^S* mutants have circadian behavioral rhythms of 19h instead of 24h; *per^L* mutants have long period rhythms of 28h; and *per⁰* mutants have no detectable circadian rhythms. Steps have been taken to gather more information about *per*'s role in the construction or maintenance of biological clocks.

By analyzing transformed *Drosophila* lines, the amount of *per* product was found to be integral to the pace of the clock. Absence of the *per* product leads to arrhythmicity; more *per* product shortens the period length; less *per* product lengthens period. In addition, single amino acid changes in the *per* product can mimic these results. DNA sequence analysis has revealed that in *per⁰* flies, a single nucleotide change resulted in a translational stop codon and hence a truncated protein. A valine-to-aspartic acid change in the *per^L* mutants lengthens period. Likewise the shortened period length in *per^S* mutants is a result of a serine-to-asparagine substitution. These combined studies suggest that *per^L* and *per^S* mutants produce hypoactive and hyperactive *per* proteins, respectively.

Using the sequence analysis of the mutants as a starting point, further amino acid changes in *per* were created, introduced back into the fly, and then evaluated for effects on biological rhythms. Five out of six amino acid changes near the *per^S* mutation also gave short period lengths. These results suggest that the region near the *per^S*

mutation acts as a domain to restrain *per* function and thereby slows the clock.

Further insight into the nature of *per* function was obtained through a cell level assay. The *per* mutations have a significant effect on intercellular communication in the salivary gland cells of third instar larvae. Dye transfer and electrophysiological experiments indicate that gap junction conductances varies inversely with the period of the behavioral rhythms. Such alterations in communication in the nervous system may explain how *per* influences biological rhythms.

Lastly, a detailed localization study of the *per* gene products during embryogenesis shows that it is expressed in particular cells in the brain and ventral nerve cord. This information should make it possible to localize the focus of *per*'s clock function to specific cells.

Introduction

Biological rhythms are observed throughout the plant and animal kingdoms. Single-celled organisms such as *Gonyaulax polyedra* display rhythms in phototaxis, photosynthesis, and bioluminescence (Johnston and Hastings, 1986; Roenneberg et.al., 1989). The fungi *Neurospora crassa* show rhythms in sporulation (Feldman and Dunlap, 1983; Dunlap, 1990). Organisms further up the evolutionary tree such as higher plants, insects, and mammals all exhibit oscillations in a variety of activities -- from leaf movements in *Phaseolus multiflorus* to sleep-wake cycles in man (Bunning and Moser, 1973; Saunders, 1977; Aschoff, 1981; Takahashi and Zatz, 1982). Even cultured cells and tissues may show persistent rhythms when isolated from the whole organism (for reviews, Jacklet, 1989).

The frequency of rhythmic behavior can vary. Ultradian rhythms are defined as having a period length of less than 24 hours. For example, calcium oscillations, in response to an agonist, occur with a period of 6 seconds in rat parotid acinar cells (Gray, 1988). On the other end of the spectrum are circannual rhythms (period ~1 year) . These rhythms underlie such seasonal responses as breeding, molting and migration in birds, and testicular growth and hibernation/activity cycles in a

variety of other organisms (Saunders, 1977). The most studied rhythms are those in time with the periodicity of the most obvious cycle -- a 24 hour day. Examples of these circadian rhythms range from the sleep/activity cycles of nocturnal hamsters to the mating activity of the fruit fly *Dacus tryoni* which occurs at dusk (Pittendrigh and Daan, 1976; Tychsen and Fletcher, 1971). The focus of the work presented here will be on circadian rhythms.

The role of all rhythms, but particularly that of circadian rhythms, is to synchronize the organism with its environment. Honeybees use circadian timing to synchronize their visits to particular flowers when these blossoms are open or producing nectar (Saunders, 1977). The selective advantage here is for both the bee and the flower; the bees are nourished and the flowers pollinated. In addition, this temporal organization allows an organism to anticipate or prepare for events, thereby providing further adaptive advantage. The body temperature in man, for example, begins to rise before he wakes and thus prepares the body in advance for activity (Winfree, 1982). Colin Pittendrigh has emphasized that circadian clocks organize "a day within" in cells and organisms. Yet, despite the universality of biological rhythms, much remains unknown about the construction of this "day within" and how it is linked to the outside world.

General characteristics about clocks

Although little is known about the organization and biochemical components of clocks, some basic principles have emerged.

All clocks possess a periodicity, a measure of how often an event occurs over a given period (i.e. day, month, year). Clocks also possess a phase, a measure of when a rhythmic event occurs, i.e. morning, night, etc. For example, a clock could run with a periodicity of 25.2h for a sleep/activity cycle, while its phase of activity could begin arbitrarily at 3AM.

A clock can have period and phase control imposed by environment cues, a process termed "entrainment". Light and temperature are the dominant environmental signals (Takahashi and Zatz, 1982). For the above example, light cues could force the period to 24h and set the phase of activity to begin at 6AM. In man, social cues may also effect entrainment (Aschoff, 1981; Takahashi and Zatz, 1982; Czeisler et.al., 1989). In the absence of environmental cues (e.g. constant conditions), a circadian clock will "free run" and reveal its natural period. This period may not necessarily be 24h. In the clock example given above, 25.2h would be considered the free running period. Therefore, the free running rhythms measured are endogenous and not mere responses to changes in light or temperature.

In addition, free running rhythms can be "phase-shifted", that is, reset by the application of a pulse of light (for example). The response of a rhythm to a flash of light given at a particular time can be plotted as a "phase response curve" (PRC). Three general features characterize phase response curves: a) little or no phase shifts occur after light pulses given during the subjective day. The subjective day is that portion of the free running cycle which would extrapolate to when it was light during entrainment. Therefore, the clock is insensitive to resetting cues given during the subjective day. b) phase delays occur after light pulses given during the early portion of the subjective night, and c) phase advances occur during the later portion of the subjective night. Even though the PRC is obtained by measuring a rhythmic function, the particular type of phase response curve for an organism (or a mutant of that organism --see later in introduction) reflects the temporal properties of the underlying clock. (Saunders, 1977; Takahashi and Zatz, 1982).

Clocks are temperature compensated. Circadian and ultradian period lengths are consistent over wide temperature ranges (Pittendrigh, 1954; Saunders, 1977). The temperature compensation is especially significant when considering organisms which do not regulate their internal body temperatures such as *Drosophila* and *Neurospora* (Zimmerman et.al., 1968; Gardner and Feldman,

1981). This parameter is also interesting when considering certain vertebrates such as hibernating species (Menaker, 1959).

Circadian oscillations are remarkably accurate. Cycle length variation in rodents can be less than 3 minutes (Pittendrigh and Daan, 1967). DeCoursey found that onset of activity showed a variation of only a few minutes per day for the flying squirrel, *Glaucomys volans* (DeCoursey, 1960).

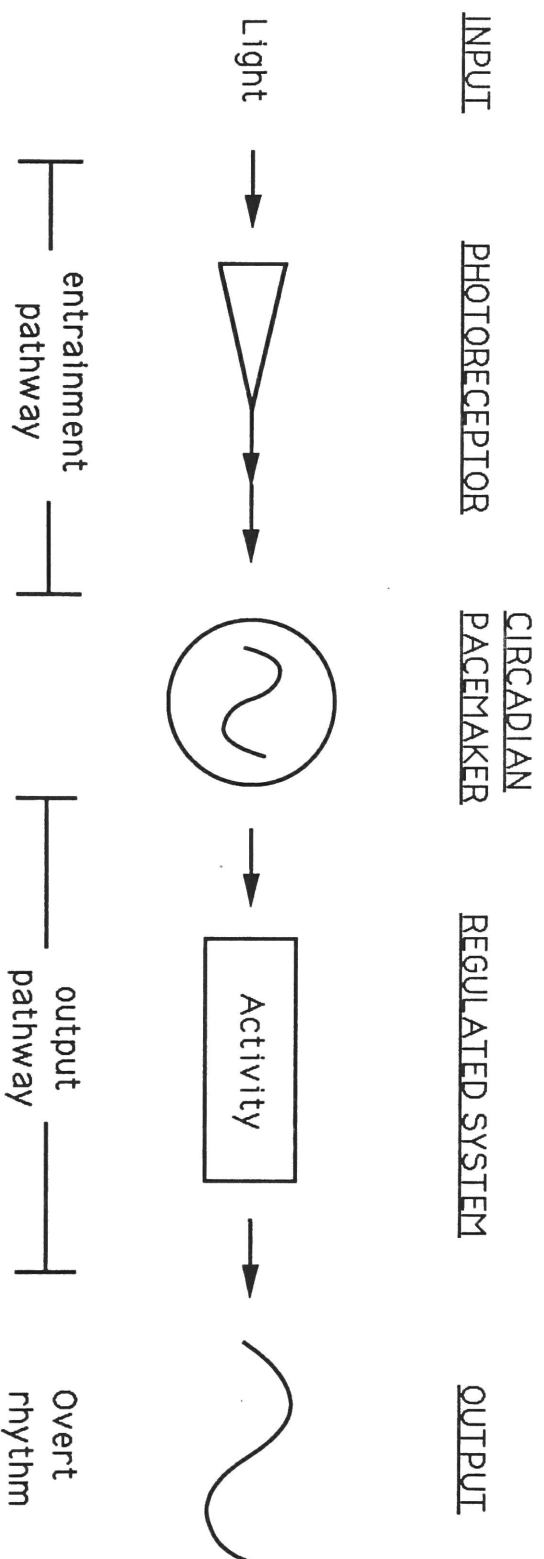
Components of Circadian Systems

As diagrammed in Figure 1, there are three major components of a circadian system (Eskin, 1979):

- 1) an input pathway for entrainment
- 2) a circadian pacemaker that generates the oscillation
- 3) an output pathway that results in the expression of the overt rhythm which can be measured.

These three elements must be linked together in order for entrainment to successfully have a full impact on an overtly rhythmic process. A simple scheme such as the one shown in Figure 1 also provides a framework for a number of questions about biological clocks. For example, where is the location of the pacemaker, of what is it composed, is it a single cell or a complex of many cells, how is

Figure 1. A Schematic Model of the Components of a Circadian System [After Eskin (1979).]



the environmental information deciphered and then relayed to the circadian pacemaker? How does the circadian pacemaker control the activity of its outputs? In addition, this outline is useful in interpreting data. For example, elimination of rhythmicity in a mutant could result from a disruption of either the pacemaker or the output pathway.

The genetic dissection of biological clocks

The genetic approach to understanding the control of biological rhythms has involved the dissection of clocks via isolation of a number of circadian mutants. The underlying principle is that the molecules composing rhythmic systems must be encoded by genes. The isolation of the gene and the subsequent determination of the structure, function, and tissue distribution of the individual gene product can be accomplished. Very complex biological processes, such as pattern formation (Reid, 1990) and tumor development (Bishop, 1991) have become better known as the molecular components are isolated and their functions are identified.

Thus far, the first steps of this reductionist approach to clock biology have been fruitful. A number of mutants that affect circadian rhythms have been found in a wide variety of species: seven in *Chlamydomonas reinhardi* (Bruce and Bruce, 1978; Feldman, 1983;

Mergenhausen, 1984), seven in *Neurospora crassa* (Feldman and Dunlap, 1983; Dunlap, 1990), one in *Lucilia cupina* (Smith, 1987), one in the *Mesocricetus auratus* (Ralph and Meneker, 1987) and eight in *Drosophila melanogaster* (Konopka and Benzer, 1971; Jackson, 1983: Konopka, 1987b, Dushay et.al., 1989, Sehgal et.al., 1990, Konopka et.al., 1991).

The *Drosophila* system

Drosophila offers the researcher other advantages in addition to its genetics (Rubin, 1988). The life cycle of the fruit fly is compacted into nine or ten days at 25°C. *Drosophila* begins its life as a rather nondescript embryo. Within 24 hours, the deceptively simple mass of cytoplasm and one nucleus develops into a newly hatched, first instar larva, complete with a complex nervous system and specific behaviors. The first instar larva will undergo a series of two molts over the next two days; the result of these molts is the second, then third, instar larva. After a further day of growth, the third instar larva will pupate and thus begin to metamorphose. During pupation the majority of larval structures are replaced with those of the adult. Approximately four days after the onset of pupation, the adult fly will emerge (or "eclose") from the pupal case, ready to begin the cycle once again. Each of these stages

of growth and differentiation are in the process of being understood both on the cellular and molecular levels. For example, the development of a subset of the nervous system can be traced to particular sets of genes which determine and then allow differentiation of particular neuroblasts (Nambu et.al., 1990; Klambt et.al., 1991). The study of rhythmic behaviors would benefit not only from such understanding of the individual facets of the organism but also the intergration of these parts into the whole organism. *Drosophila* allows for both levels of study.

Drosophila also offers its strength as a molecular system. Mutants can be created relatively easily in a variety of ways (chemical, X-ray, or by transposable elements) (Grigliatti, 1986). Subsequently, the gene affected by the mutagen may be cloned and the predicted protein sequence ascertained. Localization of the gene products -- RNA and protein -- during the development of the fly can give further information as to the biochemical process in which the gene participates. Cloned genes can be introduced back into the organism. This transformation ability allows for *in vitro* manipulation of the gene to be followed by the analysis of this manipulated gene in the fly itself. In addition, a growing number of genes which affect different aspects of growth and development in *Drosophila* have been characterized. These, too, may be applied to the study of

biological rhythms. For example, mutants affecting the visual system in the fly may aid in the dissection of the complex phenomenon of entrainment. *Drosophila* allows for the identification of not only the specific mutants which affect rhythms but also the cell type and biochemical process which the mutants alter. Taken together, the *Drosophila* system allows for the dissection and the study of the individual components of a biological rhythm such as the gene, biochemical process or cell. In addition, these components can be analyzed in the context of the whole organism such that the behavior of the organism can be comprehended.

Rhythms in *Drosophila*

Biological rhythms have been uncovered in many stages of *Drosophila* development. The best studied rhythms are in the adult: 1) on the individual level, the locomotor or activity rhythm which is analogous to our own "sleep-wake" cycle and 2) on the population level, the "eclosion" rhythm. Eclosion, as mentioned above, is the gated emergence of the adults from their pupal cases. When a population is kept on a light:dark (L:D) cycle, the flies will tend to emerge at dawn or just after lights on. For the locomotor or activity rhythm, a fly exposed to a L:D cycle shows a bimodal distribution of activity: bouts of activity are seen primarily in the

early portions and later portions of the light period. Reduced activity is seen in the middle portion of the light period. As with behaviors that are controlled by an internal clock, these two rhythms also persist in free-run (ie. no environment cues given). Other adult rhythms such as an oviposition rhythm also exist (Fluegel, 1978; Allemand, 1983). The locomotor activity and eclosion rhythms, however, serve as the primary assays for experiments done in our laboratory.

The rhythms mentioned above are circadian in nature, i.e., the periodicity of the behavior is roughly 24h. Ultradian rhythms have been studied in *Drosophila* as well. The best known example is that of courtship song rhythm which has a periodicity of approximately sixty seconds (von Schilcher, 1976; Kyriacou and Hall, 1980). Other stages and even particular tissues have distinct measurable rhythms. For example, Rensing reported that isolated salivary glands from third instar larvae display a rhythmic uptake of a voltage sensitive dye. The periodicity of this uptake was approximately 24 hours (Weitzel and Rensing, 1981). In sum, a rich variety of rhythmic behaviors are available for study in *Drosophila*.

Drosophila clock genes

An understanding of clock biochemistry can be approached in *Drosophila* by taking advantage of mutations

affecting biological rhythms. Several mutations affecting the *Drosophila* clock have been recovered and characterized in some detail. Eight clock genes have now been defined in *Drosophila melanogaster*. Four of these loci are found on the autosomes -- *phase-angle-2* (*psi-2*), *phase-angle-3* (*psi-3*), *gate* (*gat*), and *soiree* (*sre*). *psi-2* and *psi-3* cause earlier than wildtype eclosion during L:D (light:dark) cycles. Each of these mutants, in addition, have periods in free run (i.e. no imposed environmental cues) that are slightly longer than normal (24.8, 25.5h respectively). *gate* is closely linked and could be allelic to *psi-2*. The *gate* mutant causes eclosion to be poorly gated such that the flies do not emerge during the usual narrow windows of time (Jackson, 1983). A newly discovered loci, *sre*, is arrhythmic (e.g. has no discernable period) in L:D eclosion assays as well as in free running conditions of the locomotor activity assay (Sehgal et.al., 1990). Moreover, under L:D conditions in the activity assays, *sre* displays a reversal of phase: the flies are active during lights off and inactive during lights on. This night activity is opposite to that of wild-type flies and has not been seen for any of the clock mutants in *Drosophila* (Sehgal et.al., 1990).

The four X-linked loci are *Andante* (*And*), *Clock* (*Clk*), *disconnected* (*disco*), and *period* (*per*). *Andante*, as its name implies, slows down the clock -- eclosion and

activity period lengths are 25-26h (Konopka, 1987b; Konopka et.al., 1991). *Clock* has activity cycles which are slightly shorter than wild-type (22-23h) and recombination mapping indicates that it may be allelic to *period* (Konopka, 1987; Dushay et.al., 1990). *disco* was not originally isolated by virtue of its affect on circadian rhythms. Instead, *disco* was uncovered in a screen for visual system abnormalities; mutations in the *disco* gene cause disruption of the connection between photoreceptor cells of the eye and the optic lobe (Steller et.al., 1987). Eclosion and activity rhythms are abolished in *disco* flies (Dushay et.al., 1989). The last of the X-linked genes which affects circadian rhythmicity is the *period* locus. It is upon this clock gene that my thesis work has been focused.

Identification of the *period* locus

period (*per*) was the first clock gene to be recognized in *D.melanogaster* (Konopka and Benzer, 1971) and is still the most extensively studied (Konopka, 1987a; Hall and Rosbash, 1987; Rosbash and Hall, 1989; Young et.al., 1989a, Hall and Kyriacou, 1990; Kyriacou, 1990). Initial definition of *per* involved characterization of three ethyl-methane sulfonate induced mutant alleles. One mutant, designated *per*⁰, was arrhythmic; another, *per*^S, was rhythmic with a period of

19 hours; and a third, *per*¹, produced long period, 29 hour, rhythms. The mutants affected circadian rhythmicity on both the population and individual levels as measured by the eclosion and locomotor activity assays, respectively. Initial genetic analyses indicated that the three mutations affected the same functional gene on the X chromosome (Konopka and Benzer, 1971). Further genetic analyses revealed that *per* was dispensable, that is, not necessary for viability, and that the null phenotype, as determined by analysis of chromosomal deletions removing *per*, was arrhythmicity (Young and Judd, 1978). The fact that the period of the rhythm under constant conditions is altered by the mutations in *per*, implies that the gene is somehow affecting the basic oscillator.

Additional characteristics of the per mutants and circadian rhythmicity

Additional information about the relationship of the *per* locus and biological clocks has been provided by the study of dosage alterations and allelic interactions at this locus (Konopka and Benzer, 1971; Konopka, 1987; Smith and Konopka, 1982; Cote and Brody, 1986). Increased and decreased copies of *per*⁺ lead to shortened and lengthened circadian periods (Smith and Konopka, 1982). Decreasing the number of *per*⁺ genes from two to one copies lengthened period by one to two hours. Increasing

the dosage of per^+ shortened period length by one-half to one hour (Smith and Konopka, 1982). The maximum shortening observed was 1.5 hours for three copies of per^+ in a male; additional copies do not shorten the period further. The resultant period (~22.7h) still does not equal that of a per^S fly (19.0h). The observation that decreased per dosage lengthens period while increased dosage shortens period suggests that the per^L and per^S mutations alter period by respectively decreasing and increasing per gene product amount or activity. Also per^+/per^0 gave equivalent period lengths to $per^+/deletion$ of the locus, indicating that per^0 could be a loss of function of the locus (Smith and Konopka, 1982).

The mutants display partial dominance (see Table 1) (Konopka and Benzer, 1971; Konopka, 1987). Of all the per alleles, per^S exhibits the greatest amount of partial dominance over wild-type: per^S/per^+ heterozygotes have a period about two hours shorter than wild-type and about two hours longer than per^S homozygotes. per^0/per^+ and per^L/per^+ have periods about half an hour to one hour longer than wild-type. Table 1 also displays the complementation between alleles. Although per^0 has little effect on per^S period length, it causes a lengthening of the per^L phenotype by approximately one hour. This observation reinforces the idea that per^0 is null and per^L is hypomorphic. A per^S/per^L heterozygote has a

Table 1. Free running period of locomotor activity*

<u>genotype</u>		<u>N</u>	<u>Period \pm S.D.</u>	<u>Phenotype</u>
<u>First X chromosome</u>	<u>Second X chromosome</u>			
<i>per</i> ⁺ (CS) ¹	<i>per</i> ⁺ (FM7) ²	4	24.4 \pm 0.5	normal
<i>per</i> ^o	<i>per</i> ^o	4	arrhythmic	arrhythmic
<i>per</i> ^s	<i>per</i> ^s	5	19.5 \pm 0.4	short period
<i>per</i> ^l	<i>per</i> ^l	4	28.6 \pm 0.5	long period
<i>per</i> ^o	<i>per</i> ⁺	8	25.2 \pm 0.4	~ normal
<i>per</i> ^s	<i>per</i> ⁺	5	21.9 \pm 0.4	intermediate
<i>per</i> ^l	<i>per</i> ⁺	5	25.5 \pm 0.5	~ normal
<i>per</i> ^s	<i>per</i> ^o	6	19.5 \pm 0.4	short period
<i>per</i> ^l	<i>per</i> ^o	5	30.6 \pm 1.3	long period
<i>per</i> ^s	<i>per</i> ^l	6	22.9 \pm 0.4	normal-- (shortish)

*Table was adapted from Konopka and Benzer (1971). 1, CS equals Canton S strain; 2, FM7 is a balancer chromosome; N, number of flies tested; S.D., standard deviation.

period length near that of wildtype (albeit just on the short side), reinforcing the notion of per^S dominance. Taken together, the dosage and the allelic complementation studies suggest that per^0 is null--equivalent to a deletion, the per^L mutation results in less protein or a less active protein, and the per^S mutation leads to more protein or a more active protein (Konopka and Benzer, 1971; Smith and Konopka, 1982; Cote and Brody, 1986). The exact lesions associated with each of the mutants would await molecular analysis (see Part I).

One of the attributes of a biological clock is the ability to temperature compensate. In this regard the per mutants behave rather interestingly. In wild-type flies, the activity rhythms show very slight variation in period length with temperature change (15°C - 26°C) (Konopka and Benzer, 1971; Konopka et.al., 1989). However, in both per^L and per^S the period length is temperature dependent, especially for per^L (a 4h lengthening over the 11° temperature range above) (Konopka et.al., 1989). Moreover, as the temperature decreases, the mutants behave in reciprocal ways -- per^L shortens, and per^S lengthens. In other words, as the temperature decreases, the period lengths of each of the mutants move toward the wild-type period length. At high temperatures, the period differences are accentuated for both per^L and per^S (Konopka et.al., 1989).

The *per^L* and *per^S* mutants also show an altered sensitivity to light. Constant exposure to dim light in the range of 0.1 to 0.8 lux caused lengthening of the period in both *per^S* and *per^L*. For wildtype, equal lengthening required 1.5 to 10 lux. In addition, constant exposure to bright light (greater than 10 lux) has been shown to cause arrhythmia in wildtype flies. In the *per^S* and *per^L* individuals, arrhythmia could be induced; however, the intensity of light required for arrhythmia to be produced was in the the range of 0.6 to 3.0 lux, much less than wildtype (Konopka et.al., 1989). Konopka and his colleagues (1989) concluded that the photosensitivity of the pacemaker was somehow altered in the mutants. In this regard, it is worthwhile to note that the *per^S* mutant also has an altered response to phase-shifting light stimuli. The phase response curve for *per^S* has a normal subjective night (12h) but a shortened subjective day (approximately 7h) (Konopka, 1987b). Also, the amount of advance or delay is greater in the *per^S* mutant than in wildtype (Konopka, 1987b).

These studies of the original *per* mutants and circadian rhythmicity called for the determination of *per* function. In addition, the alterations in the gene caused by each of the mutations needed to be identified. This information would shed light on the relationship between

per and biological clocks and on the workings of clocks in general.

Link between circadian and ultradian rhythms and beyond -
- the pleiotropy of *per*

The *per* mutation series was discovered by Kyriacou and Hall (1980) to have an effect on the *Drosophila* courtship song. Their work indicates that *per* may affect a variety of rhythmic behaviors in the fly; therefore, *per* may be playing a more general role in clock function than just circadian timing. The *Drosophila* male produces a courtship song by a series of wing vibrations. The song consists of pulses of wing vibrations separated by intervals of relaxation. The periods of relaxation, known as the interpulse interval, average about 30mseconds (von Schilcher, 1976). Kyriacou and Hall (1980) have reported that the length of the interpulse interval for the *Drosophila* courtship song is not constant; instead the interpulse interval oscillates rhythmically around the mean of 30mseconds with a range of 29 - 35mseconds. The period for this rhythm was estimated in wildtype *Drosophila melanogaster* flies to be 55 - 60 seconds. The different *per* mutations influence the periodicity of this ultradian rhythm in the manner seen for circadian behaviors: the period for *per*^S males is shortened to about 40 seconds, for *per*^L, it is lengthened to about 80

seconds, and no rhythm is detected in per^0 flies (Kyriacou and Hall, 1980).

Despite this analogy with circadian behaviors, the pattern of allelic interactions is different when analyzing this ultradian rhythm. Both per^S and per^0 act to shorten the period of courtship song in per^+ and in per^1 heterozygotes (Kyriacou and Hall, 1980). Thus allele combinations that lengthen circadian period (i.e. per^+/per^0) often shorten ultradian period. This observation suggests that there are basic differences in the way the *per* gene regulates circadian and ultradian rhythms.

In addition to the effects of the *per* alleles on courtship song, the *per* mutations are reported to have effects on several other phenotypes. These are listed in Table 2. Some of these effects can be linked to an associated rhythm whereas others can not, at least directly. The scope of effects are wide ranging; *per* seems to affect neural structure (i.e., the placement of the neurosecretory cells) as well as neural function (i.e. learning in per^1 males). In addition, tissues other than those of the nervous system, such as those of the salivary gland, seem to be affected.

Molecular isolation of *per*

Table 2. Pleiotropic effects of the *period* mutations*.

<u>Phenomena</u>	<u>Phenotype(s)</u>	<u>References</u>
Courtship song rhythm	tau's shorter or longer than normal in <i>pers</i> ^s and <i>per</i> ^l , respectively; cycling apparently abolished in <i>per</i> ^o .	Kyriacou and Hall (1980) Hall and Kyriacou (1990)
Neurosecretory cells	In brains of <i>per</i> ^o adults, cells of a particular neurosecretory cluster tend to be displaced in anomalously dorsal locations.	Konopka and Wells (1980)
Fluctuations of salivary gland cell membrane potentials	In <i>per</i> ^o larvae, some individual cells show arrhythmic or non-circadian fluctuations.	Weitzel and Rensing (1981)
Experience-dependent modification of male courtship	Mediocre "learning scores" in <i>per</i> ^l males, though unaffected by <i>per</i> ^o .	Jackson, et. al. (1983)
Octopamine synthesis	Reduced rates of synthesis in <i>per</i> ^o , which also lowers tyrosine decarboxylase levels.	Livingstone and Tempel (1983)
Intercellular coupling in salivary glands	Larval cells exhibit strengthened coupling (by dye-fills and physiological recordings) in <i>pers</i> ^s and very weak coupling in <i>per</i> ^o .	See part II results; Bargiello, et. al., (1987)
Developmental time	Sped up or slowed down in <i>pers</i> ^s and <i>per</i> ^l , respectively; differences from normal, but not systematic ones, in <i>per</i> ^o .	Kyriacou, et. al., (1990)

*Adapted from Hall and Kyriacou, 1990.

The *per* locus was mapped to the 3B region of the X chromosome by complementation tests involving a series of chromosomal deletions (Young and Judd, 1978; Smith and Konopka, 1981). The particular chromosome region containing the *per* locus was independently cloned by two groups (Bargiello and Young, 1984; Reddy et.al., 1984). The boundaries of *per* were rather precisely delimited by the molecular mapping of nearby chromosomal breakpoints. It was evident from the physical locations of certain translocation and deletion breakpoints, that *per* must correspond to a segment of DNA approximately 7kb in length. This piece of DNA was found to encode a 4.5kb messenger RNA (Bargiello and Young, 1984; Reddy et.al., 1984). This transcription unit was subsequently proven necessary and sufficient for expression of *per*-related functions in series of P-element mediated transformation experiments (Bargiello et.al., 1984; Zehring et.al., 1984; Hamblen et.al., 1986). Transforming DNA carrying the protein coding sequences included in the 4.5kb *per* transcript were able to "rescue" the arrhythmicity associated with both a deletion of the *per*-region and the *per*⁰ mutation. Eclosion and locomotor activity rhythms with predicted periodicities were restored in transgenic flies, and courtship song periodicity appeared to be recovered (Bargiello et.al., 1984; Zehring et.al., 1984; Hamblen et.al., 1986).

The complete nucleotide sequence of the *per* transcription unit has been obtained in two strains of *D.melanogaster* (Jackson et.al., 1986; Citri et.al., 1987) and for *D.virilis*, *D.pseudoobscura*, and *D. yakuba* (Colot et.al., 1988; Thackeray and Kyriacou, 1990). Partial sequence data is available for *D. simulans* (Yu et.al., 1987; Wheeler et.al., 1991). The predicted protein sequence derived from the Canton S strain of *D.melanogaster* is shown in Figure 2. The sequence shown is from the same line of wild-type flies used by Konopka to produce the *per^S*, *per⁰*, and *per^L* alleles (Konopka and Benzer, 1971). It was derived from the analysis of several cDNA clones, primer extension experiments, and RNase protections, as well as comparison to genomic DNA (Jackson et.al., 1986). The gene encodes a protein of 1224 amino acids. The *per* protein is predominately (47%) made up of four amino acids -- serine, glycine, alanine, and proline. No hydrophobic amino acid stretches which are characteristic of membrane spanning or associated regions are found in the predicted *per* protein sequence. The conserved regions of the protein between species are shown in Figure 2 (see legend). A possible nuclear localization sequence (Dingwall and Laskey, 1986) and several potential protein phosphorylation sites (Cooper et.al., 1984; Bramson et.al., 1984; Woodgett et.al., 1986; Heald and McKeon, 1990) are also depicted. The

Figure 2. The *per* protein.

The putative *per* protein sequence derived from the genomic nucleotide sequence data of the Canton S gene, cDNA analysis, RNase protections, and primer extension experiments is shown as A) a schematic highlighting significant regions and B) the linear amino acid sequence (Jackson et.al., 1986; Sanger, 1981).

A. Schematic of the *per* protein sequence.

Open boxes denote regions of the *per* protein which are relatively conserved among *D. melanogaster*, *D. virilis*, and *D. pseudoobscura* (Colot et.al., 1988). The black box designates the region of *per* which is homologous to the *single-minded* and *arnt* proteins (Crews et.al., 1988; Hoffman et.al., 1991). Note that this region of homology falls within a conserved region. The hatched box represents the threonine-glycine repeats. The arrow head denotes the position of a possible nuclear localization sequence (Dingwell and Laskey, 1986). The remaining arrows indicate interesting sites of potential phosphorylation (see text; Bramson et.al., 1984, Woodgett et.al., 1986; Cooper et.al., 1984; Heald and McKeon, 1990).

B. The *per* amino acid sequence.

The amino acid (aa) sequence for *per* is shown. Amino acids designated by capital letters indicate conserved regions of the protein among *D. melanogaster*, *D. virilis*, and *D. pseudoobscura*. These regions are aa 1-79, 233-692, 767-842, 926-977, 1000-1014, and 1130-end (Colot et.al., 1988). Within these "conserved" regions, amino acids can be substituted or deleted. For example, serine 1202 is not present in *D. virilis* and is substituted with an asparagine in *D. pseudoobscura*.

A possible nuclear localization sequence (Dingwell and Laskey, 1986) is shown in bold print, aa 68-79. The threonine-glycine repeat is underlined (aa 698-743). Although computer programs list a number of possible phosphorylation sites, those of particular interest are shown underlined and in italics (see text and above). They are serine 585, serine 589, serine 981, serine 1202, and tyrosine 601 and 602. Double-stroke amino acids (within aa 238-496) belong to the *single-minded* and *arnt* homologous region of *per* (Crews et.al., 1988; Hoffman et.al., 1991). PEST sequences (Rogers et.al., 1986) are located at aa 131-156, 1123-1155, and 1180-1193.

significance of the potential phosphorylation sites will be addressed in Part I of my results.

per protein variants

The isolation and characterization of several *per* cDNAs derived from poly A⁺ RNA of adult *D.melanogaster* heads have furnished some proof for differential splicing of the *per* transcripts (Citri et.al., 1987). The most abundant transcript (type A) would result in a protein shown in Figure 2. Its relative abundance is estimated to be about 70%. A second class (type C) results from the lack of splicing in the 3' end of the *per* gene: the three most 3' introns which are absent from the type A message are present in the type C transcript. The last 107 amino acids of the putative type C protein, therefore, is entirely different from the 149 amino acids at the end of the type A protein. A third type of cDNA (type B) has been isolated and is missing sequences usually found in the fifth exon of *per*. This transcript could have been produced by using a novel, cryptic splice junction. Instead of the universal AG, the 3' splice junction for a type B message involves a CG dinucleotide. Type B transcripts would encode a *per* protein differing from the type A major class by a deletion of 96 amino acids. Further RNase protection experiments have failed to detect type B transcripts in total RNA isolated from *D*.

melanogaster heads (Weiner and Young, unpublished). These experiments would detect the type B transcripts if they composed as little as 1-2% of the total *per* RNA population. Nonetheless, the splicing experiments do suggest that there are potentially different variants of the *per* protein. The functional importance of these *per* proteins will have to be considered as further biochemistry on the *per* protein is completed.

per, the proteoglycan, and other homologies

Portions of the *per* protein coding sequence are conserved in other species as revealed by probing "zoo" blots -- genomic DNAs from yeast, birds, rodents, cats, and man -- with *per* DNA. One homologous region was studied in some detail because of its similarity to certain mammalian proteoglycan core proteins and its unusual structure (Shin et.al., 1985; Jackson et.al., 1986; Reddy et.al., 1986, Ishida et.al., 1988). This region in the *per* protein consists of alternating amino acid repeats of threonine and glycine (TG). In fact, twenty-three uninterrupted tandem copies of this TG repeat are located near the center of the *per* protein (see Figure 2). Outside this region of strict tandem repetition can be found additional threonine-glycine as well as serine-glycine pairs. The serine-glycine motif has been shown to be the site for attachment of sulfated

glycosaminoglycans in a rat chondroitin sulfate proteoglycan (Bourdon et.al., 1985). In this light, the initial biochemistry performed with the *per* protein has indicated that the native protein extracted from adult heads is of a size expected for a heavily glycosylated *per* protein (> 1 million daltons) (Reddy et.al., 1986; Bargiello et.al., 1987). After treatment with heparinase II, which removes the glycosaminoglycan side chains from heparin sulfated proteoglycans, the *per* protein was smaller (molecular weight ~100kd) and more closely resembled the predicted size from the sequence data (Bargiello et.al., 1987). Reddy and her colleagues (1986) have also reported a change in mobility on a DEAE-Sephacel column of the *per* antigen after treatment with another enzyme, heparitinase. Thus, the types of carbohydrate linkages to the *per* protein may be more diverse than just heparan side chains.

Interestingly, sequence analysis of the *Neurospora* clock gene *frq* indicates a similarity to *per* in this region (McClung et.al., 1989; Dunlap, 1990). The sequence at *frq* predicts a protein carrying a cluster of threonine and glycine and serine and glycine amino acids repeats. A seventeen amino acid long segment of the *frq* protein is largely composed of a mixture of these repeat pairs. In addition, high stringency screens of mouse genomic libraries with the threonine-glycine repeat reveal the existence of this repeat in a number of mouse genes

including B-major globin gene (Shin et.al., 1985; Ishida et.al., 1988).

The purpose of the threonine-glycine repeats at *per* has been analyzed in transgenic *Drosophila* and through comparison of the repeating TG motif in different *Drosophila* strains and species (Yu et.al., 1987; Costa et.al., 1991). Separate *Drosophila* species had distinct numbers of threonine-glycine repeats. In contrast to the twenty-three uninterrupted repeats in the Canton S strain of *Drosophila melanogaster*, the comparatively close relative, *D.pseudoobscura* had seven TG repeated pairs, whereas the more distantly related *D.virilis* had only two repeats. *D.simulans*, an even closer relative to *D.melanogaster* than *D.pseudoobscura* had twenty-three pairs of the TG motif (Yu et.al., 1987). The polymorphism in length of the threonine-glycine region also exists within *D.melanogaster* strains and, as a result, separates the strains in three size classes. The differences between the size classes are in increments of three TG repeats or eighteen nucleotides: Canton S -- 23 pairs, Seto -- 20 pairs, and Chieti V. -- 17 pairs (Yu et.al., 1987). These variations may reflect the amplification or deletion of this particular sequence during evolution.

An in-frame deletion removing the threonine-glycine amino acid repeats at *per* has been constructed for use in transgenic *Drosophila* (Yu et.al., 1987). Although no significant effects were seen on circadian rhythmicity,

strong effects were detected on courtship song periodicity. The deletion shortens the period of courtship song from 55-60 seconds to 40-45 seconds. In addition, replacement of this region plus flanking regions with that from *D.simulans* resulted in a *D.melanogaster* male singing a *D.simulan's* shorter period song. That is, instead of a 55-60 second song rhythm characteristic of *D.melanogaster*, the transgenic male sang its song with a periodicity of the 30-40 second rhythm characteristic of *D.simulans*. Recent work (Wheeler et.al., 1991), however, has shown that it was unlikely that the length of the threonine-glycine repeat had the powerful effect on song; rather, the data suggests that the 122 amino acid stretch downstream of the threonine-glycine repeat is the most likely candidate to encode the species-specific song information. Interestingly, a species comparision of this 122 amino acid region reveals 4 candidate amino acids which are unconserved. One or more of these may account for the species-specific song rhythm differences. In sum, these experiments define a region of the *per* gene which may be especially important to ultradian rhythms and indicates that the pathways by which *per* controls circadian and ultradian rhythms may differ.

In addition to the homology of the threonine-glycine domain of *per* and certain proteoglycan core proteins, a region of similarity to the *Drosophila single-minded*

(*sim*) gene (Crews et.al., 1988) and the mouse aryl hydrocarbon (dioxin) receptor transporter (*arnt*) gene (Hoffman et.al., 1991) has been found. The *sim* gene encodes a nuclear protein necessary for the proper development of the central nervous system (CNS) of the embryo (Thomas et.al., 1988; Crews et.al., 1988). The homologous regions of proteins are 259 amino acids long in *per* and 269 amino acids in *sim* (Crews et.al., 1988). Within the region of homology in both proteins, there are two copies of a shorter, 52 amino acid segment. The overall similarity of *per* and *sim* across the ~260 amino acid regions is 23%. The importance of this homology is unclear as the function of these regions in the respective proteins remains unknown. Both proteins, however, are expressed in an overlapping set of the ventral midline cells of the embryonic CNS (discussed in Part III of the results) and, at least in some cell types, are associated with nuclei (discussed later in this introduction).

The two 52 amino acid segments found in *per* and *sim* are also contained within a 258 amino acid region of *arnt* (Hoffman et.al., 1991). The similarity to the *per* protein across the region is 24.4%. No specific function has been assigned to this particular region of *arnt*; the *arnt* protein has been implicated in the translocation of the ligand-bound aryl hydrocarbon (dioxin) receptor to the nucleus by either shuttling the receptor to the nucleus

or increasing the affinity of the receptor for the nucleus (Hoffman et.al., 1991). In light of the nuclear and cytoplasmic localizations of *per* (see later in introduction and Part III), this homology to a nuclear "shuttle" or "magnet" is tantalizing. Further speculation on the role of this region awaits a dissection of *arnt* as well as *sim* function. As an added point, it should be noted that, unlike the polymorphisms seen with the threonine-glycine region of *per*, the *sim/arnt* homologous portion of *per* is well conserved in other *Drosophila* species (Figure 2 and Colot et.al., 1988).

Patterns of *per* expression during *Drosophila* development

Mosaic and transplantation data have indicated that the focus for *per*'s action for circadian behavior is in the brain of the fly (Handler and Konopka, 1979; Konopka et.al., 1983). For song rhythm, additional mosaic studies have shown that *per* is necessary in the thoracic ganglion (Hall, 1984). With the development of RNA and antibody probes, the *per* gene products could be localized throughout *Drosophila* development. *per* was found to be expressed in a number of tissue types in addition to the sites predicted from genetic studies mentioned above.

The 4.5kb *per* RNA transcript is first detected at midembryogenesis by Northern blot analysis (Young et.al.,

1985; James et.al., 1986). RNA localization by *in situ* hybridization has pinpointed *per*-expressing cells in the central nervous system (CNS) (James et.al., 1986 and part III of the results) and the developing salivary glands (Bargiello et.al., 1987) during embryogenesis. Antibodies generated against *per* fusion proteins and synthetic *per* peptides also detect *per* protein in salivary glands (Bargiello et.al., 1987) and the CNS (Siwicki et.al., 1988 and see part III). In addition, flies have been transformed with a reporter gene, the B-galactosidase gene, placed under the control of the *per* promoter. During embryogenesis, this *per* B-gal fusion protein can be detected in the embryonic CNS (Liu et.al., 1988).

In the larval stages of development, RNA blot analysis reveals very low levels of the *per* transcript (Bargiello et.al., 1987). *per* RNA and protein, however, have been detected in third instar larval salivary glands by RNA blot analysis, *in situ* hybridization, and immunocytochemistry (Bargiello et.al., 1987; L.Vosshall, unpublished). No clear expression of *per* RNA or protein has been detected in the larval CNS (Siwicki et.al., 1988).

During pupal development *per* RNA and protein as well as *per* B-gal fusion protein expression have been found in a number of tissues. Most significantly, as predicted by the genetic mosaic experiments, *per* is expressed in the brain and thoracic ganglia (Liu et.al., 1988, Saez and

Young, 1988). *per* is also encountered in the ring gland (Liu et.al., 1988; Saez and Young, 1988), a complex which secretes hormones such as eclosion hormone, and in the ovaries and testes (Saez and Young, 1988). In adults, *per* expression has been observed again in the nervous system -- the central brain, the optic lobes, the photoreceptor cells of the eyes, the thoracic ganglion, the ocelli (also involved in photoreception), and the antennae (Liu et.al., 1988, Siwicki et.al., 1988; Saez and Young, 1988). However, *per*-expressing cells are also located in the ovaries and testes (Saez and Young, 1988) and in the esophagus, gut, rectal papillae, Malpighian tubules, and proboscis (Liu et.al., 1988).

Furthermore, the rich variety in *per*-expressing tissues is matched by the different subcellular compartments in which *per* is found. For example, *per* protein was seen primarily in the cytoplasm of the embryonic nervous system (see Part III results, Liu et.al., 1988) and at cell borders in the embryonic and larval salivary glands (Bargiello et.al., 1987). The cytoplasmic locale of *per* was also detected for pupal and adult ovaries (Saez and Young, 1988) and in certain cells of the adult central brain (Siwicki et.al., 1988). A nuclear localization of *per* was found in the photoreceptor cells, the cells of the optic lobes (Siwicki et.al., 1988; Saez and Young, 1988), the cells composing the pupal ring gland complex (Saez and Young,

1988), and in the gut and Malpighian tubules (Lui et.al., 1988).

The significance of these varied subcellular locations and whether *per* is active in all or some of these sites are unknown. Likewise, the mechanism responsible for the subcellular localization of *per* in each of the different tissues remains to be uncovered.

Cycling of the *per* products

Siwicki and her colleagues observed cycling in *per* immunoreactivity in the *Drosophila* visual system as well as in the central brain (Siwicki et.al., 1988; Zerr et.al., 1990). The peak of the oscillation was during the middle of the night, at approximately circadian time 18 (CT18). Circadian time 0 (CT0) corresponds to "lights on", whereas circadian time 12 (CT12) corresponds to "lights off". The rhythmicity associated with the immunoreactivity persists in constant darkness and therefore "free runs" as expected of a clock-controlled or associated process. In addition, more recent experiments showed that this cycling of the *per* protein is anticipated by a dramatic fluctuation of the *per* RNA (Hardin et.al., 1990). The peak of the mRNA cycling is approximately six hours ahead of the protein peak (CT 14). Moreover, the RNA oscillation is altered in a predicted manner in the mutants : the peak for *per^S* RNA

occurs earlier than the peak for wildtype. Likewise, the peak for *per*^L RNA occurs later than the peak for wildtype. There is no circadian fluctuation in the *per*⁰ mutants. The authors suggest that the oscillation of the *per* products may be a central component of the clock's function and that the activity of the *per* protein may feedback on the cycling of its own mRNA (Hardin et.al., 1990).

However, one may question how central this cycling is to clock activity since damping of the rhythm is seen within 4-5 days of exposure to constant darkness (Hardin et.al., 1990). Although the cycling of the transcript ceases in these conditions, the flies remain perfectly rhythmic. In addition, transgenic flies carrying a *per* gene under the control of the heat shock promoter showed restored rhythmicity upon chronic exposure to elevated temperature (Ewer et.al., 1988; Ewer et.al., 1990). Due to the promoter employed (heat shock promoter) and the conditions necessary for rescue (constantly elevated temperatures), constant levels of *per* RNA would be continually produced and rhythmicity was still achieved. However, the stability of the *per* RNA is not known; thus cycling may be achieved through this mechanism. Nonetheless, the oscillation of the *per* products is intriguing. The significance of this aspect of *per* function remains to be discerned.

The initial genetic and molecular data concerned with the *per* locus are intriguing; a gene intimately involved with the manifestation of a biological clock has been isolated. Different mutations in the *per* gene alter the periodicity of the clock, indicating that this gene is a necessary component of the clock itself rather than just an output of the clock. The molecular analysis gave few insights into the exact biochemical role by which the *per* gene influences clocks. Although the *per* protein can be glycosylated, the deletion studies challenge whether this form of *per* actually plays a primary role in the generation of circadian rhythmicity. Homology searches with the *per* conceptual amino acid sequence have not uncovered any similar protein that has a known biochemical function. The oscillation experiments indicate that *per* activity may exert some influence on the metabolism of its own transcript. The localization studies did not define a unique place of *per* activity. The varied tissue and subcellular locales seem to indicate a *per* activity not associated just with circadian rhythmicity, but with any place where synchronous, rhythmic function is necessary.

The goal of my work has been to gather more information about *per* function. This was first

accomplished through the analysis of *per* regulation and structure. In collaboration with others in the lab, I molecularly characterized the influence of *per* dosage on the period length. This was accomplished through the manipulation of transgenic flies carrying a copy of the *per* gene and the subsequent analysis of *per* RNA titre. In addition, I examined the known mutants of the *per* locus, *per*⁰, *per*¹, and *per*^S, which have aberrant period lengths but equivalent RNA titres, to determine the alterations in each. By doing so I determined regions of functional significance. Based on what I found for the mutant series, I generated a series of new mutations in *per*. These new substitutions in *per* altered amino acid size and charge as well as secondary structure and possible modification sites. In this way, domains of the *per* protein important for its clock function could be better defined. In addition, a cell level-phenotype for *per* was sought. This cellular assay could give insights into the particular biochemical mechanism by which *per* acts. Lastly, a more precise study of *per*'s localization in the embryo was pursued. More information about *per*'s role in the formation or running of a biological clock from the earliest developmental stage was obtained. With answers to the above in hand, part of the *per* puzzle could be solved.

PART I --REGULATION AND STRUCTURE/FUNCTION ANALYSIS
OF THE PER GENE PRODUCT

INTRODUCTION

Despite the abundance of information that is known about the *per* locus and its products, we have very little information on how *per* actually affects biological rhythms. The biochemical mechanics of its function have remained elusive. Sequence analysis gave very few hints to the actual process in which *per* partakes. Moreover, the localization data did more to obfuscate the issue. *per* was localized in a number of tissue types. Some of these were expected, such as the nervous system; others such as the salivary gland, the gonads, and the gut, were not expected and were thought to have little overt influence on biological rhythms. The subcellular localization also did not clarify the situation. In some tissue types, the *per* protein was localized to the nucleus; in others, the cytoplasm; and still in others, the cell border. As a first step towards understanding *per* and its role in biological clocks, changes in the regulation and structure of the *per* gene products were analyzed. Characterization of these alterations in *per* would give further insight into *per*'s unique function in biological clocks.

RESULTS

A. The effect of *per* dosage on period length

Through the analysis of several chromosomal breakpoints, a 4.5kb mRNA was determined to be the likely candidate for the *per* transcript. Final proof that this RNA was indeed the *per* transcript was obtained through P-element mediated germline transformation of *Drosophila* (Bargiello et.al., 1984; Zehring et.al., 1984; Hamblen et.al., 1986 and see experimental procedures). When the 7.1kb genomic DNA encoding the 4.5kb transcript was introduced into *per*⁰ flies, the arrhythmic behavior of these flies was rescued: periodicities of locomotor activity behavior in the range of 24h were obtained (Bargiello et.al., 1984). Therefore, the *per* gene is contained within the boundaries of this 7.1kb genomic DNA fragment.

More independent strains of *per*⁰ flies carrying the same 7.1kb *per* construct were desired, initially for a more complete analysis of the rescuing periodicities. However, when the transformation construct containing the 7.1kb *per* DNA was "mobilized" from its original insertion site and "hopped" to various new positions in the genome (Rubin, 1985), an interesting observation was made. Instead of generating periodicities in the locomotor assay of 24-25h (see experimental procedures), a

range of periodicities from 24 to 40h was obtained from the thirty lines created.

Seven of these strains, representing the entire range of derived periodicities, were selected for further molecular and behavioral analysis. *In situ* polytene chromosome analysis using the *per* DNA as a probe showed that each strain carried one copy of the 7.1kb construct. In addition, each strain has this copy inserted in a different site within the *Drosophila* genome (Table 3). Locomotor assays on at least fifteen flies from each strain were performed. A wide range of period lengths between individual lines was again generated. However, the period length within a strain was similar (see standard errors, Table 3). The only exception to this is line P1/30 which has a mean period length of 37h. Period lengths up to 45h as well as arrhythmia are obtained with this line. This variation is reminiscent of the behavior seen with a *per* mutant created by a translocation of the X-chromosomal region containing *per* to the 4th chromosome. In this case, the majority of the *per* gene (minus some of the noncoding 3'end sequences) was fused to sequences from the 4th chromosome. The resultant "fusion" gene produced an 11.5kb *per* mRNA which gave long, variable period lengths (Jackson et.al., 1986). Perhaps the movement of the transforming *per*

Table 3. Periodicities of transformed *D. melanogaster* lines.

<u>Line</u>	<u>Period (h)</u>	<u>N</u>	<u>insertion site</u>
P1/14	26.9 ± 1.1	20	47E-F
P1/23	27.3 ± 2.6	20	34E-F
P1/25	28.2 ± 1.8	20	97E-F
P1/29	24.8 ± 1.7	21	ND
P1/30	37.6 ± 11.1	15	54A-B
P1/35	31.3 ± 2.0	18	87A-B
P1/36	27.2 ± 1.2	17	93A

Insertion site refers to polytene chromosome band location; N, number of flies tested for each line; h, hours; ND, not determined.

construct to the site in P1/30 did something to alter proper regulation provided by the 3' end sequence of the 7.1kb *per* DNA. The remaining six lines show a high degree of penetrance; 75-90% of the flies tested give rhythmic behaviors.

P-element mediated transformation in *Drosophila* generally does not act by homologous recombination (Craig 1990); therefore, the site of insertion in the genome may affect transcription of the inserted, "rescuing" DNA (Spradling and Rubin, 1983; Goldberg et.al., 1983). Given this possibility as well as the prior genetic work on the dosage of *per* and period length (Smith and Konopka, 1982; introduction), a molecular analysis of the abundance of the *per* transcript in each of these strains was undertaken.

It was previously shown that *per*⁰ flies, despite their arrhythmic behavior, still make a 4.5kb *per* transcript. Therefore, the strains in their present state (i.e. in a *per*⁰ background) would not be amenable to transcript analysis: the endogenous *per*⁰ transcript could obscure any differences, if present, between the lines. Hence, a derivative strain was created for each of the lines. A 10kb, X-chromosomal deletion containing the *per* locus can be produced in females homozygous for *Df(1)TEM202/Df(1)64j4* (Young and Judd, 1978; Smith and Konopka, 1981). Strains were crossed to produce

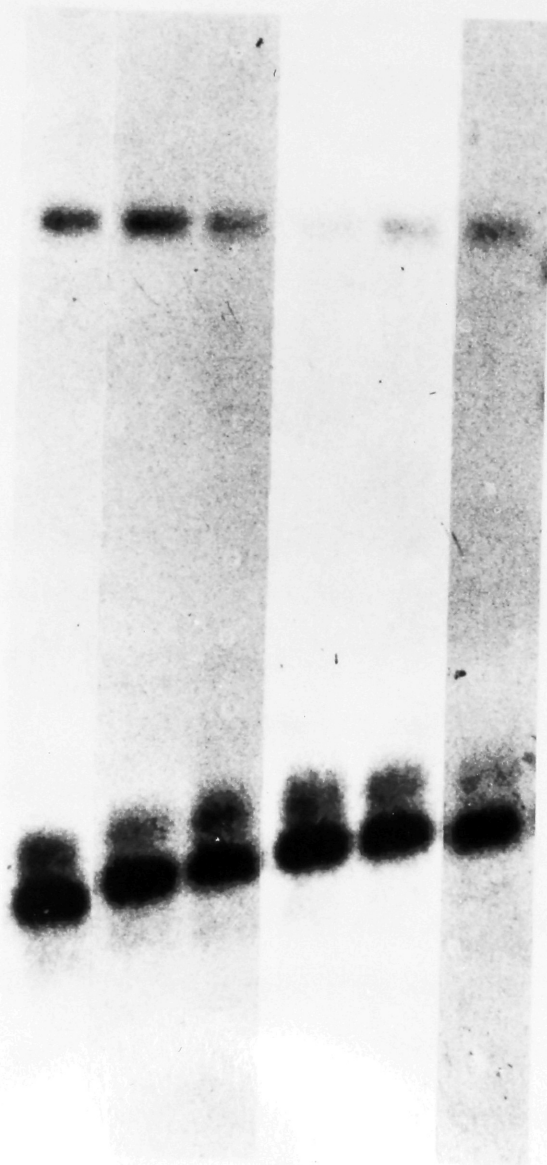
these *Df(1)TEM292/Df(1)64j4* females carrying the autosome-linked transforming *per* DNA. The only *per* transcript seen would be that derived from the transforming *per* DNA. The transcript analysis of several of the derivative lines is shown in Figure 3. Poly A⁺ RNA derived from whole adult flies was probed with a *per* specific probe. To control for the amount of RNA loaded in each lane, an actin probe was hybridized to the blots. The relative level of the *per* transcript was assessed by comparing the abundance of the 4.5kb *per* message with that of the actin mRNA. All the transformed lines produced the 4.5kb *per* transcript, but the levels of the transcript varied among the strains (Figure 3).

Two to three independent RNA preparations from each line was tested as in Figure 3. The relative levels of *per* RNA for each preparation from each line was plotted as a function of period length as in Figure 4 (open triangles). An inverse correlation between period length and *per* RNA titre is found: the lower the amount of *per* RNA, the longer the period length. For comparison, the relative levels of *per* RNA found in homozygous flies (*per*⁺/*per*⁺) and heterozygous flies (*per*⁺/*per*^{def}) are also shown (filled triangles). The period lengths for these flies are 24h for the homozygous flies and one hour longer, 25h, for the heterozygous flies.

Figure 3. Abundance of the 4.5kb *per* RNA in different lines of transformed flies

Northern analysis of P1/14, P1/23, P1/30, P1/35, and P1/36 transformed strains in a *Df(1)TEM202/Df(1)64j4* background was completed using a *per* riboprobe (see experimental procedures). 5ug of poly A⁺ RNA was loaded in each lane. The 4.5kb transcript detected is the *per* mRNA. The Northern analysis was also probed with actin DNA as a control for the amount of *Drosophila* poly A⁺ RNA loaded in each lane.

P1/14
P1/23
P1/25
P1/30
P1/35
P1/36



• per, 4.5 kb

• actin, 1.9 kb

Interestingly, the relative RNA value for the flies carrying one copy of *per* (per^+/per^{def}) is similar to that of the shortest period transformed line (P1/29). Both strains have period lengths of approximately 25h.

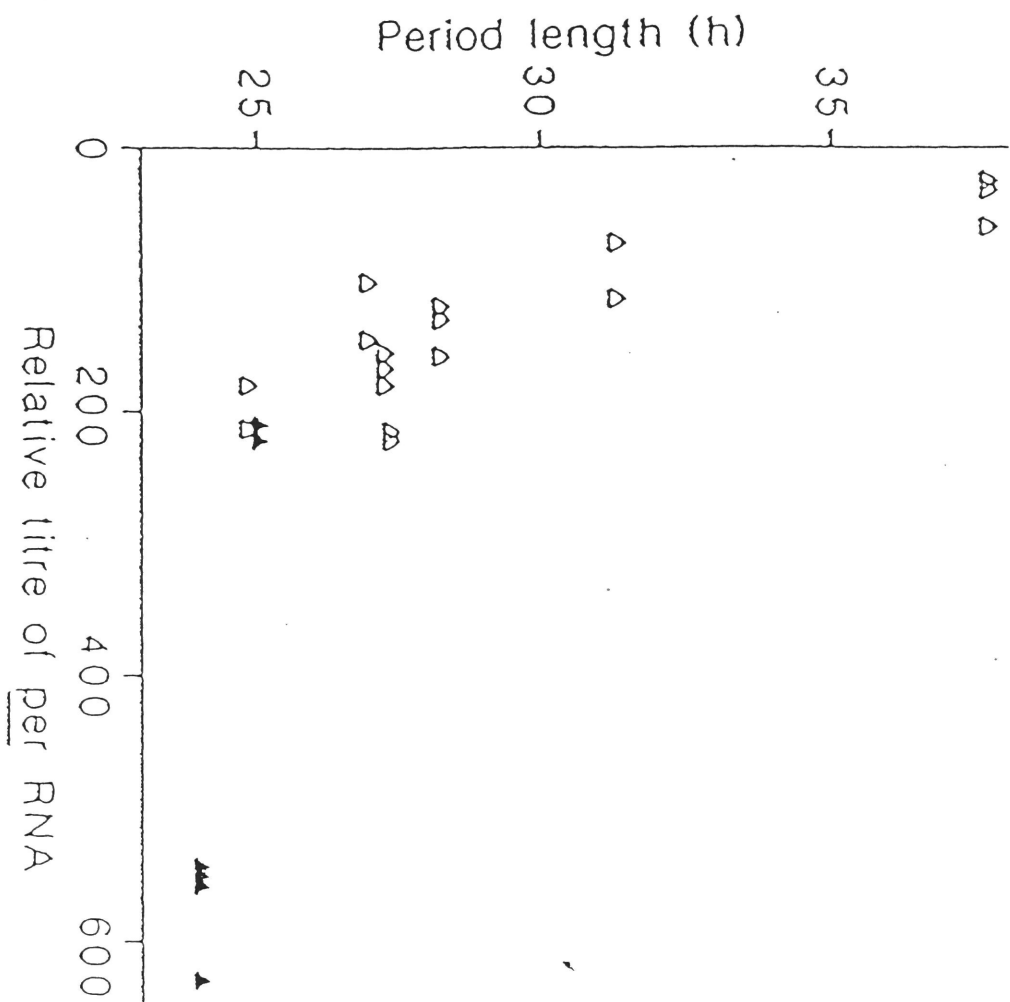
These studies of transformed flies indicate that the level of *per* gene product sets the period length of the *Drosophila* circadian rhythm. More *per* product yields a shorter period length; less *per* product results in a longer period length. The effects can be dramatic. A twenty fold drop in RNA titre yields a lengthening in period from 24h to almost 40hr (Figure 4).

B. Determination of the molecular lesions responsible for the mutant phenotypes, per^L , per^S , and per^0 .

Alterations in period length can also be brought about by chemically induced mutations of the *per* locus. Konopka and Benzer's screen of EMS mutagenized flies for altered circadian behaviors led to the isolation of the three *per* alleles, per^S , per^L , and per^0 (Konopka and Benzer, 1971). EMS treatment, usually, is associated with point changes or small, undetectable (by chromosomal *in situ* analysis) deletions (Grigliatti, 1986). Considering the results of the RNA titre experiment, the mutants

Figure 4. Correlation of *per* RNA titre and period length

Autoradiograms showing the results of several experiments like that in Figure 3 were analyzed by densitometry. Relative levels of accumulation of the 4.5kb *per* RNA were determined for transformed lines after normalization with actin mRNA levels (Figure 3). Each triangle represents the level of *per* transcript observed in a single preparation. Two or three independent RNA preparations were analyzed for each transformed line and are represented on the same abscissa. Filled triangles represent *per* RNA present in wild-type (Oregon R) or *per*⁺/*per*^{def}. Oregon R and *per*⁺/*per*^{def} periodicities were 24.0 and 25.0h, respectively. For transformants, period lengths are on Table 3. The data as plotted suggest an exponential relationship of RNA titre and period length.



were analyzed by Northern analysis to determine if the abundance of 4.5kb *per* RNA was altered. No change in the abundance or processing of the 4.5kb *per* transcript was detected in *per*⁰, *per*¹, or *per*^S (Bargiello and Young, 1984; Young et.al., 1985, Hall and Rosbash, 1987; T. Bargiello, personal communication).

To ascertain the lesions responsible for the mutant phenotypes, sequencing of each of the mutant *per* genes became necessary. Transformation experiments have shown that a ~6.0kb EcoRI - Hind III fragment is capable of restoring rhythms with periodicities in the wild-type range (Zehring et.al., 1984; Hamblen et.al., 1986). Sequence analysis of wild-type *per* as well as cDNA analysis, RNase protections, and primer extension experiments showed that all the protein-coding regions of the gene are contained in this fragment (Jackson et.al., 1986; Citri et.al., 1987). Therefore, this biologically active fragment was examined for alterations in sequence for each of the mutants. Moreover, the parental Canton S stock from which all three mutants were derived was sequenced previously in the laboratory (Jackson et.al., 1986). The sequences from each mutant in this interval, then, could be compared to the parental wild-type sequence as well as to each other. Any unique change in a

mutant could then be associated with the particular mutant phenotype.

This 6.0 kb segment of DNA from each of the mutants was subcloned into M13 vectors and a series of nested deletions created (Dale et.al, 1985; see experimental procedures). Sequence data was then obtained for the entire segment from each of the mutants and was compared to the wild-type sequence. Figure 5 displays a portion of the original sequencing gels containing the alterations associated with each mutant. A single, unique nucleotide change was found in each of the mutants and hence, could be correlated with the appearance of the mutant phenotype. No third base or "silent" changes had accumulated in the 15 years since the mutants were first isolated from the parental strain. Figure 6 records the resultant change in the *per* amino acid sequence. For *per*^L and *per*^S, a single nucleotide substitution results in an alteration of an amino acid. In *per*^L, a T-to-A transversion leads to a valine (V243) being replaced by an aspartic acid. A serine (S589) is replaced by an asparagine in *per*^S due to a G-to-A transition. In *per*⁰, a C-to-T transition results in the substitution of a glutamine (Q464) by an amber stop codon. This nonsense change in *per*⁰ would lead to a protein of approximately one-third the size of the

Figure 5. Base Changes associated with the *per* mutants.

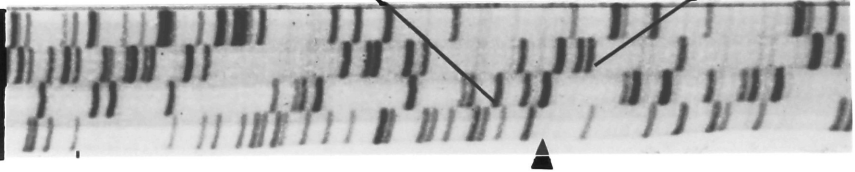
The Eco RI - Hind III fragment from each of the mutant genes -- *per*⁰, *per*^l, and *per*^s -- was subcloned into m13mp18 and mp19, and a series of nested deletions made (Maniatis et.al., 1982; Dale et.al., 1985; see experimental procedures). DNA sequencing was performed as described (Jackson et.al., 1986; Sanger, 1981).

The portion of the sequence containing the single base change for each mutant is shown. The interval containing the single change in each case is bracketed by lines and the corresponding "read" sequence listed vertically to the right of the sequence under the corresponding mutant designation -- *per*⁰, 0; *per*^l, L; and *per*^s, S. + indicates the wildtype sequence in the corresponding interval. The bases in **bold** are those altered in the mutant from the wildtype sequence.

For *per*⁰, G4355 in the wildtype sequence was changed to an A. In *per*^l, T3629 was changed to an A, and in *per*^s, C4793 was changed to a T.

per0

GATC

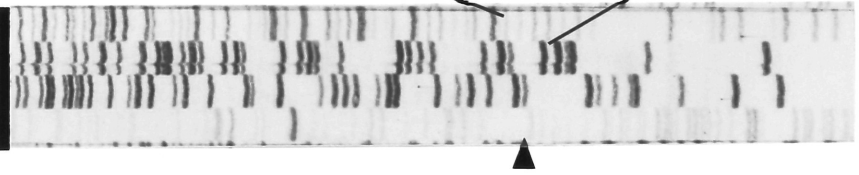


+ G T A G G T C T T

O G T A G A T C T T

per1

GATC

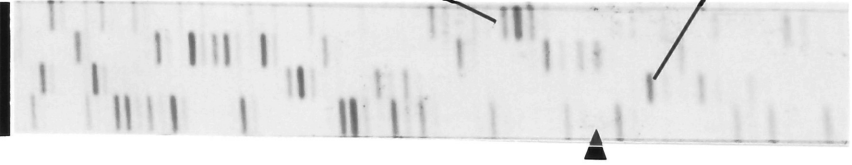


+ C T A C T G C G T

L C T A C A G C G T

pers

GATC



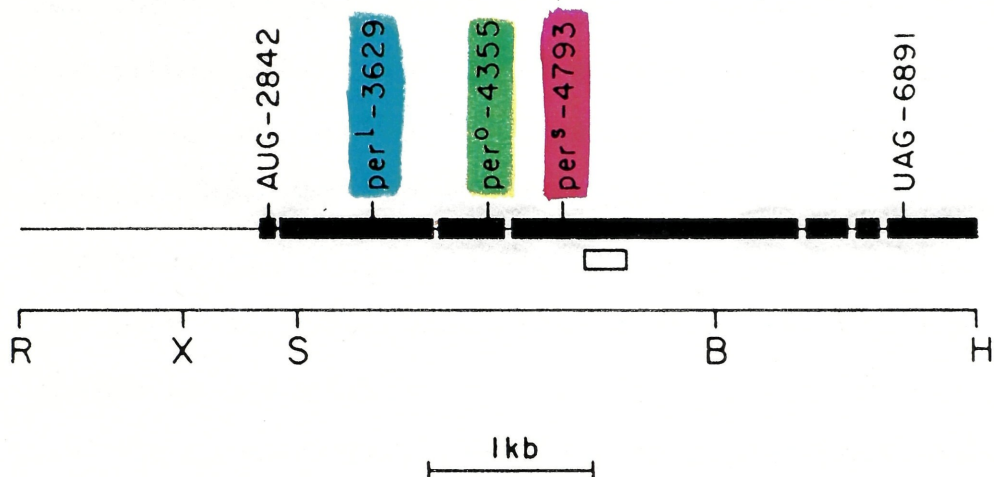
+ C C C T G T C G C A

S C C C T G T T G C A

Figure 6. Location associated with the *per* locus point mutations

The structure of the Eco RI - Hind III fragment sequenced for each of the mutants is shown at the top. This genomic DNA fragment contains all the protein coding regions of the *per* gene (Jackson et.al., 1986; Citri et.al.,1987). Nucleotide positions for the predicted translation start and stop codons in the wild-type gene are given, and the locations of the single base changes found in *per^s*, *per^l*, and *per⁰* are shown and highlighted in red, blue, and green, respectively. The open box marks the position of the alternating threonine-glycine and serine-glycine repeats found in *per* (Jackson et.al., 1986; Citri et.al., 1987). R, Eco RI; X, XbaI; S, Sall; B, BamHI; H, HindIII.

Below are the *per* DNA and protein sequences of the mutants. The regions of the Canton S wild-type DNA and protein altered in *per^l* (upper, blue), *per⁰* (center, green), and *per^s* (bottom, red) are shown. For each mutant, the predicted change in the protein sequence is indicated above and the change in the DNA sequence below the corresponding wild-type sequence. Nucleotide numbers are from Jackson et.al., 1986.



perl

Arg	Val	Lys	Glu	Asp	Ser	Phe	Cys	Cys	Asp	Ile	Ser	Met	His	Asp	Gly	Ile	Val
CGG	GTG	AAG	GAG	GAC	AGC	TTC	TGC	TGC	CTC	ATC	TCC	ATG	CAC	GAC	GGC	ATC	GTC
									A								3654

pero

Gly	Ala	Ser	Phe	Cys	Ser	Lys	Pro	Tyr	Arg	Phe	Leu	Ile	AM	Asn	Gly	Cys	Tyr
GGC	GCC	TCC	TTC	TGC	AGC	AAG	CCA	TAC	CGC	TTC	CTC	ATC	CAG	AAC	GGT	TGC	TAC
													T				4369

pers

His	Glu	Asn	Glu	Leu	Thr	Val	Ser	Glu	Arg	Asp	Asn	Val	Met	Leu	Gly	Glu	Ile
CAC	GAG	AAC	GAG	TTG	ACC	GTC	TCG	GAG	CGG	GAC	AGC	GTG	ATG	CTC	GGC	GAG	ATT
											A						4812

wild-type protein (i.e 463 amino acids instead of 1224 amino acids. Jackson et.al., 1986).

The behavioral phenotype of a deletion of the *per* locus and *per*⁰ is the same --- arrhythmia. Thus, the finding that *per*⁰ would produce a truncated, disfunctional protein is logical. Since flies carrying the *per*⁰ mutation still produce wild-type levels of the 4.5kb *per* RNA (Young et.al., 1985), *per* must influence biological rhythms through the action of its protein.

It is much more difficult to explain how the single amino acid substitutions in *per*^l and *per*^s generate the respective phenotypes. The RNA titre experiments, shown in Figures 3 and 4, indicate that the abundance of *per* RNA is inversely related to the period length: underproduction of the wild-type RNA gives longer period lengths whereas overproduction of wild-type RNA results in shorter period lengths. *per*^l and *per*^s flies have the same amount of *per* RNA as wild-type flies. Taken together, these observations would indicate that the *per*^s protein would be more active or more stable than the wild-type protein. The *per*^l protein containing that single valine to aspartic acid change, in turn, would be less active or less stable than the wild-type protein.

How the particular amino acids altered in *per*^L and *per*^S cause this change in *per* protein activity remains for further investigation. The serine which is replaced by an asparagine in the *per*^S protein may be a site for post-translational modification such as glycosylation or phosphorylation. Loss of this modification may lead to greater activity of the *per* protein and hence, short period phenotypes. Why the valine to aspartic acid substitution leads to a long period phenotype is less clear. This amino acid substitution would result in a size and charge change in this position. In addition, this mutant maps within the region homologous to the *Drosophila single-minded (sim)* protein and the mouse aryl hydrocarbon (dioxin) receptor transporter (*arnt*) protein (Crews et.al., 1988; Hoffman et.al., 1991; introduction). The function of *sim* is unknown, although recent experiments indicate that it may be a transcriptional regulator (Nambu et.al., 1990; also Part III). The role of *arnt* may be to direct the ligand-bound receptor to the nucleus, either by shuttling the receptor-ligand complex to the nucleus itself or by increasing the affinity of the receptor-ligand complex for the nucleus (Hoffman et.al., 1991). Further experimentation is necessary to determine how the *sim* and *arnt* observations relate to the alteration caused by *per*^L.

C. Rationale for in vitro mutagenesis of the per products

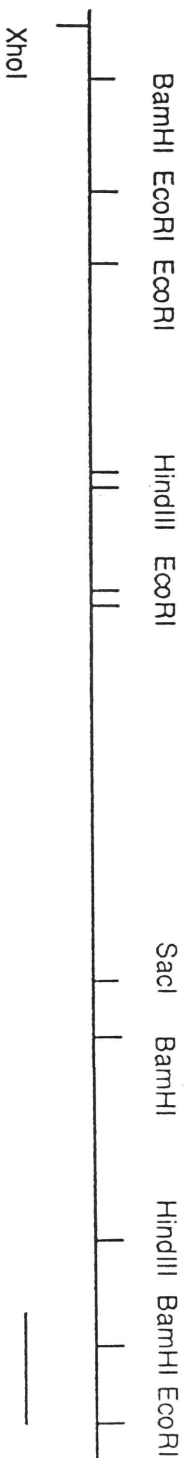
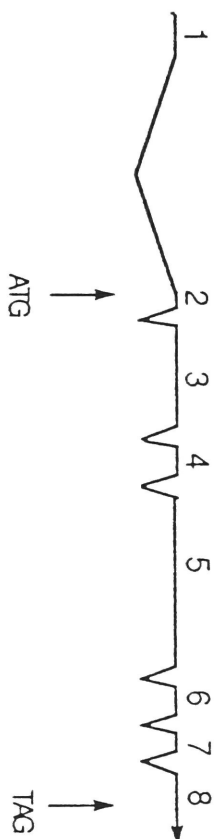
The actual changes of the *per*^l and *per*^s mutations gave little insight as to how these single amino acid alterations might affect the activity of the *per* protein. Moreover, the biochemical mechanism by which *per* affects the periodicity of biological rhythms still remains unknown. Therefore, a new series of mutations were created in the *per* gene. By defining domains of function important for *per*'s activity, further understanding of the actual function of *per* could be obtained. This information would ultimately be used to determine how *per* influences biological rhythms and the "clocks" which underlie them.

D. Establishment of the parameters for in vitro synthesized mutations of per

The results from the RNA titre experiment reinforced the observation that position of insertion affects transcription from the transforming *per* DNA (Spradling and Rubin, 1983; Goldberg et.al., 1983). This position effect would have to be eliminated or at least reduced before introducing new mutations into the *per* gene. Such variation in period length would not allow for

Figure 7. Diagram of the *per*-containing genomic DNA fragments used to transform *Drosophila* lines.

The top line shows a schematic of the *per* transcription unit. As drawn, transcription goes from left to right; introns are depicted by slanted lines; exons by horizontal lines. The start of translation (ATG) and stop site of translation (TAG) are noted. The second line shows a limited restriction map of genomic DNA flanking and including the *per* gene. The bottom two lines show two fragments of genomic DNA used to transform *Drosophila* lines. The 7.1 kb HindIII fragment was used to generate the initial lines that restored rhythmic activity in *per^o* flies (presented in Table 3 and Figures 3 and 4). The 12.3 kb XhoI fragment was used in all subsequent experiments because it gave fewer position effects (see text and experimental procedures).



1.0 Kb



accurate characterization of the effects of a new mutation as short, wild-type, or long.

Inspection of the *per* genomic fragment used in the RNA titre experiment reveals the following. Although this fragment contains all the coding sequences necessary to produce a wild-type *per* protein (Jackson et.al., 1986; Citri et.al., 1987) and has been shown to "rescue" arrhythmic flies (Bargiello et.al., 1984), it contains little promoter and 5' and 3' cis acting regions (see Figure 7, construct labelled 7.1kb). These upstream and downstream regions are necessary to ensure the proper amount and location of transcription (Jones et.al., 1988) and to buffer against position effects (Spradling, 1986).

Figure 7 shows the genomic fragment, labelled 12.3kb, which was employed for all the subsequent transformation experiments. This construct contained not only all the necessary coding regions for the wild-type *per* protein but also more 5' and 3' flanking regions (approximately 6.5kb upstream sequences and < 1.0kb downstream regions). In addition to buffering position effects, a construct of this size would allow for efficient transformation frequencies (Spradling, 1986).

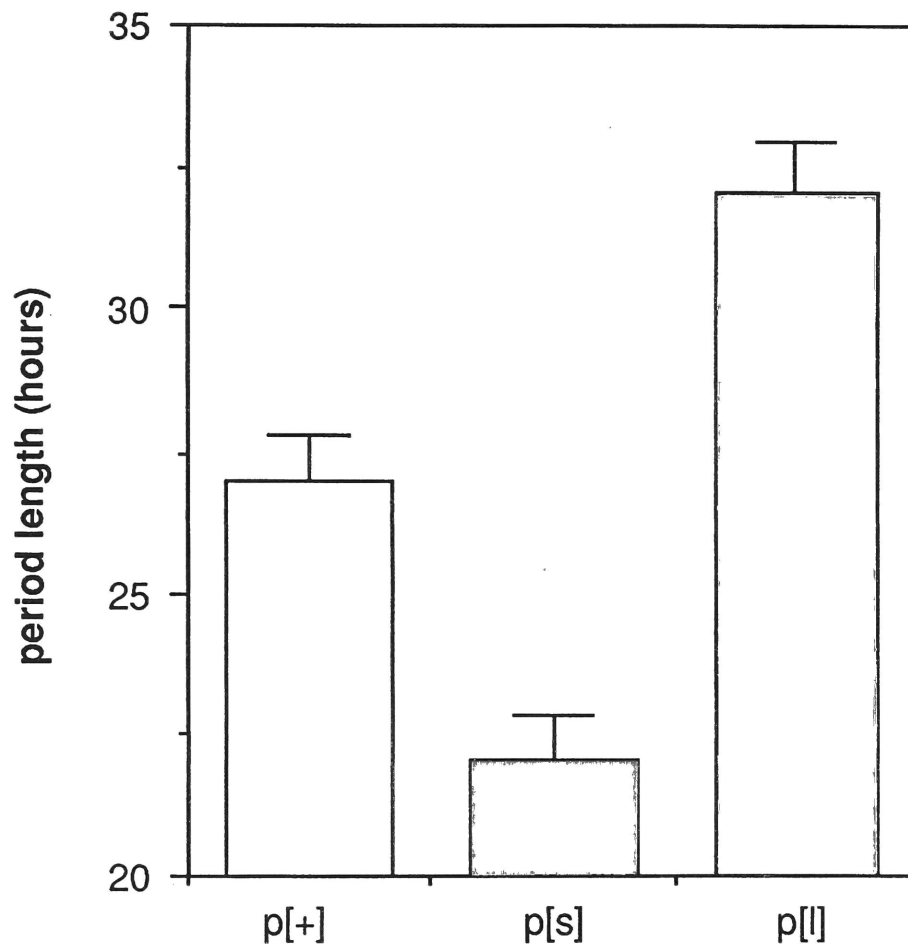
Two additional constructs were also created using this same construction strategy. One of these

Figure 8 and Table 4. Period lengths from control heterozygous transformed lines.

Table 4 shows the mean period length or tau in hours \pm the standard error (S.E.) (Strickberger, 1968) determined for each of the controls : p[+] is the construct containing wild-type *per* DNA; p[s] is the construct containing the *per^s* mutation; and p[l] is the construct containing the *per^l* mutation. n = rhythmic number of flies tested. N = number of independently transformed lines. Period length \pm the standard deviation (S.D.) (Strickberger, 1968) for each independent line carrying one copy of the construct was determined. Range of period lengths for the lines of each construct is shown.

Figure 8 presents the mean period length \pm S.E. for the heterozygous lines of each control in a bar graph.

Control Transformant Periods



control periods

genotype	tau	S.E.	range -- tau \pm S.D.	N (lines)	n (flies tested)
p[+]	26.9	0.8	26.65 \pm .88 - 27.20 \pm .50	6	38
p[s]	22.1	0.7	21.35 \pm .53 - 22.90 \pm .57	6	30
p[l]	32.1	0.9	31.10 \pm .65 - 32.60 \pm .57	7	40

constructs contained the single nucleotide change which defined the *per^S* mutation and is designated p[s]. The second contained the single nucleotide change associated with the *per^L* mutation and is named p[l].

Six to eleven independent, rhythmic lines were created from arrhythmic *per⁰* flies by P-element mediated transformation for each construct as described (Rubin and Spradling, 1982; Spradling, 1986; experimental procedures). All the lines were maintained as heterozygous stocks with respect to the transforming DNA. Seven to fifteen flies of each line were tested in the locomotor behavioral assay (experimental procedures) and the periodicities of the flies' circadian behavior were determined. Table 4 and Figure 8 display the pertinent data for each of the constructs. For the construct containing the wild-type *per* DNA (p[+]), the period length or tau was 26.9h. The period length for the *per^S* construct (p[s]) was 22.1h and for the *per^L* construct (p[l]) was 32.1h. As shown by the range and standard error in Table 4, little variation in period length was observed. This indicates that the additional 5' and 3' flanking sequences could adequately buffer the constructs from position effects. Moreover, the data from p[s] and p[l] corroborate the sequence analysis; indeed, transformed DNA containing the

base changes found from the "brute force " sequence analysis gave period lengths that were altered as expected. It should be noted that the period lengths obtained with this construct strategy are slightly longer than those obtained with the *per* allele in its correct chromosome locale (Smith and Konopka, 1982) or with other constructs transformed into flies (Bargiello et.al., 1984; Zehring et.al., 1984; Hamblen et.al., 1986). This lengthening is most likely due to the disruption of regulatory elements in the 3' or 5' portions of the gene. The number of hours separating the mutant from the wildtype phenotype, however, is maintained with the transformants using this construct strategy. For example, there is a 4.9 hr difference between the original EMS-induced *per^S* and *per⁺* compared to a 4.8hr difference between *p[s]* and *p[+]*.

These experiments also define the parameters from which all *in vitro* created mutations will be judged. Any changes made in the *per* gene, constructed with scheme outlined above and then re-introduced into flies via the same methodology can be properly evaluated as short, wildtype, or long.

E. In vitro synthesized mutations in the per gene

i) Construction of a *per* gene containing both the *per*^L and *per*^S mutations

Earlier work has shown that females carrying one copy of the *per*^S mutant gene and one copy of the *per*^L mutant gene in *trans* were rhythmic with a period length of approximately $22.9\text{h} \pm 0.4$. For reference, females homozygous for wild-type *per* had period lengths of $24.4\text{h} \pm 0.5$ (Konopka and Benzer, 1971; also Table 2). Therefore, the mutant proteins, long and short, are somehow able to counterbalance one another. The activity of the *per*^S protein was able to modify the activity of the *per*^L protein in some way so that a near wild-type phenotype would result. Furthermore, as the above would indicate and other experiments show, *per*^S exhibits the greatest amount of partial dominance of any of the *per* alleles (Konopka, 1987b). What would happen to the counterbalance between the mutants if, instead of being placed in *trans*, the mutants were now placed within the same gene? Could *per*^S exert its influence now that another portion carried the effects of the *per*^L change?

The construct containing both the *per* mutations was prepared according to the scheme outlined for the controls. Seven independent lines were established. The results from the locomotor behavior assays are shown in Table 5; the mean period length

Table 5. Period lengths of heterozygous transformed lines.

<u>genotype</u>	<u>tau</u>	<u>S.E.</u>	<u>range (tau ± S.D.)</u>	<u>N</u>	<u>n</u>
p[+]	26.9	0.8	26.7 ± 0.9 - 27.2 ± 0.5	6	38
p[s]	22.1	0.7	21.4 ± 0.5 - 22.9 ± 0.6	6	30
p[l]	32.1	0.9	31.1 ± 0.7 - 32.6 ± 0.6	7	40
p[l/s]	29.2	0.7	28.9 ± 0.6 - 30.1 ± 0.1	7	56
p[3'P*site]	26.9	0.8	26.0 ± 0.4 - 27.5 ± 1.4	6	27
p[D588V]	22.5	0.4	21.2 ± 0.7 - 23.2 ± 0.7	4	48
p[D588F]	24.3	0.6	23.9 ± 1.1 - 24.8 ± 1.6	2	30
p[G593S]	21.3	0.8	20.3 ± 0.6 - 22.1 ± 0.6	8	58
p[G593C]	30.7	1	29.7 ± 1.0 - 31.7 ± 0.8	10	80
p[S589L]	22.1	0.5	21.3 ± 0.4 - 23.9 ± 0.5	7	72
p[E586V, V590D]	22.7	0.6	21.5 ± 0.7 - 23.6 ± 0.4	9	78

tau, refers to the period length; S.E., the standard error (Strickberger, 1968); S.D., the standard deviation (Strickberger, 1968); N, number of independent lines tested; n, total number of flies tested. Control (p[+], p[s], p[l]) data added for comparison (see Table 4).

of $29.2\text{h} \pm 0.7\text{ h}$ was obtained (homozygous period length is $26.9 \pm 0.8\text{ h}$, Table 8). No significant variation was found within or between the established lines. This intermediate phenotype between wild-type and long indicates that the *per^S* mutation is still able to exert its effects on the system -- it continued to shorten the period length from an otherwise long period. However, due to the *per^L* mutation being in the same protein, the full shortening to wild-type or just short of wild-type period length could not be obtained.

ii) Construction of a mutant with a deleted potential phosphorylation site at the carboxy terminus of the *per* protein

Phosphorylation is known to regulate a number of cellular events from entry into the cell cycle to release of synaptic vesicles (Lewin, 1990; Levin et.al., 1990; DeCamilli et.al., 1990). The *per* protein not only includes many potential sites for phosphorylation but also has been shown to be phosphorylated on serine and tyrosine in cell culture (Saez and Young, unpublished). This raises the possibility that the action of the *per* protein could be regulated post-translationally. The kinase(s) and exact amino acid sites of phosphorylation are, as of yet, unknown; however, a possible site for phosphorylation by cyclic AMP-

dependent protein kinase exists near the carboxy terminus (amino acids 1192 -1207) of the *per* protein. This amino acid sequence resembles the phosphorylation site in troponin I from rat skeletal muscle (Bramson et.al., 1984). A synthetic peptide containing this peptide from the *per* sequence was found to be an efficient substrate for phosphorylation *in vitro* (Jackson et.al., 1986; Gasic, 1988). Various chemical agents commonly used to alter cyclic nucleotide levels can induce a phase shift in circadian clocks (Eskin et.al., 1982; Eskin and Takahashi, 1983; Jacklet, 1989b). Thus, agents that affect cyclic nucleotide levels could alter the cAMP-dependent phosphorylation of the *per* protein, thereby changing *per*'s activity.

To investigate whether this cAMP-dependant phosphorylation site had any affect on *per* function in biological rhythms, a mutant was created that removed this phosphorylation site (see experimental procedures). Table 6 shows how the phosphorylation site was changed. The serine which could be phosphorylated was removed and replaced by a valine. Furthermore, the serines and threonine following the preferred serine were also replaced. This alteration also resulted in a *per* protein having one less amino acid than wild-type.

Table 6. Potential phosphorylation site by cAMP protein kinase.

<i>per</i> ⁺	E K D P <u>K H R K L K S M</u> S T S E S
<i>p[3'P*site]</i>	E K D P <u>K H R K L K</u> V L D R ... E S

Legend: Figure shows sequence of amino acids (1192-1207) near the carboxy-terminus of the wildtype *per*⁺ and mutant *p[3'P*site]* proteins. Underlined amino acids are homologous to the phosphorylated site in troponin I from rabbit skeletal muscle (Bramson et.al., 1984). Amino acids in bold are those affected by mutation in *p[3'P*site]*. Single amino acid code is used: V, valine; L, leucine; P, proline; M, methionine; S, serine; T, threonine; D, aspartate; E, glutamate; K, lysine; R, arginine; H, histidine.

Several independent strains of transgenic *Drosophila* were made and flies carrying one copy of the construct were tested in the locomotor behavior assay. The results are shown in Table 5 for this construct which is designated p[3'P* site]. Wild-type period lengths were generated in the p[3'P* site] flies. Therefore, this site has no functional significance in the assays for *per* function which were tested. Moreover, sequence analysis from *D.pseudoobscura* and *D.virilis*, two distantly related species, indicate little conservation of this potential site (Colot et.al., 1988). The lack of conservation reinforces the idea that these sequences do not play the pivotal role in the regulation of *per*'s control of circadian rhythmicity.

iii) Construction of mutants containing alterations in the vicinity of the *per*^S mutation

The region surrounding the *per*^S mutation was selected for further mutagenesis for four reasons.

1. The *per*^S mutation was localized to this region of the *per* protein. Previous work (above; Smith and Konopka, 1982; Cote and Brody, 1986) suggests that the amino acid substitution in *per*^S leads to greater stability or greater activity of the resultant *per* protein.

2. This region of the *per* protein is highly conserved between species of *Drosophila* that are evolutionarily separated by millions of years. Only six changes in amino acid sequence were found in the sixty amino acids surrounding the *per^S* change (Colot et.al., 1988). These substitutions, however, would be considered conservative changes (Shulz and Schirmer, 1979). For example, aspartate in the *D.melangaster* sequence is changed to a glutamate in the *D. virilis* and *D. pseudoobscura* sequence. Another difference is an alanine in *D.melanogaster* changed to a glycine in *D. virilis*. Other regions of the gene are far less conserved; in fact, approximately 33% of the protein sequence is nonconserved, and within the conserved regions, 38.2% of the amino acids have been substituted (Colot et.al., 1988). Low variability at a given residue indicates structural and functional importance of this position (Shulz and Schirmer, 1979). Thus, the high degree of sequence conservation in the *per^S* region could reflect a functionally significant domain for *per* activity.

3. The conserved region surrounding the *per^S* mutation contains possible sites for post-translational protein modification. In particular, sites for phosphorylation by protein kinase C and a tyrosine kinase are located in this region (Woodgett

et.al., 1986; Cooper et.al., 1984). Dr. Lino Saez has shown that the *per* protein in cell culture is phosphorylated on serine and tyrosine (Saez and Young, unpublished). It is not known at this time what the exact sites of phosphorylation are.

4. The predominant secondary structure in this region is the alpha helix. Chou-Fasman (Chou and Fasman, 1974) and Garnier (Biou et.al., 1988) analyses both predict, with striking similarity, an alpha helix which breaks at I595 (see Figure 9). The alpha helix then would include the amino acid altered by *per*^S (S589N). The programs are in less agreement as to the secondary structure which preceeds and follows the alpha helix. The predicted alpha helix could define a region of unitary function in *per*. In addition, mutations in this region may reveal that the secondary structure rather than the particular amino acid sequence is the important aspect of this region's activity.

By employing random mutagenesis (Ner et.al., 1988; Hubner et.al., 1988; experimental procedures), a wide variety of changes were produced in the *per*^S region. Figure 9 lists the initial six alterations which were introduced into *per*⁰ flies via P-element mediated transformation. The changes listed affect not only the amino acid that was altered by the original *per*^S mutation but also amino acids on

Figure 9. Mutagenesis in the *per^s* region.

The initial six mutants created in the *per^s* region which were subsequently transformed into *y per⁰; ry⁴²* flies are listed. The wild-type amino acid sequence for the 24 amino acids surrounding the site of the *per^s* mutation is shown (derived from Jackson, et.al. 1986). Highlighted is serine 589 which is mutated to asparagine to give the *per^s* phenotype. The asparagine (asn) is shown above that serine and is labelled **per^s**. The broken bar line indicates the α -helical secondary structure of this region as determined by Chou-Fasman (Chou and Fasman, 1974) and Garnier (Biou et.al., 1988) (also see text). The alpha helix continues in the N- terminal direction for another 29 aa (to approximately arginine 552). Astericks indicate potential sites for phosphorylation. Mutants listed in the column on the left hand side of the figure have been named as follows: (Single letter amino acid code is used.) The first letter is the wild-type amino acid, the number following indicates its position in the per protein, and the subsequent letter is the amino acid derived from the mutagenesis. For example, in the first mutant, G593S, glycine (G) at position 593 is changed to a serine (S).

pers region -- Mutagenesis I

amino acids 581-604

pers

* asn * *

581 glu leu thr val ser glu arg asp ser val met leu gly glu ile ser pro his his asp tyr tyr asp ser604

G593S
D588V
G593C
D588F
S589L
E586V,V590D

val phe leu asp

val ser

cys

* possible phosphorylation site
— alpha helix (aa 522-595)

either side of the original change. In addition, the types of substitutions made reflect a range in size and charge alterations. As shown in Table 7, substitutions in size span from practically no difference (the glycine to serine change G593S) to large size changes (serine to leucine, S589L). Likewise, a charged amino acid was exchanged for a neutral amino acid (D588V) as well as vice versa (V590D). A mutant having two changes (E586V, V590D) also was produced. In addition to switching the charge structure of this area, these two changes in one mutant may cause greater damage, akin to a deletion of this region. Table 5 also records the predicted alterations to secondary structure of the region. Some substitutions have no effect on the helicity of the region (S589L) whereas others break the helix at an earlier position (D588V). Lastly, potential modification sites which are altered are listed. In sum, the wide variety of amino acid substitutions could result potentially in a spectrum of phenotypic responses. This would allow an initial assay of the *per^S* region.

Transgenic flies were made according to the procedure outlined for the controls. Depending on the construct, two to ten independent lines were maintained. Flies heterozygous for the transforming DNA were tested by the behavioral locomotor assay

Table 7. Mutations in the *pers* region.

<u>mutant</u>	<u>size</u>	<u>charge</u>	<u>α-helix</u>	<u>other changes</u>	<u>period length \pm S.E.</u>
S589N*	same	same	no change	lose potential modification site	22.1 \pm 0.5*
G593S	same	same	+1 aa	--	21.3 \pm 0.8
G593C	larger	same	+1 aa	possible disulfide bond formed	30.7 \pm 1.0
D588V	larger	-1 to 0	-5 aa	--	22.5 \pm 0.4
D588F	larger	-1 to 0	-2 aa	--	24.3 \pm 0.6
S589L	larger	same	no change	lose potential modification site	22.1 \pm 0.5
E586V + V590D	larger smaller	-1 to 0 0 to -1	-6 aa	double mutation	22.7 \pm 0.6

Table shows the effect of amino acid changes engineered into the *per* gene on various properties of the *pers* region. * denotes the original *pers* mutation. For this chart, the period length of *pers* was measured in lines transformed with the original *pers* gene. Size and charge indicate the size and charge of the new amino acid relative to the wildtype amino acid it replaced. α -helix, refers to disruptions in the size of the α -helical region that encompasses the *pers* region.

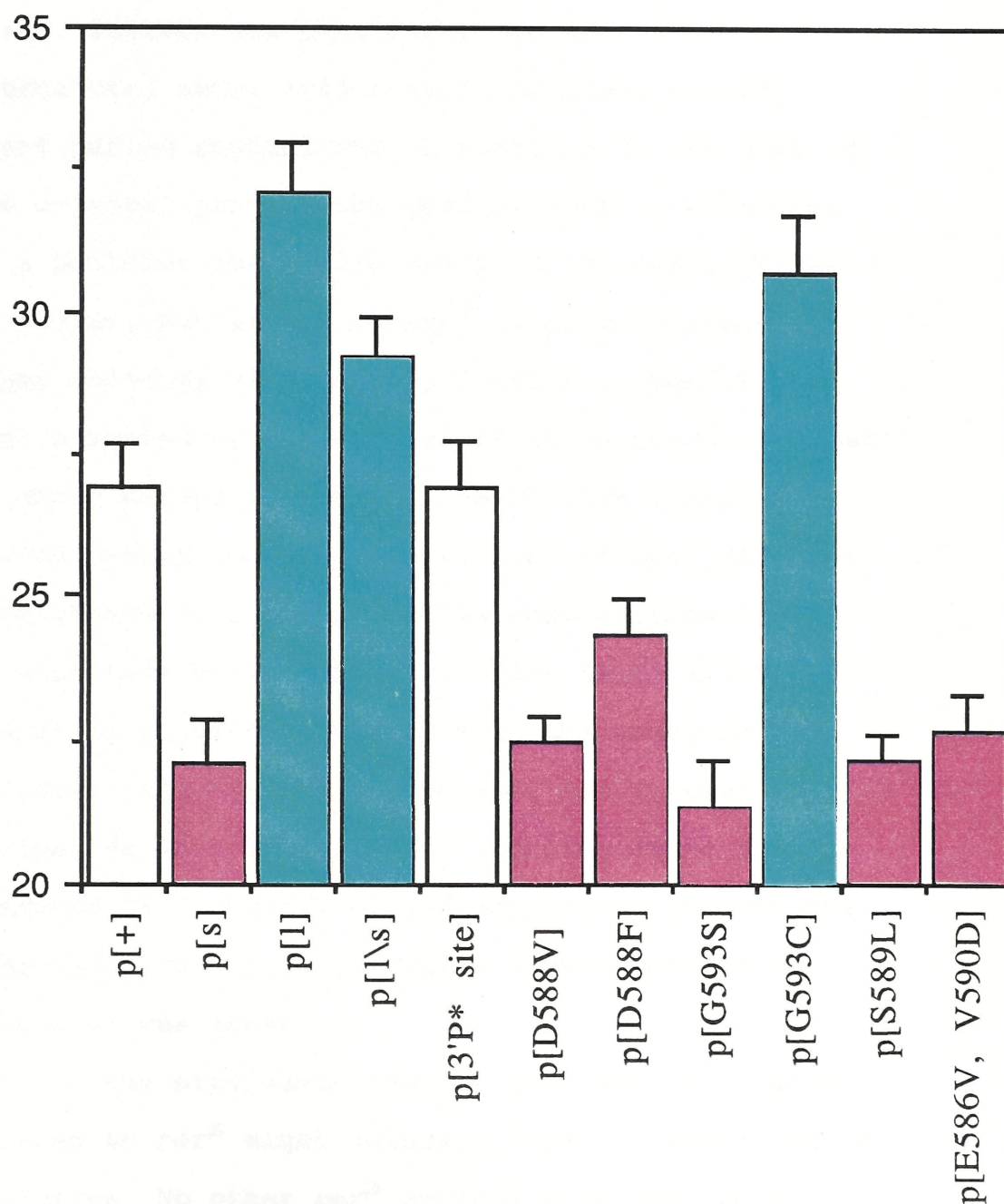
(experimental procedures). Table 5 lists the mean period length or $\tau \pm$ the standard error for all the mutants described above. The control information is included for comparison. In addition, the period length data can be plotted in the form of a bar graph to easily visualize the results (Figure 10).

All of the amino acid changes in this region with the exception of one resulted in short period phenotypes. The only mutant which did not give a short period phenotype in this region was G593C (see Table 5 and Figure 10). This glycine-to-cysteine change resulted in a long period phenotype. Given that disulfide bonds have a role in determining the tertiary structure of proteins (Schulz and Schirmer, 1979), this additional cysteine perhaps generates a new, less stable or less active conformation of the *per* protein. A long period length would be the phenotypic result. In addition, when this same glycine is changed to a serine it results in a short period rhythm (G593S, see Table 5). Other mutations (Tables 5 and 7,) which cause large size changes do not cause long period phenotypes. Therefore, it is the specific change to the amino acid cysteine which is responsible for the long period phenotype. An additional mutation in this region (D588N,S589C) which alters a serine to a cysteine is now being analyzed and may shed more light on this question.

Figure 10. Period lengths of heterozygous transformed lines

Mean period length \pm standard error (S.E.) for each of the constructs detailed in Table 5 is plotted. Transformants having a similar period length to that of one of the controls (the first three bars) are shown in the same color as the corresponding control. Note that for the mutants made in the *per^s* region (the last six bars) nearly all the constructs gave short period lengths.

Transformant Period Analysis



Most interesting, however, was that the majority of the changes (five out of the six) in this region resulted in short period phenotypes. The double change and the four remaining single changes gave short period lengths (see Figure 10, last six bars). Neither the particular position nor the substituted amino acid seem to uniquely specify short period rhythmicity. A position to the left of the original *per^S* change gave as short a phenotype as a position four amino acids to the right (D588V vs G593S). Particular changes in size, charge, or alpha helicity were not predicative in the sense that practically all classes of amino acids resulted in short period phenotypes. D588F gave a short period length intermediate to that of *p[s]* and *p[+]*. This change to a phenylalanine must somehow result in a protein whose activity, while still short, is nearer to wildtype activity than the other changes produced. However, this same position changed to valine, an equally large and nonpolar amino acid, resulted in a shorter period phenotype. In sum, the overriding phenotype generated from mutations in this area was short.

It was previously thought that the amino acid altered by *per^S* might uniquely confer a short period phenotype. No other *per^S* mutants were uncovered in subsequent EMS screens whereas other *per* alleles

Table 8. Period lengths of homozygous transformed lines.

<u>genotype</u>	<u>tau</u>	<u>S.E.</u>	<u>range (tau \pm S.D.)</u>	<u>N</u>	<u>n</u>
p[+]	25.0	0.7	24.1 \pm 0.5 - 26.1 \pm 0.2	6	20
p[s]	20.6	0.6	20.3 \pm 0.4 - 21.0 \pm 0.8	5	27
p[l]	30.7	0.4	28.9 \pm 0.3 - 31.7 \pm 0.1	5	20
p[l\s]	26.7	0.8	26.4 \pm 0.4 - 27.2 \pm 0.6	3	10
p[3'P*site]	N.D.				
p[D588V]	N.D.				
p[D588F]	23.0	0.6		1	5
p[G593S]	20.4	0.2	20.1 \pm 0.4 - 20.7 \pm 0.1	2	12
p[G593C]	28.9	0.2	28.5 \pm 0.2 - 29.6 \pm 0.2	3	14
p[S589L]	20.4	0.7	19.9 \pm 0.1 - 20.9 \pm 1.0	2	10
p[E586V, V590D]	21.9	0.5	21.7 \pm 0.5 - 22.0 \pm 0.9	2	10

tau, refers to period length; S.E., standard error (Stickberger, 1968); S.D., standard deviation; (Stickberger, 1968) N, number of independent lines tested; n, total number of flies tested.

giving per^L and per^O -like behaviors have been uncovered (reviewed in Hall and Kyriacou, 1990). Moreover, given preliminary evidence that per protein can be phosphorylated, one could easily speculate how a post-translational modification of this serine affected by per^S could result in short period phenotypes (Hall and Kyriacou, 1990). However, the mutagenesis studies contradict these assumptions: a number of amino acids instead of a particular single amino acid generates short period rhythms.

The results from locomotor studies performed with flies which are homozygous for each particular construct are shown in Table 8. As predicted from the RNA titre experiment discussed previously and gene dosage experiments (Smith and Konopka, 1982), the period lengths were shortened by doubling the gene dosage in every case tested by approximately one to two hours. Thus, per^S/per^+ is not as short as per^S/per^S for each new per^S allele.

iv) Eclosion analysis of the new per mutants

The original per^L , per^O , and per^S mutations were discovered by virtue of their distinct and dramatic alterations in eclosion behavior (Konopka and Benzer, 1971). The same effects were later noted for the locomotor behavior, that is per^S shortens

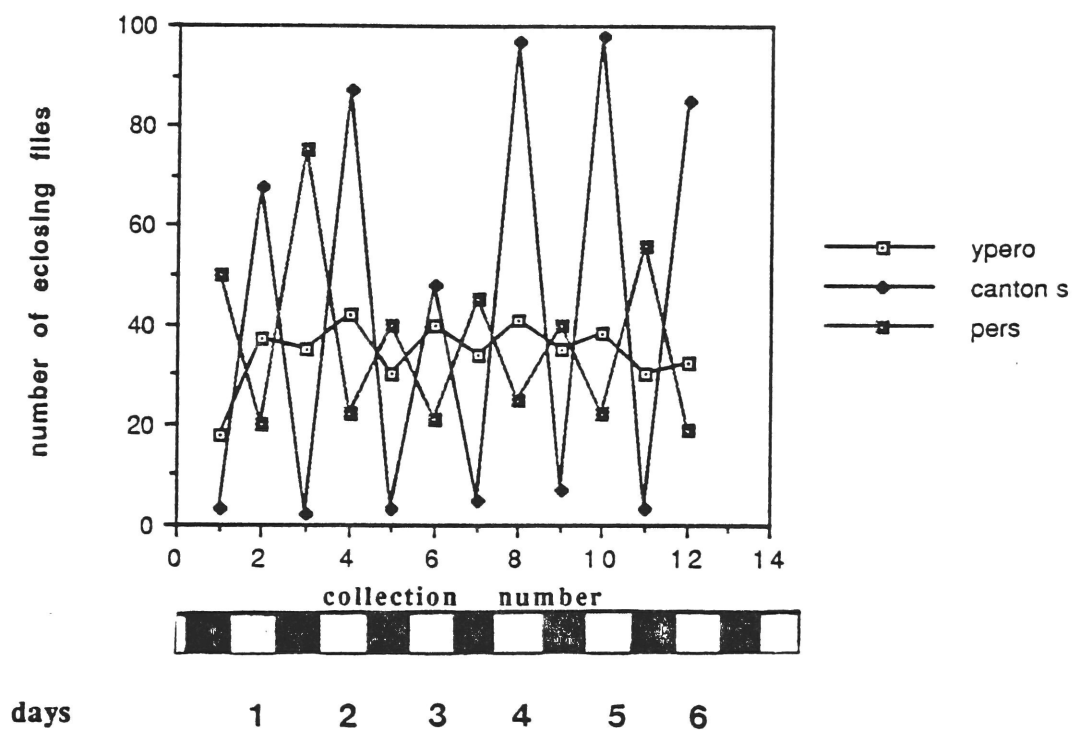
the rhythm to 19h, per^L lengthens the rhythm to 28h, and per^O abolishes this rhythm. It was therefore predicted that the new per mutants should behave in the eclosion assay as they did in the locomotor assay.

Instead of an around-the-clock eclosion assay, a diagnostic, two point eclosion assay was performed on the majority of new mutants (Konopka and Benzer, 1971; Jackson, 1983; experimental procedures). This assay can clearly distinguish between per^O , per^S and per^+ flies. The eclosion profiles for the controls, per^S , per^O , and per^+ are shown in Figure 11. In these experiments, flies are kept on a 12h Light: 12h Dark cycle. Collections of newly emerged flies are made twice a day: 45' before lights on (odd numbered collections) and 2-3h before lights off (even numbered collections). per^S flies are considered "night emergers" as they tend to emerge from the pupal cases before lights on. The peak of eclosion for per^S flies, then, should be in the first collection of each day (the odd numbered collections). per^+ flies are "day emergers" as they tend to eclose after lights on; therefore, the peak of emergence should be in the second collection of each day (the even numbered collections). per^O flies show no preference for day or night emergence. Likewise, in this two point assay, per^L flies emerge

Figure 11. Two Point Eclosion Experiment -- Controls

Eclosion profiles from a two point experiment are shown for *per⁰*, *per^s*, and Canton S (*per⁺*). In this experiment, flies are continuously exposed to a 12 Light : 12 Dark cycle. Emerging flies are collected at two points in the 24 hour cycle-- 45 minutes before lights on, and 2-3 hours before lights off. The black and white bar shows lights off and lights on, respectively. The day is indicated below the bar. Odd numbered collections occur during lights off; even numbered collections occur during lights on. The daily peak of emergence for the *per^s* population is in the odd numbered collection of each day; therefore, *per^s* flies are known as "night emergers". The Canton S (*per⁺*) peak of emergence occurs in the even numbered collection of each day; hence *per⁺* flies are described as a "day emergers". *per⁰* flies emerge randomly and as a result are "day/night emergers".

Two Point Eclosion Experiment Controls



both during the day and night; however, they do show greater preference for day emergence. Figures 12a and 12b are examples of the eclosion profiles from 3 of the mutants. Table 9 is a record of the mutants tested and the results. In each case, the new *per* mutant behaves as expected -- the flies which are classified as short in the locomotor analysis also behave as "shorts" in the two point eclosion assay. The long period mutant's (G593C) eclosion profile is shown in Figure 12b. Although its profile is similar to *per*⁺ (preference for day emergence), a significant number of flies emerge at night. This would allow for a preliminary classification as a long period mutant in the absence of other supporting evidence. Note that the profile is distinctly different from the short period mutant.

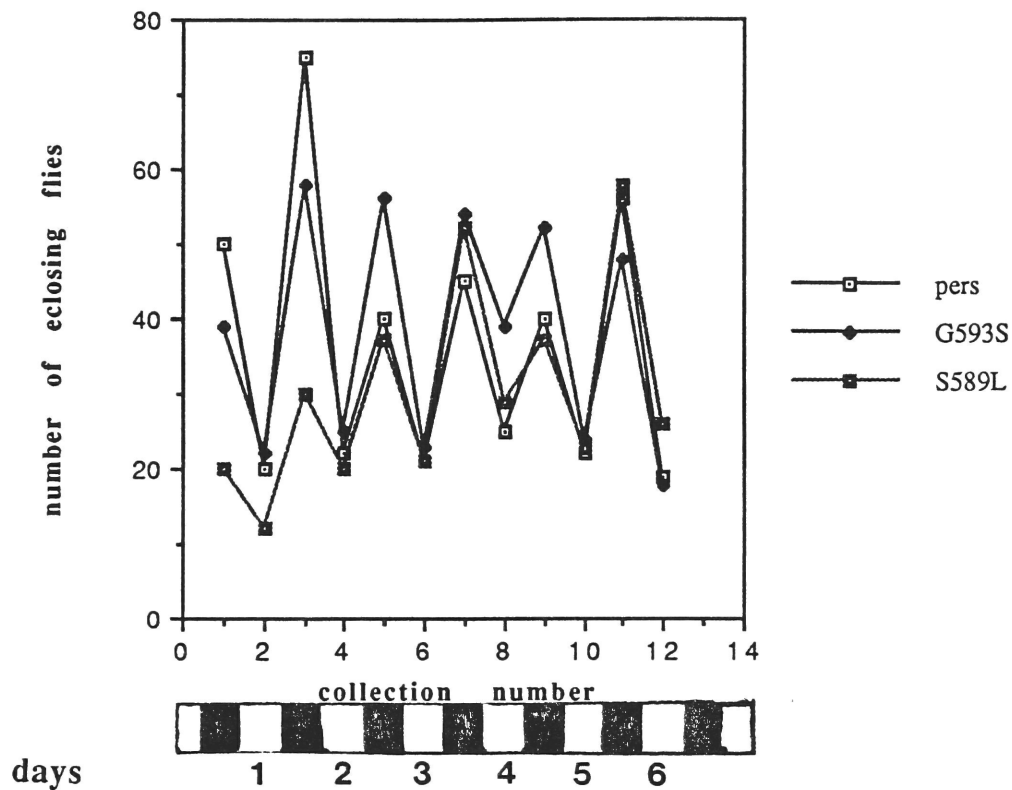
v) Further mutagenesis in the *per*^S region

The results from the mutations made in the *per*^S region indicate that a number of amino acids can be changed to give a short period length. This would seem to indicate that a domain in which short period phenotypes predominate exists. To investigate and define the limits of this putative domain, another series of mutations were created. An example of the sequence analysis used to confirm the changes is shown in Figure 13. The base changes are noted. The

Figure 12a and b. Two Point Eclosion Study -- New Mutants

Plots 12a and 12b are examples of eclosion profiles from the mutants generated in *per*. The number of eclosing flies is plotted for each collection under the conditions described in Figure 11. In Figure 12a, G593S and S589L both show eclosion profiles similar to *per^s*. This result is predicted from the period length data gathered from locomoter activity records (see Table 5). In the graph shown in Figure 12b, the profile for the mutant G593C is shown. The G593C flies show a greater tendency to emerge after lights on---much unlike that of *per^s* flies. Since a significant number of flies from the G593C line also emerge during the lights off period, G593C would be classified as a long period mutant in this assay. This result also follows from Table 5.

Mutant Eclosion Study -- G593S and S589L



Mutant Eclosion Study -- G593C

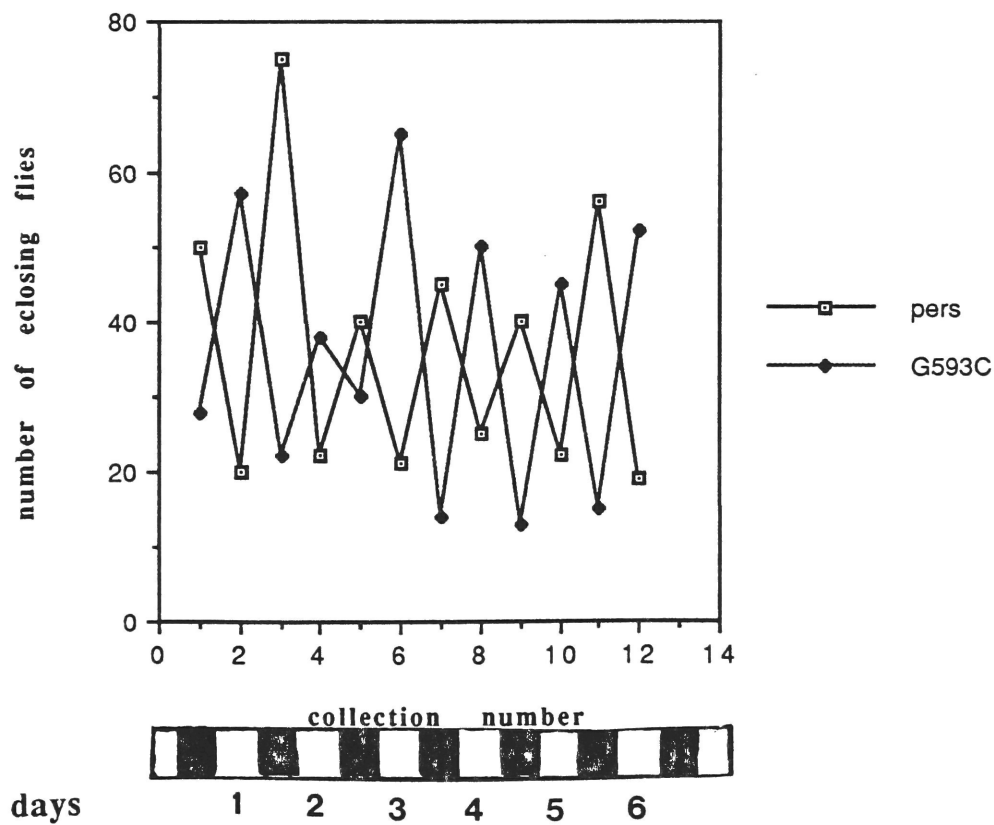


Table 9. Two point eclosion test.

<u>Mutant</u>	<u>Behavior</u>
p[l/s]	day/night emerger
p[G593S]	night emerger
p[G593C]	day/night emerger
p[D588V]	night emerger
p[D588F]	N.D.
p[S589L]	night emerger
p[E586V, V590D]	N.D.
p[3'P*site]	N.D.

Behavior in eclosion test was determined by comparing eclosion profiles to that of *pers*^s, *per*^o, *per*^l and *per*⁺ controls. *pers*^s, night emerger; *per*^l, day/night emerger; *per*⁺, day emerger; *per*^o, day/night emerger; N.D., not determined.

subsequent amino acid alterations made in this round of mutagenesis are shown in Table 10. The amino acid residues highlighted in pink in this table reflect those which were altered and discussed previously. Another thirty-five mutations have been made by random mutagenesis. These new changes, as before, vary in size and charge as well as alter potential phosphorylation sites (highlighted in green) and the alpha helicity of the region (for example, E575G breaks the helix). Multiple changes also have been produced (for example, E586G,D588A,M591L). In all, a span of 40 amino acids in the *per*^S vicinity has been mutated.

A subset of these have been reconstructed with the same construct strategy as the controls and transformed into *per*⁰ flies. These mutants include the two mentioned in the paragraph above as well as L574M; D600E,S604G; and D600V. These experiments are in progress.

Discussion

Early genetic analyses suggested that period length is sensitive to *per* locus dosage (Smith and Konopka, 1982). Decreased dosage of *per*⁺, *per*^S, or *per*^L slightly lengthens period (1/2-1h) for circadian locomotor rhythms. Increased dosage of *per*

Figure 13. New mutations in the *per^s* region.

Further mutations in the *per^s* region were created by random mutagenesis as described (Ner et.al., 1988; Hubner et.al., 1988; see experimental procedures). Mutagenesis was assessed by DNA sequencing (see experimental procedures). This figure shows an example of such an analysis. An interval of DNA sequence from the *per^s* region containing the mutations is shown. For comparison, a wild-type (*per⁺*) sequence is shown. Arrows indicate induced base alterations in the mutants **P577R**, **H578Q** (G to C, G to T); **E581L**, **T583P** (C to G, T to A, T to G); and **L576R**, **H578N** (C to A, A to C, G to T).

per⁺

P577R,
H578Q

E581L L576R
T583P H578N

AGCT AGCT AGCT AGCT

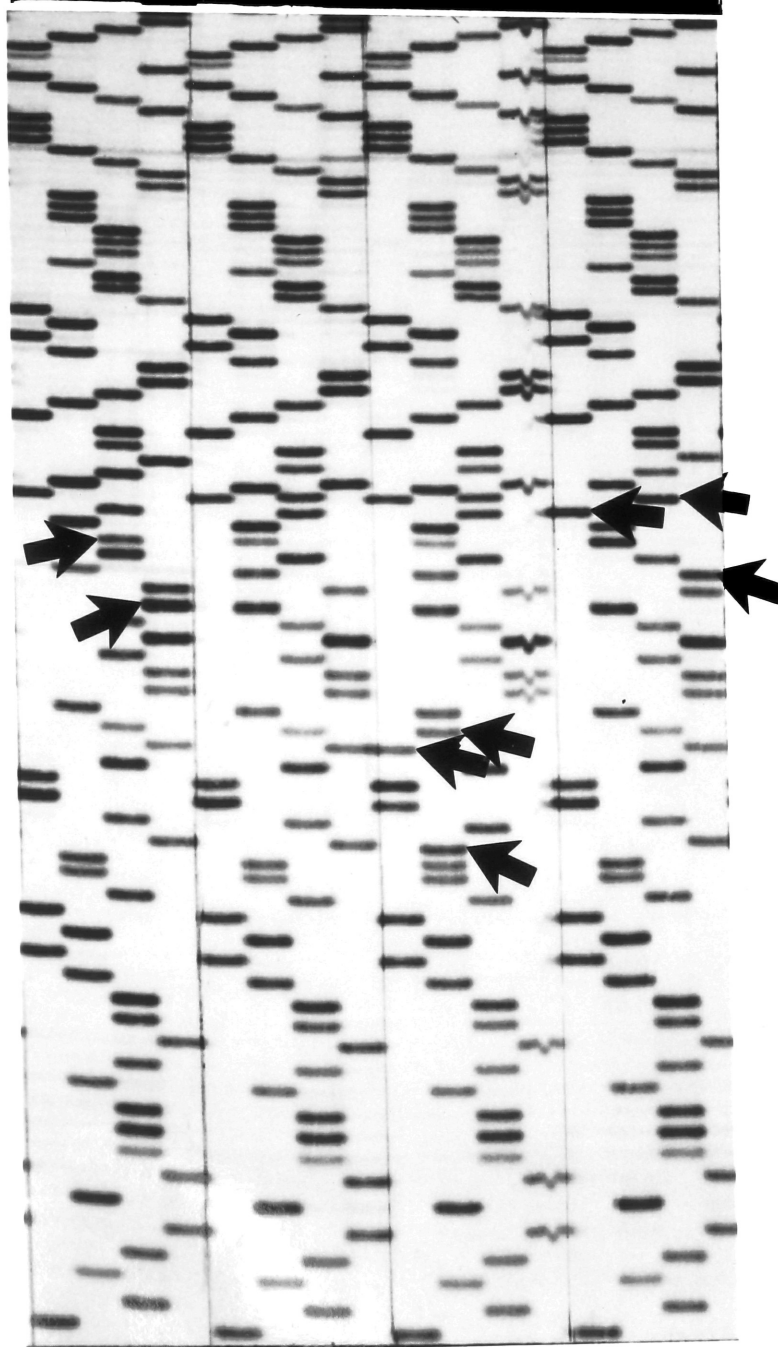


Table 10. Further Mutagenesis in the *per^s* region

The sequence of the 40 amino acids in the *per^s* region are listed horizontally across the table (derived from Jackson, et.al. 1986). Amino acids mutated and discussed previously in this section are highlighted in red. Potential phosphorylation sites are highlighted in green. The second serine (from the left) highlighted in green is changed in the original *per^s* mutant to asparagine. These mutants were all generated by *in vitro* random mutagenesis and sequenced to verify these changes (see experimental procedures and examples in Figure 13).

pers region -- Mutagenesis II

[illegible]

(three or more copies) shortens period length by a maximum of 1.5h. Our results strengthen and extend these observations; changes in the level of *per* RNA correlate with changes in period length. The lower the RNA titre, the longer the resultant period. The dose response curve indicates that the period varies according to the log of the RNA titre. While decreasing the levels of *per*⁺ RNA below the wild-type threshold has a dramatic effect on period length (a twenty fold difference almost doubles the period length), the period is relatively insensitive to overexpression of *per*⁺ RNA. Extrapolating from the level for *per*⁺ RNA, an estimated 10 fold overproduction of the wild-type transcript would only shorten period length by five hours (see Figure 4).

The analysis of Konopka's three *per* alleles indicates that the structure of the *per* protein can also fix period length. In light of the titre experiment, the single amino acid change associated with the *per*^S mutation results in a protein with higher activity. The *per*^L associated amino acid alteration leads to a protein with lower activity. The significance of these particular amino acid changes is under investigation. The position of the *per*^L change lies within a region homologous to the *Drosophila single-minded (sim)* protein (Crews

et.al., 1988) and the mouse aryl hydrocarbon (dioxin) receptor transporter (*arnt*) protein (Hoffman et.al., 1988). The functions of *sim* and *arnt* are currently unknown. Recent data indicate that *sim* may be acting as a transcriptional activator of genes required for the development of the central nervous system (Nambu et.al., 1990). The *arnt* protein may be involved in translocating the ligand-receptor complex to the nucleus (Hoffman et.al., 1991). Further study is needed to identify how this region of homology is functioning in *sim* or *arnt* and if that function is playing the same role in *per*. Given the homology between *sim*, *arnt* and *per*, it would be interesting to replace the *per* domain, for example, with the *sim* homologous domain (and vice versa) and ask if wildtype *per* (and *sim*) function could be obtained. Possibilities for the *per*^S change include being a site for post-translational modification. Further mutagenesis of *per*^S has been completed, and the implications will be discussed below.

The nonsense change associated with *per*⁰ mutation leads to a truncated protein. Behavioral studies imply that this *per*⁰ protein is dysfunctional; the arrhythmia found in free-running conditions is the same in *per*⁻ as well as *per*⁰ individuals (Smith and Konopka, 1981; Bargiello and

Young, 1984). Moreover, the behavior of the *per*⁻ and *per*⁰ tissues (see next section) in a cellular assay is the same.

To address the nature of the activity of *per* protein and how the single amino acid changes in *per*^S and *per*^L have such a dramatic effect on it, a new series of mutations were made. These provide additional information about the *per* protein function and regulation.

Potential sites for post-translational modification of the *per* protein

Phosphorylation of the *per* protein may regulate the activity of *per* function. Cell culture and *in vitro* biochemical studies of the *per* protein indicate that it is phosphorylated on serine and tyrosine (Saez and Young, unpublished). The particular serines and tyrosines which are phosphorylated are not known at this time; however many potential sites for both tyrosine and serine/threonine kinases exist in the predicted *per* sequence.

Since increased levels of cAMP can lead to alterations in clock behavior in other systems (Eskin et.al., 1982; Eskin and Takahashi, 1983; Jacklet, 1989b), one possible aspect of cAMP-

mediated regulation of *per* was investigated. A potential target for cyclic AMP-dependent protein kinase is a sequence located at the C-terminus of the *per* protein (amino acids 1196-1207). This site has homology to a known phosphorylation site in troponin I of rat skeletal muscle (Bramson et.al., 1984) As stated in the results, a synthetic peptide having this *per* sequence is phosphorylated *in vitro*. However, transformant flies carrying the deleted site (p[3'P*site]) have perfectly wild-type period lengths, indicating that phosphorylation of this site does not modulate *per* activity in circadian behaviors.

Phosphorylation by protein kinase C may also serve to regulate *per* activity. Possible substrates for protein kinase C phosphorylation are found in the *per^S* region (Woodgett et.al., 1986) . In particular, one potential site is the serine altered by *per^S* (S589). By changing this serine to either asparagine or leucine, a short period phenotype results. The short period length could be the result of the loss of phosphorylation of this serine. Biochemical data are necessary to show that this particular serine is phosphorylated. If this post-translational modification of the serine is required for wild-type *per* function, transgenic flies carrying a *per* construct with this serine changed to

threonine, another amino acid capable of being phosphorylated by the same kinases, would be predicted to have wild-type rhythms.

Another serine in this region (S585) could also serve as a target for protein kinase C. Mutants have been created which eliminate this target (i.e. changing that serine to alanine); however, the period data is not available at this time.

A known visual mutant, *norpA* (*no-receptor potential A*) has been shown to encode a gene for phospholipase C (Bloomquist et.al., 1988). This enzyme is a key player in the signal transduction cascade which results in the activation of protein kinase C (Berridge, 1986; Berridge and Irvine, 1989; Majerus and Ross, 1990). It is interesting to note that the *norp A* mutant is also a clock mutant having a short (22h) period length (Dushay et.al., 1989). Could this mutant then, result in loss of protein kinase C activation and a subsequent loss of phosphorylation in the *per^S* region? This loss of phosphorylation could, in turn, lead to higher activity of the *per* protein and, therefore, to shorter period lengths.

A consensus sequence for tyrosine kinase (Cooper et.al., 1984) also exists in the *per^S* region, and as with the other sites in the *per^S* region, is highly conserved among distantly related

Drosophila species. Mutants have been created which destroy this site. Analysis of these lines is presently underway. A combination of mutagenesis and biochemistry will give more insight into the regulation of the *per* protein function. This, in turn, will provide more information about the workings of a biological clock.

Identification of a *per*^S domain and the paradox about short period phenotypes

The mutational analysis of the *per*^S region indicates that a domain of function exists in which short period phenotypes predominate. Any of a number of amino acids can be changed to a variety of other amino acids and still generate a short period length. Amino acid replacements in size, charge, or secondary structure all lead to short period phenotypes. This apparent mutability with the same resultant phenotype would seem to indicate that the short period phenotype is a consequence of a loss rather than a gain of function.

This outcome, however, would appear to produce a conflict, because previous experiments have shown that increasing the abundance of the *per* product shortens period length (Smith and Konopka, 1982; Cote and Brody, 1986; above). This observation led

us to conclude that the original *per^S* mutant was a "gain-of-function" mutant or hypermorph.

The paradox of the short period phenotype can be solved by intergrating the two opposing views. The mutagenesis study would be consistent with a loss of function in this particular domain. Due to the loss of function in this domain, the overall activity of the protein is greater. The *per^S* mutant, therefore, appears like a "gain-of-function" mutant. The increase in overall activity of the *per* protein could be achieved in two ways:

- 1) increase the specific activity of the *per* protein. In the wildtype protein the *per^S* region could be responsible for restraining the activity of *per*. Mutations in this region would lead to the loss of this restraint and thereby a *per* protein with greater specific activity. By analogy to enzyme biochemistry, the rate at which *per* functions would be greater.

- 2) increase the stability of the *per* protein. Much in the spirit of PEST sequences which mark proteins for rapid turnover (Rogers et.al., 1986) this region in the *per* protein may be responsible for determining the half-life of the *per* protein. Mutating this region disrupts the cues necessary for determing the normal half-life of the protein and as a result, the *per* protein would be

more stable. Although the mutated *per* protein would function at the same rate as the wildtype *per* protein, the overall amount of the protein is greater and hence, its activity.

Modulation of *per* function by the *per*^S domain

Modulation of overall *per* function by increasing either specific activity or stability can be accomplished through post-translational modification including glycosylation (Jackson and Tijan, 1988), myristoylation (Cross et.al., 1985; Kamps et.al., 1985), and phosphorylation (Prives, 1990). As examples, release of synaptic vesicles at the nerve terminal is due in part to the proper phosphorylation state of synapsin (DeCamilli et.al., 1990), and EGF receptor loss at the membrane surface can be triggered by the phosphorylation of a particular threonine in the EGF receptor (Lin et.al., 1986). By analogy, proper phosphorylation of the *per*^S region (perhaps the site or sites discussed previously) may lead to wild-type levels of activity or stability of the *per* protein and hence a 24h clock. Mutations in this site would lead to a loss of restraint in this region and therefore greater activity of the *per* protein. In turn, shorter period lengths would result. This model of *per* regulation

would predict that the *per^S* region in the wild-type protein would be phosphorylated. In the short period mutants, this region would either have no phosphorylation or a different pattern of phosphorylation. Phosphopeptide analysis of the wild-type and *per^S* proteins will reveal these changes, if they exist.

Another manner of regulation would involve the interaction between two domains of the protein. For example, protein kinase C has a pseudosubstrate domain which interacts with the catalytic site of the kinase, thereby preventing kinase activity. Only upon the proper phosphorylation of the kinase itself is this repression of activity relieved. Mutations in the pseudosubstrate region or its binding site in the catalytic domain lead to constitutive kinase activity (Hardie, 1988; Soderling, 1990). If the *per^S* domain were acting as a pseudosubstrate, then mutations in this region would lead to a constitutively active or more stable protein (as it seems it does). However, this model also predicts that another region of the *per* protein, when mutated, for example, to disrupt the interaction between *per^S* and itself, should result in short period phenotypes.

A third example of regulation involves the interaction of another protein. GAL4 is a

transcriptional activator of genes required for utilizing galactose in yeast (Johnston, 1987). In the absence of galactose, GAL80, which does not bind DNA, inhibits the activity of GAL4 (Lue et.al., 1987). Further studies have indicated that GAL80 forms a complex with GAL4 so that, although still bound to DNA, GAL4 can not activate transcription. The activating surfaces of GAL4 are masked by the binding of GAL80 (Ma and Ptashne, 1987; Johnston et.al., 1987). If a variation on this theme were happening for *per*, a protein could bind to the *per^S* region thereby modulating *per* specific activity or stability to a level which would result in a 24h period length. In short period mutants, this protein could not bind to the *per^S* domain. Therefore, higher than normal levels of *per* protein activity would result in shorter than normal period lengths. Using *Drosophila* genetics, the predicted interacting protein could be isolated by looking for second site suppressors of *per^S*.

The actual mechanism of modulation by the *per^S* region by *per* need not be as clear cut as presented above. A combination of two or all three mechanisms has precedence. For example, the tyrosine kinase activity of pp60^{C-src} is regulated by phosphorylation as well as intramolecular interactions. pp60^{C-src} can be phosphorylated on

tyrosine-527. If phosphorylation occurs, this phosphotyrosine will associate with the SH-2 domain of the same pp60^{C-src} to form a structure with low protein-tyrosine kinase activity (Matsuda et.al., 1990). If the phosphorylation site is missing (as it is in pp60^{V-src}) (Cartwright et.al., 1987) or if the SH-2 region is mutated (Matsuda et.al., 1990), higher levels of pp60^{C-src} kinase activity results.

In the case of the retinoblastoma gene product (pRB), phosphorylation and intermolecular protein-protein interaction regulate its function. Activation of pRB's growth suppressor function occurs by dephosphorylation in a cell cycle dependent manner (Buchovich et.al., 1989; Chen et.al., 1989). However, this modulation can be perturbed by the transforming proteins of several DNA tumor viruses including SV40 large T antigen. It has been demonstrated that large T antigen binds exclusively to the dephosphorylated form of pRB, thereby causing unregulated growth of the infected cells (Ludlow et.al., 1990). By analogy, perhaps the phosphorylation state of the *per*^S region may govern the ability of another region of the *per* protein or another protein to interact with it. This two step process, then, would result in the regulation of *per* activity.

These models offer a convenient way to imagine how the *per^S* domain is exerting its control on the *per* protein. Although we are still left not knowing the exact function of the *per* protein and how this activity controls biological clocks, more clues about the *per* protein were obtained. By taking a reductionist's approach, specific regions (*per^S* and *per^L*) of the protein have been highlighted as functionally significant. Other possible regulatory sites have been dismissed. And finally, the regulation of *per* activity has been explored. The initial skeleton of information provided here will need to be fleshed out before further speculations can be made. In the next section, Part II, the cellular mechanism by which *per* acts will be explored.

PART II -- CELL LEVEL ASSAY OF THE PER GENE PRODUCTS

INTRODUCTION

The effects of the *per* mutations on both circadian and ultradian rhythms have been well documented (for review, Hall and Kyriacou, 1990). In addition, these mutations have been reported to affect several other phenotypes. Some of these seem related to "clock" phenomena such as alterations in developmental time (Kyriacou et.al., 1990); others, at least overtly, do not, such as alterations in octopamine synthesis (Livingstone and Tempel, 1983) (see Table 2).

per is found in a number of tissues in the developing fly. For example, *per* products localize to the nervous system, as is predicted from mosaic and transplantation studies (Handler and Konopka, 1979; Konopka et.al., 1983; Hall, 1984). However, *per* is also present in unanticipated places such as the gonads (Saez and Young, 1988), the malpighian tubules (Liu et.al., 1988), and the third instar larvae salivary glands (Bargiello et.al., 1987). The broad tissue distribution of *per* may be a bit surprising, yet the localization of the *per* protein might explain the diversity of phenotypes affected by the mutants.

The pleiotropy and widespread distribution of *per* would seem to indicate that *per* influences the "clock" in a manner that does not involve its product in an intrinsically circadian biochemical process. It seems likely that, whatever the mechanism of its action, *per* is functioning similarly in all the tissue types in which it is present. The specific components of the particular tissue and/or developmental stage which interact with *per* determine the output which we measure. Although these outputs can vary in time scale (one minute to 24 hours), tissue (nervous system versus gonads) and developmental stage (embryo versus adult), *per*'s biochemical function remains the same. To illustrate, cAMP-dependent protein kinase functions to phosphorylate a number of proteins in a number of tissues. However, given the tissue, the effect of the phosphorylation can be quite different. For example, phosphorylation of connexin 43 in cardiac myocytes by this kinase leads to an increase in junctional conductance whereas in sertoli cells, the same phosphorylation event leads to a decrease in junctional conductance (Bennett, 1991). In each case the function of the kinase is the same (and even the substrate is the same), however, given the unique environment afforded by the tissue, the output is different.

Therefore, to investigate the biochemical mechanism by which *per* exerts its effects, a cell-level assay was sought. By focusing on a cellular phenotype versus a behavioral one, further insight into how *per* functions could perhaps be ascertained. This work was done in collaboration with Dr. Ted Bargiello, Dr. David Spray, and Dr. Vito Verselis.

Two observations lead to the analysis of *per* in third instar salivary gland cells. The first was the localization studies done by Dr. Lino Saez. The third instar salivary glands proved to be among the sites of *per* expression (Bargiello et.al., 1987). In addition, the 4.5kb *per* message can be detected by Northern blot analysis of RNA isolated from dissected third instar larval salivary glands (Vosshall and Young, unpublished). The second observation was the work of Rensing and coworkers (Weitzel and Rensing, 1981). This group has reported a rhythmic uptake of a fluorescent dye, 3,3'-dihexyloxacarbocyanine iodide in isolated salivary glands. The periodicity of this rhythm, as measured by the amount of fluorescence accumulated within the cells over time, was approximately 24h and thus, circadian in nature. This type of fluorescent probe is a voltage-dependent, membrane-permeable dye which can serve to measure periodic changes in electrical

potential across cell membranes (Hoffman and Laris, 1974). Hence, the rhythmic accumulation of fluorescence would imply an oscillation in membrane potential of the cells of the salivary gland. Most cells in the wildtype gland behave similarly in these experiments: the period as well as the phase of the uptake were in synchrony.

Interestingly, isolated salivary glands from *per*⁰ larvae showed a different behavior in these experiments. Few cells displayed the circadian rhythmicity of dye uptake seen for the wildtype glands. Moreover, the overall amount of dye concentrated within the individual cells was lower indicating that the fluctuations in membrane potential were not robust (Weitzel and Rensing, 1981). These results could be interpreted to mean that the membrane potentials of each of the cells within the *per*⁰ gland at any one time was different. The synchronization of membrane potential somehow afforded in the wildtype salivary gland was lost in the *per*⁰ gland. Secretory glands are known to coordinate the secretion from the gland through the synchronization of all of the cells of the gland. In the case of the insect and mammalian salivary glands, this coordination is provided by intercellular coupling, mediated by gap junctions. Perhaps the lack of synchrony in the *per*⁰ salivary

glands could be due to a *per*-dependent alteration in cell communication.

RESULTS

A. Establishment of the cell level assay -- the link between per and intercellular communication

Intercellular communication in *per* mutants and wild-type flies was analyzed first by a series of dye coupling experiments. Gap junction channels are able to transport dyes up to a molecular weight of about 1500 - 3000 daltons, thus allowing communication between cells to be traced (Bennett, 1973). The standard fluorescent tracer used in the following experiments was Lucifer Yellow. This dye is small (molecular weight 457.2 daltons) and membrane impermeable; Lucifer Yellow would therefore spread from one cell to another only through a channel such as a gap junction.

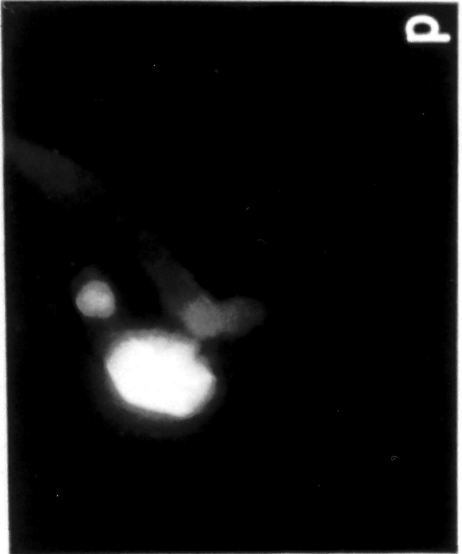
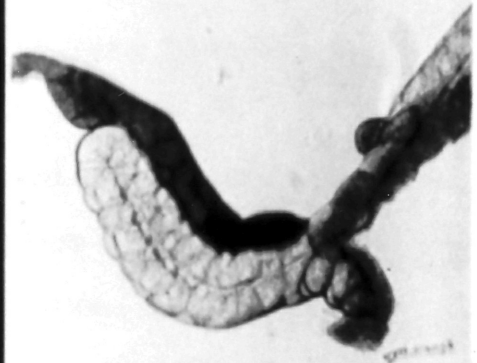
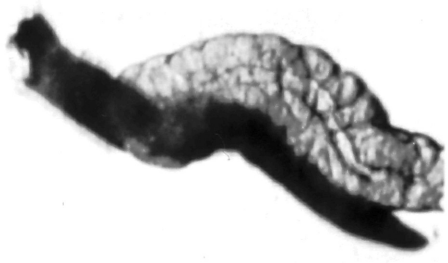
Dye spread was monitored in wildtype (*per*⁺) third instar salivary glands (see Figure 14 legend and experimental procedures for details). As shown in Figure 14, the dye diffuses readily from the site of injection in these wildtype glands. This degree of diffusion is in contrast to that seen in *per*⁰ third instar salivary glands. Nearly all the dye is confined to the cell which was injected in the *per*⁰ gland (Figure 14). The difference seen between the *per*⁰ and the *per*⁺ glands is not a function of the developmental age of the respective glands. Dye

coupling is seen throughout the third as well as the second instar larval stages. In addition, the decrease in dye spread measured in the per^0 glands is not due simply to genetic background. Three different per^0 strains ($per^0 w spl$; $per^0 sn m$; $y per^0, ry^{42}$) all yield the same results in this assay: little or no dye spread. Also, a transformant carrying the wildtype per gene in a $y per^0, ry^{42}$ background (transformant strain P1/25, cf Part I results) showed greater dye coupling than per^0 . This transformant differs from per^0 only by virtue of the transforming DNA. Rescue is obtained in this transformant of not only behavioral rhythmicity but also dye coupling. Moreover, the transformant data further strengthen the idea that the differences in coupling seen in per^0 are not due to genetic background.

In contrast to per^0 glands, cells from salivary glands of per^S strains exhibit a more rapid exchange of Lucifer Yellow than wild-type. The dye spreads to second and third order cells (see figure 14). A continuum of dye coupling has taken form, with per^0 exhibiting little or no coupling, a long period transformant showing more spread, per^+ having even more dye spread, and per^S showing the greatest dye coupling. In addition, these results suggest an inverse relationship between the amount of dye

Figure 14. Dye coupling among the salivary glands of the *per* mutants

Lucifer yellow CH was iontophoresed into an individual salivary gland cell of *per*⁰ (a,b), wild-type (*per*⁺) (c,d), and *pers*^s (e,f). a,c,e are phase contrast micrographs of the injected glands. b,d,f are fluorescence micrographs of the corresponding glands taken after dye was allowed to spread for a period of one minute in each case. Illustrated examples are representative of at least 30 experiments in each group. In the photomicrographs (a,c,e) the black band of cells is the fat body. The lighter group of cells is the salivary gland. Under the conditions used to take the fluorescent micrograph (b,d,f), the fat body is autofluorescent. The *per*⁺ salivary gland illustrated shows two dye injections, with the faint staining on the left being residual dye from an injection 10 minutes previously. Cell sizes are about 40 microns.



spread and period length of the *Drosophila* behavioral rhythms. The shorter the period length, the more the dye spread. The implication is that greater cell communication exists in the short period mutant due to the increased activity of the *per* product.

The results from the dye coupling experiments can be quantified through measurements of electrical coupling by electrophysiological recordings. The strength of electrotonic coupling among the salivary glands of wildtype (*per*⁺) and the *per* mutants was assessed in two ways -- on intact salivary glands and between isolated cell pairs. The results of these experiments with *per*⁰, *per*⁺, and *per*^S glands and cell pairs are described in Figures 15 and 16 and summarized in Table 11. In each case, the differences between the mutants and between the mutants and wildtype were significant.

Table 11 lists the various parameters of electrotonic coupling calculated from these experiments. The coupling coefficient (*k*) is a measure of communication or synaptic effectiveness which has a range from 0 (very low, inefficient coupling) to 1 (very high, quite efficient coupling) (Spray et.al., 1985, Jaslove and Brink, 1987). Salivary gland cells from *per*⁰ larvae have a low *k* value (0.14 ± 0.03) indicative of reduced current

Table 11. Strength of electrical coupling between salivary gland cells and *per* mutants.

<u>Parameter</u>	Fly genotype		
	<u><i>per</i>^o</u>	<u><i>per</i>⁺</u>	<u><i>per</i>^s</u>
Coupling coefficient (κ)	0.14 \pm 0.03 (38)	0.44 \pm 0.04 (25)	0.77 \pm 0.03 (22)
Apparent space constant (λ')	32 \pm 15 μ m (19)	120 \pm 40 μ m (14)	420 \pm 100 μ m (14)
Junctional conductance (g_j)	0.17 \pm 0.05 μ S *	1.57 \pm 0.7 μ S (6)	10.3 \pm 4.2 μ S (4)

Values are given as mean + s.e.m., with the number of determinations, each performed on a separate salivary gland, given in parentheses.

Coupling coefficients were determined with current and voltage electrodes in each of two adjacent cells (1 and 2) in salivary glands.

The apparent space constants were determined in the same way except that separate current and voltage electrodes were placed in one distal gland cell and voltages were recorded in that cell and two others serially adjacent to it (see Figure 14). Apparent space constant was obtained for each experiment from plots of electrotonic decay as a function of cell number (Caveney and Blennerhassett, 1980, Van Venrooij, et.al. 1974 ,see Figure 15). Cells were assumed to be similar in size (40 μ m) and isopotential in all experiments (Van Venrooij, et.al. 1974).

Junctional conductance was determined on dissociated cell pairs (see experimental procedures) with current and voltage electrodes in each cell; g_j values were calculated from input and transfer resistances obtained in response to current pulses passed alternately in the two cells (Spray, et.al. 1985, Bennett, 1966).

*Because values for *per*^o (n=4) and *per*⁻ (n=7) were similar, they were combined in calculations of g_j .

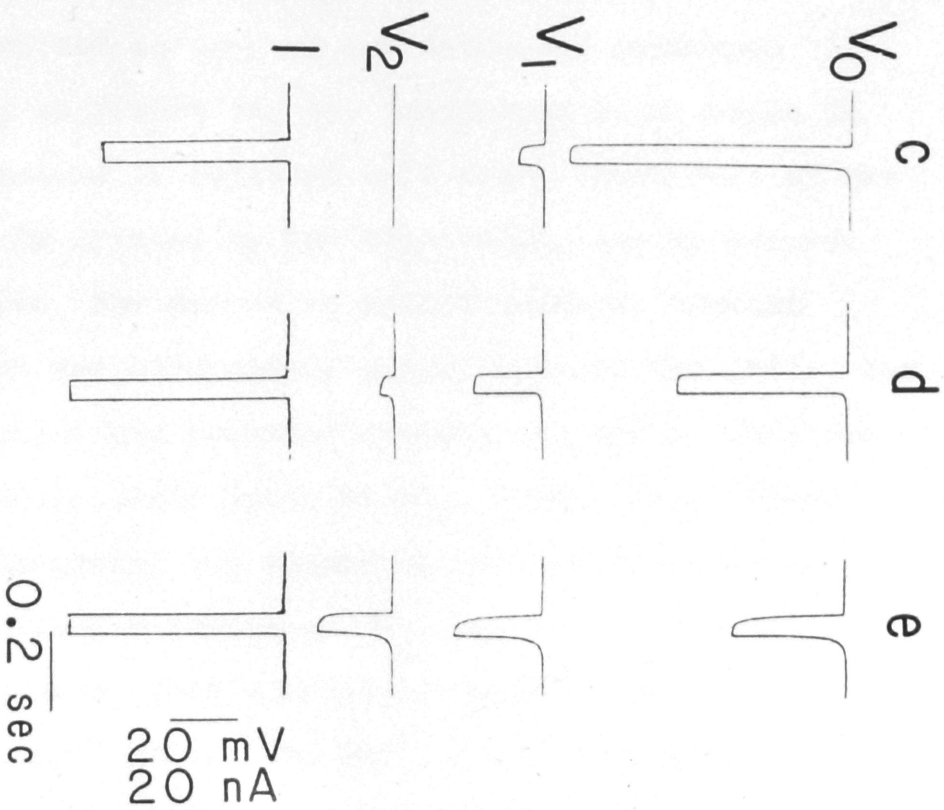
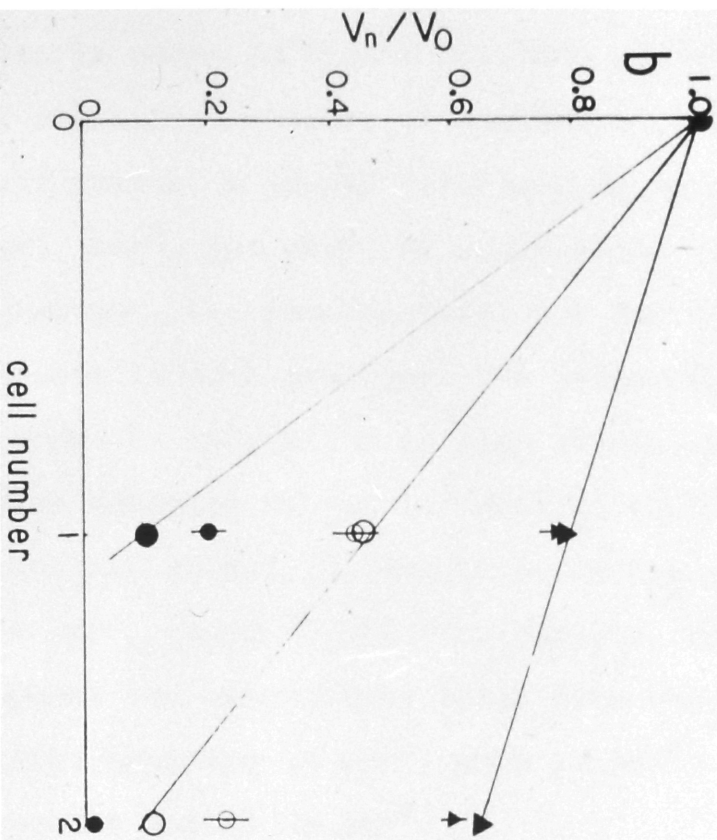
transfer due to poor coupling. An intermediate coupling coefficient (0.3 ± 0.05 , $n=7$) was calculated for the transformed line $y\ per^0, ry^{42}$, P1/25. Recall that this transformed line show period lengths intermediate between per^0 and per^+ and differs from per^0 only by the addition of the transforming per DNA. per^S shows the highest value for the coupling coefficient (0.77 ± 0.03) as would be predicted from the dye spread experiments. For comparison, one of the highest k values calculated is 0.95 at the earthworm septal junction. This high value is due to myelination of the nonjunctional membrane and therefore leads to very little current loss in the circuit (Brink and Barr, 1977) . An example of a typical coupling coefficient for invertebrate neurons is about 0.45 (Johnston and Ramon, 1981; Jaslove and Brink, 1987).

The apparent space constant, another attribute of electrotonic coupling, was measured for all the genotypes. This parameter assesses the extent of current transfer from a selected cell to neighboring cells (see Figure 15 and Van Venrooij et.al., 1974; Caveney and Blennerhassett, 1980). The premise underlying these experiments is that a current encountering little resistance, on account of increased communication between cells, will be able to spread to cells farther away. An example of this

Figure 15. Electrotonic spread within the salivary glands in the *per* mutants.

a: Arrows show positions for current injection and measurement of electrotonic decay. Two electrodes in cell at farthest right allow for current delivery (I) and potential measurement (V_0). One electrode in each of the remaining two cells allows for measurement of electronic spread (V_1 , V_2).

b: Voltages in the three linearly arrayed cells due to current injected into the first (most right hand) of the cells illustrated in A are plotted semilogarithmically as a function of cell number for representative *per*⁰ (●), wild-type (○), and *per*^s (▲) glands. Sample records from which these values were obtained appear in c, d, and e (*per*⁰, wild-type, *per*^s, respectively). Each panel is read vertically. Injected current (I) is shown at the bottom of each panel. Voltage changes in the first (V_1) and second (V_2) cells of the linear array due to current injection (I) are shown. Data from these types of experiments are plotted as in b. In b, small symbols with error bars represent mean and s.e for experiments from which apparent space constant was determined (see Table 11 for n); large symbols represent data in c-e. Values for apparent space constants obtained from a number of such experiments are given in Table 11.

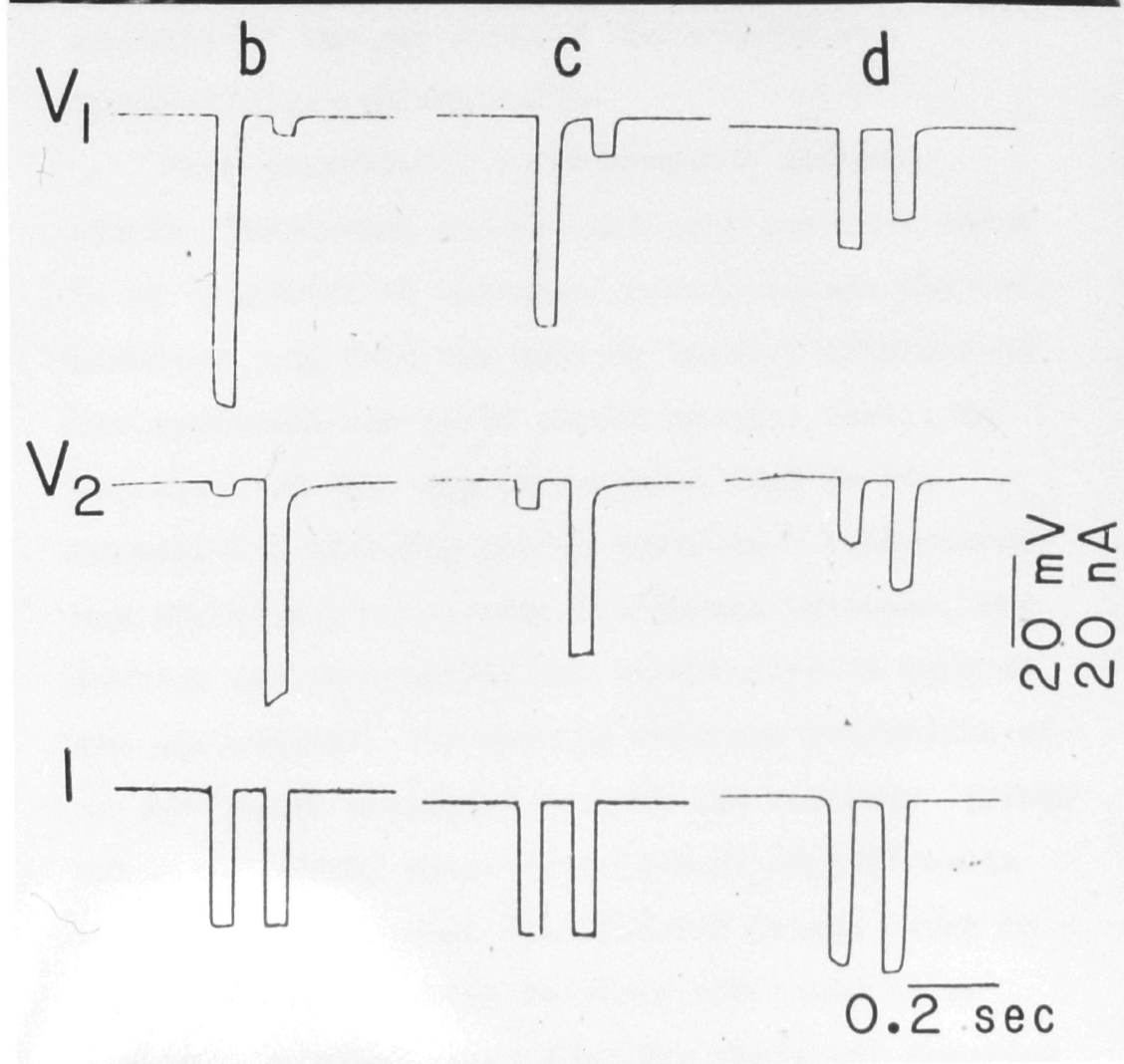
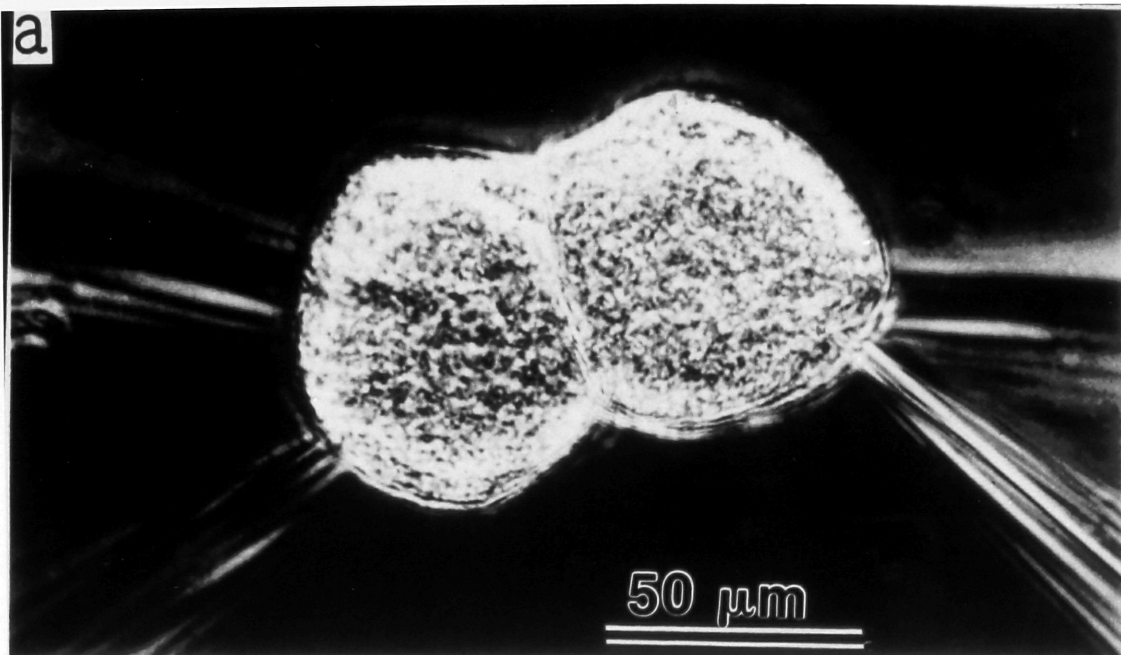


data is shown in Figure 15. When the voltage change or electrotonic decay is plotted as a function of cell number, a linear relationship is obtained for per^+ , per^0 , and per^S . By calculating the X-axis intercept, one can determine how far the current spreads in each genotype. The cells of the gland are assumed to be similar in size (40um). The apparent space constant listed in Table 11 would then translate into 1, 4, and 10 cells for per^0 , per^+ , and per^S , respectively. The results, once again, support the conclusions drawn from the dye coupling: little coupling in per^0 , more in per^+ , and the greatest amount in per^S .

The junctional conductance (g_j) was also determined in each of the different genotypes. As shown in Figure 16, the junctional conductance is determined in isolated cell pairs. Each cell of the pair is impaled by two electrodes: one to deliver current, the second to record voltage. Current pulses are alternately passed between the cells, and the input and transfer resistances can be obtained (Bennett, 1966; Spray et.al., 1985). Using these measurements, the amount of current transfer or junctional conductance (g_j) can be unequivocally determined. Weak electrical coupling is seen in $y per^0$, ry^{42} glands and per^- , $Df(1) TEM202/Df(1) 64j4$ glands. The values for junctional conductance (g_j)

Figure 16. Electronic coupling between dissociated pairs of salivary gland cells of the *per* mutants.

Each of the two cells in a, giving voltages V_1 and V_2 respectively, was impaled with separate electrodes for passing current (I) and recording voltages. Current pulses passed alternatively in the two cells allowed the measurement of input and transfer voltages as well as the subsequent calculation of junctional conductances (g_j). Values for g_j in *per*⁰ (b), wild-type (c), and *per*^s (d) cell pairs shown here were 0.04, 0.13, and 3.2 μ S (see Table 11).



were $0.045 \pm 0.012\mu\text{S}$ (n=4) and $0.21 \pm 0.08\mu\text{S}$ (n=7), respectively, which are not significantly different. This similarity in g_j indicates no activity remains in the truncated *per* protein which would be produced in these *per*⁰ larvae (cf Part I results). In addition, a tenfold increase in junctional conductance is observed between *per*⁰ and *per*⁺ and between *per*⁺ and *per*^S genotypes. Taken together, the electrophysiological and dye coupling studies indicate a relationship between alterations in the activity of the *per* protein and anomalous intercellular communication.

From experiments on *Chironomous* salivary glands, junctional conductance (g_j) has been shown to be sensitive to voltages imposed across the cell membrane, and from the cell or channel interior to the extracellular space (Obaid et.al., 1983). To ascertain whether the differences seen in the mutants are directly due to junctional conductance and not simply to different membrane voltages, the resting membrane potentials were tested in each of the *per* mutants. The resting membrane potentials of 10 *per*^S cells exhibiting strong dye transfer (range -35mV to -50mV, mean -42mV) and of 10 *per*⁰ cells characterized by weak dye transfer (range -40mV to -55mV, mean -45mV) were measured and found to be similar. This indicates that the different coupling

shown in Figure 15, 16, and Table 11 is related to alterations in junctional conductance and not to nonjunctional membrane related phenomena such as altered sodium or potassium channels.

To test whether the percentage of available open channels in per^0 is simply less than that in per^S , glands from each genotype were hyperpolarized and junctional conductances assessed.

Hyperpolarization favors opening of all available channels and should result in higher conductances. In both per^S and per^0 cell pairs, the junctional conductance (g_j) increased by 30 - 50%. Nonetheless, the g_j values for per^0 did not approach those of per^S . These data indicate that the quality of cell communication is altered in the per mutants, perhaps by alterations in the organization or number of functional gap junction.

The dye permeability and electrophysiological data indicate an alteration in intercellular communication, as mediated by gap junctions, in the per mutants. These experiments rule out other forms of cell communication. For example, intercellular cytoplasmic bridges could show dye transfer with Lucifer Yellow; however, they would not respond in the same manner electrophysiologically. Hyperpolarization, in this case, would not have an effect. All the characteristics, such as g_j and k ,

are within the expected range for gap junctions (Bennett and Spray, 1985), and insect salivary gland cells have been shown morphologically to contain gap junctions (Weiner et.al., 1964, Caveney and Berdan, 1982; T. Bargiello, personal communication). Moreover, these results indicate that *per*, by modulating gap junctions in some manner, plays an important role in regulating intercellular communication.

B. New mutants and new conditions

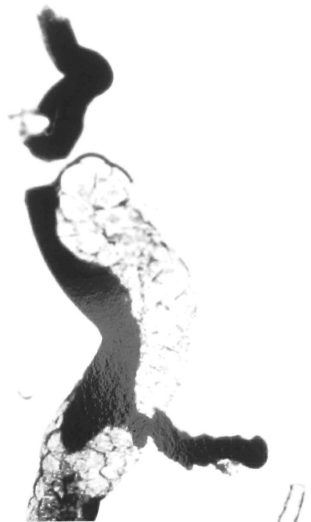
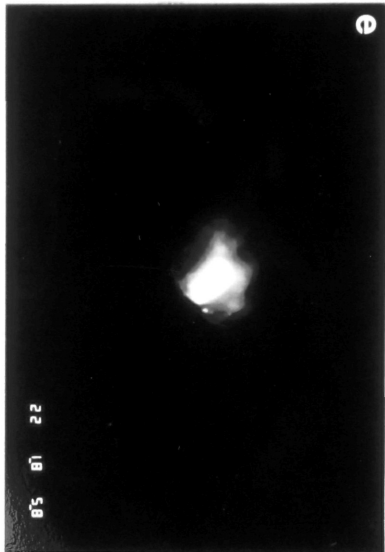
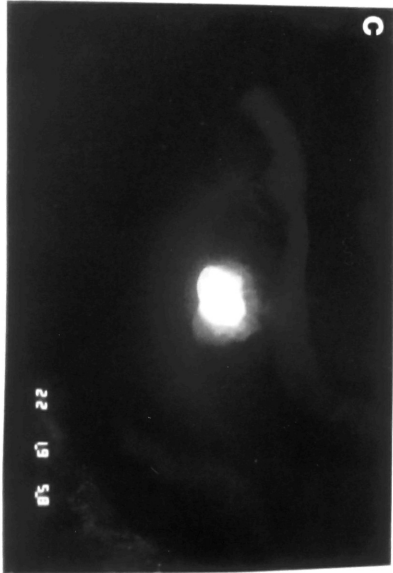
i) New mutants

Dye permeability was assessed in two new *per* mutants, G593S and D588F which were discussed in Part I (see Table 5). It would be predicted from the circadian behavioral results that the new short mutants, G593S and D588F, would behave much like *per^S* in the cell level assay.

As anticipated, preliminary results indicate that the short period mutants behave much like *per^S*. Figure 17 is an example of the data obtained with G593S. Panel b,e and c,f show a salivary gland from G593S and *per^S* respectively. The amount of spread was comparable in each.

Figure 17. Dye coupling among salivary gland cells in the mutant G593S.

The dye spread assay was performed as described previously (Figure 14, experimental procedures). Illustrated examples are from at least 8-10 experiments in each group. Panels b, d, f are phase contrast micrographs of Lucifer yellow CH injected glands of *per⁰*, G593S, and *per^s*, respectively. a, c, e are fluorescence micrographs of the corresponding glands. G593S shows similar spread to that of *per^s*.



ii) New conditions

Since the original experiments were performed (Bargiello et.al., 1987), further work has been done to define the components of this assay. Drs. Ted Bargiello and Vito Verselis have characterized, by electrophysiological means, gap junction channels in third instar salivary glands (Verselis et.al., 1991). Their studies have revealed a complex, voltage-dependent channel sensitive to alterations in membrane potential and uncovered some variability in the range of conductances seen for *per*⁰ and *per*^S flies. For example, increased dye spread is often seen in *per*⁰ glands. Therefore, in order to further characterize the *Drosophila* gap junctions and how they might be differently regulated by the *per* mutants, we carried out the dye permeability assay under various conditions. In this manner, we would learn more about the properties of the intercellular system responsible for conductance and the influence which *per* exerts on it.

Our preliminary data identify a temperature component of the assay. By subjecting larvae to temperature pulses (for example, 0.5h at 37°C, and then allowing them to recover at room temperature for 2-6h), we find a greatly exaggerated dye coupling effect -- that is, no dye transfer in *per*⁰ glands in contrast to significant spread in *per*^S

glands. For comparison, experiments performed at the same time with *per*⁰ larvae grown at 25°C, generally show some transfer of dye from the site of injection. For *per*^S glands, transfer is similar under both conditions. The transfer in *per*⁰ at 25°C is still not that of *per*^S at 25°C with cells of equivalent resting membrane potentials. In addition, measurement of dye transfer immediately after heat shock (no recovery period) shows no transfer at all in any of the genotypes tested. These preliminary results may indicate that the *per* mutants cope differently with the aftereffects of the heatshock. Initially, the response is equivalent -- no intercellular communication; however, *per*^S salivary gland cells are able to recover faster than the *per*⁰ salivary gland cells. Further experiments are currently underway to establish the conditions of the heat shock, to pinpoint the effect of the heat shock, and to address how the *per* mutants operate differently in these conditions.

DISCUSSION

The data indicate that the product of the *per* locus modulates intercellular communication in *Drosophila* salivary glands, thereby synchronizing the cells within the gland. A unitary response to a

stimulus (e.g. a hormone) could therefore be achieved.

Perhaps *per* functions analogously in other tissues in which it is present. For example, it may be surprising to find that *per* is expressed in the gut. However, careful synchronization of these cells is necessary for peristalsis to occur properly. Moreover, gap junctions have been shown to exist in a number of tissues in which *per* is expressed: the ovaries (between follicle cells), in the Malpighian tubules, in the rectal papillae, and in the nervous system (Caveney and Berdan, 1982; Lane, 1982).

Gap junctions play an important role in the nervous system of organisms ranging from hydra to man (DeMello, 1987). As the major component of an electrical synapse, gap junctions assure rapid and mutual excitation of neurons. Therefore, this type of synapse is found in neural circuits requiring high speed and/or synchrony (Jaslove and Brink, 1987). Electrical synapses, moreover, are not merely passive connections; they are able to be modulated by a number of different extracellular and intracellular mechanisms (Bennett and Spray, 1985; Jaslove and Brink, 1987; Bennett et.al., 1991). These multiple ways for adjustment would allow for greater plasticity and variable outputs from a circuit containing gap junctions. Within the

particular cells containing the gap junctions, calcium (Rose and Lowenstein, 1975; Obaid et.al., 1983), pH (Moreno et.al., 1987), calmodulin (Peracchia, 1987), and cAMP (Hax et.al., 1974; Obaid et.al., 1983) can affect the permeability of the junctions, thereby modulating the degree of coupling. The levels of these effectors can, in turn, be regulated by external mechanisms such as hormones. Given the synchrony as well as the plasticity of the response made available by gap junctions, it is not difficult to imagine that electrical synapses would be important components of a circadian clock. Indeed, the *Aplysia* eye circadian neural oscillator which mediates both entrainment and synchronization of the sleep-waking cycle is composed of neurons which are both electrical and chemically synapsed (Strumwasser, 1974).

The effect of *per* on gap junctions, therefore, would offer a level of control for clock function. By affecting the quality of intercellular communication, the circuit constituting the clock may be manipulated. In order to adjust for a phase advance, for example, the central oscillator may need to change the frequency of its output by altering junctional conductance. In addition, this consequence of *per* function may affect input (the coupling of the entrainment pathway to the clock) as

well as output (the coupling of the clock to the behavior) since communication between these pathways is essential to clock function.

We see alterations in intercellular communication with all the *per* mutants tested. The null, long, wildtype, and short period mutants of *per* form a continuum of intercellular coupling. This relationship between the mutants is correlated with results of the mutants in the other available assays. Indeed, the altered junctional conductance may explain the behavior of the mutants. For example, as the temperature drops, the period length of *per^S* flies approaches that of wildtype (i.e a longer period than 19h results). The period length of wildtype flies is unaffected by this same treatment. These data indicate that unlike wildtype *per*, *per^S* cannot temperature compensate (Konopka 1987a, introduction). Hence, it seems likely that a consequence of a decrease in temperature would be the reduced overall activity of the *per^S* protein. Decreased activity in *per^S* would lead to decreased junctional conductance which could, in turn, cause the lengthening in period length seen under these temperature conditions.

How *per* actually modulates intercellular communication is unknown. It is unlikely that *per* encodes a structural component of a gap junction.

Comparisons between the amino acid sequence of *per* and that of gap junction proteins from other species indicate no homology (Bennett et.al., 1990). The *per* protein has none of the requisite membrane spanning domains necessary to form this type of channel. In addition, gap junctions have been found in tissues where *per* is not expressed such as the epidermis (Eichenberger-Glinz, 1979). The recent preliminary results, however, suggest that *per* may be playing a physiological role. Heat shock has been shown to raise the calcium concentration four-fold and to drop pH levels from 7.38 to 6.91 in third instar salivary gland cells (Drummond et.al., 1986). Increases in calcium or a drop in pH of these magnitudes are known to reduce junctional conductance through the closure of gap junctions (Obaid et.al., 1983). Perhaps *per* activity leads to the sequestering of calcium. In the *per*^S mutant, more calcium could be sequestered due to the higher activity of the protein. Therefore, after exposure to heat shock, the junctions recover more rapidly and open so that more dye is spread. In the *per*^O mutant, the release of intracellular calcium stores upon heat shock likewise tips the balance towards the critical zone and gap junction closure results. However, with no *per* protein to compensate in *per*^O, higher levels of calcium would be present for a

longer period of time. Even after the set recovery period, closure of gap junctions would continue and thus, no dye transfer occurs in the *per*⁰ mutant. This lack of "fine tuning" of calcium levels in the *per*⁰ mutant could also explain some of the variability which can be seen with the assay (i.e. the variable, low conductances in *per*⁰ glands). Depending upon the level of calcium set by the "coarse adjustment" in these *per*⁰ cells, the amount of dye transfer may vary dramatically from cell to cell.

This hypothesis about *per* function can be tested. The salivary gland cells can be treated with agents such as serotonin (Rapp and Berridge, 1981) or calcium ionophores (Weingart, 1987) which will specifically raise the level of intracellular calcium. If this were done in the *per*⁰ and *per*^S glands and measurement of calcium levels was monitored (with fura-2 for example) (Grynkiewicz et.al., 1984), one would first predict an abrupt cessation in junctional conductance in all mutants and then a more rapid recovery to pre-treatment levels of calcium in the *per*^S cells. Calcium levels in the *per*⁰ cells would be expected to remain higher for a longer period of time. Likewise, similar experiments can be performed to test pH recovery in the mutant salivary glands.

It is most reasonable to hypothesize that the net effect of *per* would be on the "fine adjustment" of calcium levels. As *per*⁻ flies are perfectly viable and fertile, redundant systems must exist for "coarse adjustment" of calcium levels. Loss of all calcium control, given calcium's regulatory role in a number of cellular processes (Berridge, 1987, Berridge and Irvine, 1989; Majerus and Ross, 1990) would prove lethal.

By carefully setting the level of intercellular calcium or pH, *per* may be modulating a number of cell activities, including that of intercellular communication. Calcium and pH are known to be involved in a number of secondary messenger systems (Berridge, 1987; Berridge and Irvine, 1989; Majerus and Ross, 1990), hence a variety of cell functions may be affected by alterations in *per* function. This, too, may explain the pleiotropic effects of *per* (cf Table 2, introduction). For example, reduced octopamine synthesis in the *per*⁰ mutant could be explained by this lack of calcium level refinement. Recent studies reveal the involvement of a calcium-calmodulin dependent kinase in the regulation of catecholamine biosynthesis. Phosphorylation of the enzymes in this biosynthetic pathway is known to increase the kinetics of the reaction in mammals (Cooper et.al., 1986). Thus, due to altered calcium

levels, the biosynthetic enzymes required for octopamine synthesis could be functioning at reduced rates, leading to an overall reduction in octopamine in *per*⁰ flies.

However enticing, *per*'s effect on calcium and then secondarily on intercellular communication awaits further experimentation. Why cells requiring synchronous behavior evolved such a mechanism which is dispensable remains to be seen. Nevertheless, this cellular assay offers an avenue for the dissection of the biochemical mechanism of *per* function.

PART III EXPRESSION OF PER GENE PRODUCTS DURING EMBRYOGENESIS

INTRODUCTION

Although *per* is expressed throughout the development of the fly, much of the localization work has focused on the pupal and adult stages (Saez and Young, 1988; Siwicki et.al., 1988; Lui et.al., 1988;). These analyses would allow for a direct correlation between *per* localization and *per* phenotypes. For example, mosaic and transplantation analyses have indicated that the brain controls circadian behavior. *per* expression, therefore, has been characterized extensively in the adult brain in order to gain more insight into the cells controlling this behavior (Zerr et.al., 1990).

Embryonic expression of *per* has been only superficially described (James et.al., 1986; Bargiello et.al., 1987) due primarily to a lack of an embryonic assay for *per* function. Early reports indicate some nervous system expression (James et.al., 1986). Given improvements in localization techniques -- *in situ* mRNA detection with digoxigenin-labelled probes (Tautz and Pfeifle, 1989) and the fillet technique (M.Bate, personal communication; Asburner, 1989), I wanted to further characterize the nervous system expression of *per* during embryogenesis. Perhaps with this more

complete picture, we may be able to derive additional information on *per* function. This work was completed with the assistance of Dr. Simon Kidd in the laboratory.

Results

A) *RNA localization during Embryogenesis*

i) probe specificity

The probe used in these experiments was generated from the 5' end of a *per* cDNA. The 515bp EcoRI/BglIII fragment contains the sequences for the first and second exons of the *per* gene (see experimental procedures).

per⁻ embryos were analysed to verify that the signals detected with this probe are bonafide *per*. Control embryos were generated from the strain *Df(1)TEM202/Df(1)64j4* (see experimental procedures). This strain produces females that are deleted for *per* locus. The males from this strain, however, are wild-type with regard to *per* function because they carry a translocation of the X chromosome containing the *per* locus on their Y chromosome. Thus, this strain should produce some embryos which show hybridization to the above probe, but at a reduced frequency when compared to *per*⁺, Canton S strain. This expectation was realized: 50% of the tested

embryos showed no nervous system labelling. This result indicated that the expression seen with this probe does correspond specifically to the *per* RNA.

ii) Early embryonic expression of *per*

Significant expression of *per* RNA is seen at syncytial blastoderm (stage 5, ~1.5-2.5h). Expression is seen throughout the cytoplasm of the developing cells. (Figure 18A). Northern analysis fails to detect expression of a 4.5kb *per* transcript at this developmental stage (Young et.al., 1985). However, a 2.0kb transcript homologous to *per* is produced at this time. RNase protection data of this transcript indicate an overlap in exon usage (at least for exons 2-5) with the 4.5kb transcript (T. Bargiello, unpublished). The 5' end and promoter of this transcript, however, have not been mapped.

Upon cellularization (3hr), all *per* message has disappeared. No further expression of *per* is seen until approximately 2.5h later.

iii) Mid embryonic expression of *per*

By the middle of stage 10 (~5.5h), *per* expression is found in single cells which dot the midline of the extended germband (Figure 18b). One cell per segment is seen; the location within the

Figure 18. Distribution of *per* RNA during embryogenesis.

Embryonic stages are based on Campos-Ortega and Hartenstein (1985). Where appropriate, anterior is left and dorsal is up. A mid-sagittal plane of focus is shown unless noted. Whole mount *in situ* mRNA detection with a digoxigenin-labelled *per* probe was performed as described (see experimental procedures) on embryos (a-j).

a) Embryo at Blastoderm formation (stage 5). *per* RNA expression is seen on the ventral surface (arrow) in the cytoplasm as the cell membranes grow inward.

b) Late stage 10, early stage 11 embryo. Single *per*-expressing cells (arrow, for example) dot the midline of the extending germband of the embryo. One cell in each segment is seen.

c) Germ band retracted, stage 13 embryo. Note the increase in number of *per*-expressing cells in the ventral nerve cord (vnc) and brain (br). Inset shows close-up of the brain. In both c) and the inset, arrow heads indicate *per*-expressing cells in the brain. Small arrows indicate possible sensory organ (Bolwig's organ) or peripheral nerves. Full arrows point to clusters of *per*-expressing cells in the vnc.

d) Close-up of ventral view of an Early stage 12 embryo. *per* clusters have increased in size from one-two cells to three to six cells.

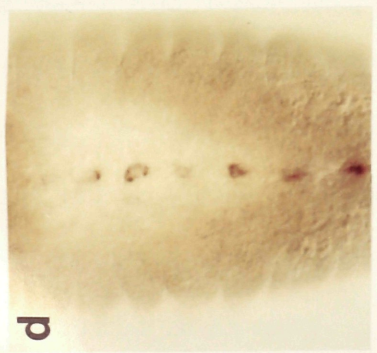
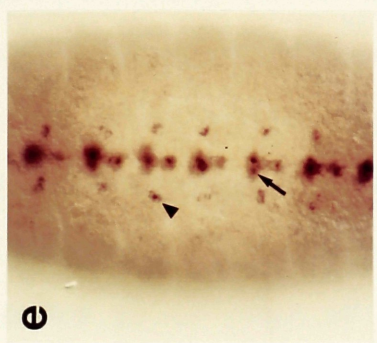
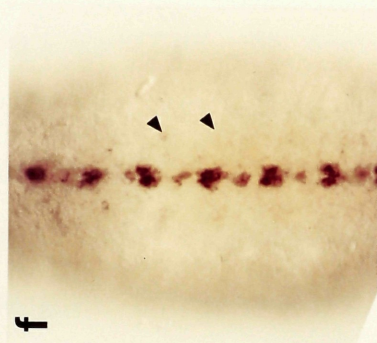
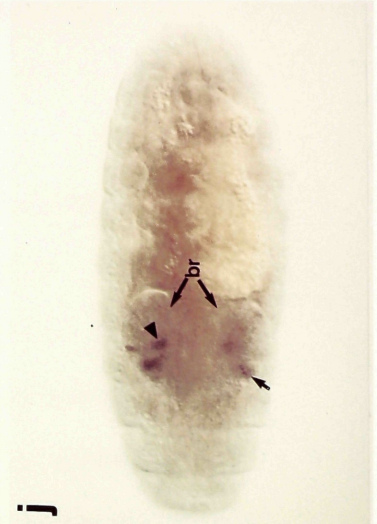
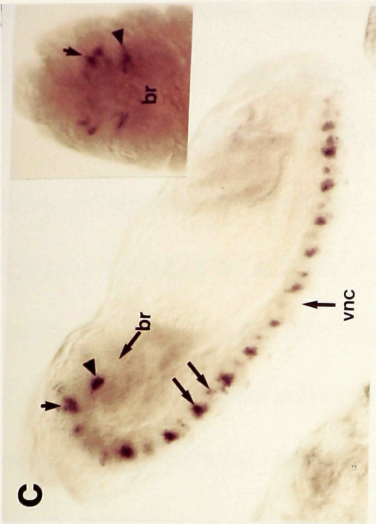
e) Close-up of ventral view of a stage 13 embryo. Note, as in 18c, the increase in the number of *per*-expressing cells. Full arrow indicates clusters of midline expressing cells. Arrow head indicates lateral pairs of cells which express *per*.

f) Close-up of ventral view of a stage 14-15 embryo. The lateral clusters (arrow heads) of *per*-expressing cells disappear during these stages. The ventral midline cells still express *per*.

g) Lateral view of a stage 15 embryo. Expression of *per* continues in the vnc as well as in and around the brain.

h-j) A stage 16 embryo. h) A lateral view showing the shortened vnc and br. *per* expression seems more condensed at this time. i)

A ventral view of the same embryo showing the vnc. This view also shows the condensed "butterfly" pattern of *per* expression. j) A dorsal view of the same embryo showing *per* labelling in the br. *per* expression is present in the anterior portions of the br (arrows) and perhaps in the sensory organs (arrow heads).



embryo suggest that these cells correspond to ventral midline cells.

These midline cells, known also as mesectodermal cells, develop from a single-cell wide strip on each side of the blastoderm embryo. They form a boundary between the cells which will give rise to the mesoderm and those of the neurogenic region (Thomas et.al., 1988; Crews et.al., 1988). Upon gastrulation, these strips of cells come to lie together at the ventral midline and are situated between the two bilaterally symmetric neuroblast plates. Eventually, these midline cells give rise to both neurons and specialized nonneuronal cells of the central nervous system (Thomas et.al, 1984; Thomas et.al, 1988; Crews et.al., 1988; Jacobs and Goodman, 1989a). By the time that *per* is first expressed in a subset of these cells, the mesectodermal cells have delaminated along with the neuroblasts.

During stages 11 and 12 (6-10h) of *Drosophila* development, the germ band has extended to its final point, the establishment of the germ layers is complete, and retraction of the germ band is initiated. The midline cells have undergone at least one round of division. Several of the midline cells will undergo further mitotic activity.

The single *per*-expressing cells have now become a pair during stage 11 and early stage 12. Both cells are in the same layer as seen by the similar plane of focus. Each segment has one pair. Towards the end of stage 12, the *per*-expressing pairs have given way to small clusters of 3-6 cells (Figure 18d).

At this point in development, several progeny of the midline cells can be recognized by location and morphology, as well as antibody and enhancer trap lines (Bate and Grunewald, 1981; Thomas et.al., 1984; Jacobs and Goodman, 1989b; Klambt et.al., 1991). These include three pairs of midline glial cells, a single median neuroblast (MNB) and its several support cells, a group of six ventral unpaired median (VUM) neurons, and a bilateral pair of MP1 neurons that arise from a single MP1 precursor. We speculate that *per* is expressed in a subset of these cells.

During Stage 13 (10h30' to 11h30') the germ band has retracted and nerve differentiation has given form to the central nervous system (CNS). Differentiating neurons have established a ladder-like network of pioneering axons. This network consists of a two bilaterally symmetrical longitudinal connectives extending the length of the CNS and, in each segment, two commissures (the "rungs") which

connect the two sides of the nerve cord. These pioneering axon fibers form the basic skeleton upon which the later-developing neurons will grow. (Bate and Grunewald, 1981; Jacobs and Goodman, 1989a,b). In addition, the first groups of sensory progenitor cells appear at this time in regions surrounding the brain and ventral nerve cord (Campos-Ortega and Hartenstein, 1985).

The *per* expression pattern "blossoms" during this time of development. First, the clusters of cells are no longer in the same plane; instead, *per* expression is seen in a group of cells which span from the dorsal surface towards the ventral surface of the nerve cord. Secondly, in each segment *per* RNA is detected in 1-2 pairs of bilaterally symmetric cells located dorsally and anteriorly to the original clusters. Thirdly, two additional pairs of cells appear in a similar anterior locale as the pairs above, but these new pairs are located ventrally and much more laterally in the CNS in each segment (Figure 18c and 18e). Lastly, expression of *per* is found in clusters consisting of 1-3 cells located in and around the brain (Figure 18c and inset). Some of these clusters may be cells located within the brain; others appear to be located within sensory organs surrounding the brain. It is difficult, at this time, to determine the particular

sensory organs and brain cells in which *per* is expressed. Based on morphological criteria, some of the cells anterior to the lobes of the brain could correspond to Bolwig's organ at this stage (Campos-Ortega and Hartenstein, 1985).

iv) Late embryonic expression of *per*

Stages 14 and 15 of embryonic development (11h-15hr), are marked by the completion of two important morphogenic movements, dorsal closure and head involution. The expression of *per* at this time is found still among the cells of the midline described for stage 13 (Figure 18g). The number of these midline cells remains unchanged. However, during stage 14, expression in the lateral pairs of cells gradually disappears (Figure 18f). At stage 15 these cells no longer express *per* RNA. *per* expression in and around the brain continues in 4-6 clusters seen at stage 13 (Figure 18g). At this later stage, however, Bolwig's organ (by morphological criteria) do not seem to express *per* RNA.

The hallmark of the final stage of embryogenesis is the condensation of the ventral nerve cord (stage 16, 15h-hatching). The contraction experienced by the ventral cord is reflected by the change in the *per* expression pattern. Although it appears that the same cells in the nerve cord still

express *per*, their organization has changed. The *per*- expressing cells seem to tightly group or compress along the midline of the segment (Figure 18h and 18i). Another interpretation is that the pattern of cells labelling at this point has changed. Experiments are currently in progress to differentiate between these alternatives.

The arrangement of *per* expressing cells in or near the brain remains the same. The slight difference in locale would reflect completion of head involution (Figure 18h and 18j).

B) per Protein distribution during Mid to Late Embryogenesis

i) specificity of the antibody

The "fillet" or "flat prep" technique allows for detailed visualization of the embryonic *Drosophila* CNS. This technique is easily performed on embryos from stage 13 to stage 16 ; therefore, I could analyze *per* protein expression in these stages. The antibody used in the following experiments was generated against the carboxy-terminus of the *per* protein (Saez and Young, 1988).

The control for *per* protein localization was embryos generated from *y per^O; ry⁴²* flies. These embryos produce a truncated, disfunctional *per* protein. Since the antigen for the antibody is more

carboxy-terminal of the truncation, this protein is not recognized by the antibody. Therefore, staining seen in *y per⁰;ry⁴²* embryos would be considered background. Fig 19a shows an example of a control embryo fillet. Although background staining is observed in the head region, no staining is seen along the ventral nerve cord.

ii) *per* protein expression during mid to late embryogenesis

In general, the *per* protein expression observed mimics that of the RNA expression. Initially six, then approximately ten, cells produce *per* protein along the midline of the ventral nerve cord. However, a time lag for the protein expression is detected. For example, the protein expression seen along the mid-line at stage 13 resembles the RNA expression seen in late stage 12 (Figure 19c). Moreover, the lateral cells which express *per* RNA at stage 13 now express *per* protein during stage 14. The protein remains expressed in these cells throughout stages 14 and 15 and may extend to the final condensation of the CNS. (see figure 19b and 19d). I am unable to comment on *per* protein expression in and around the brain of the embryo due to background.

Figure 19. Distribution of the *per* protein during mid to late embryogenesis.

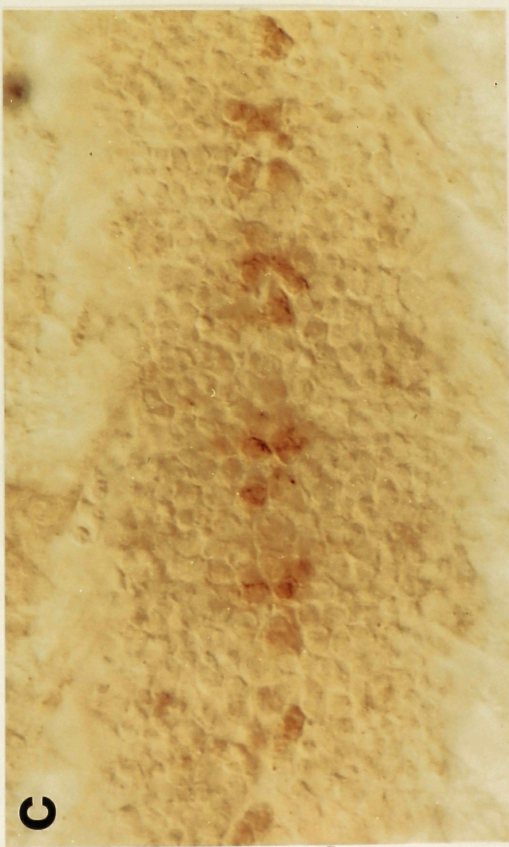
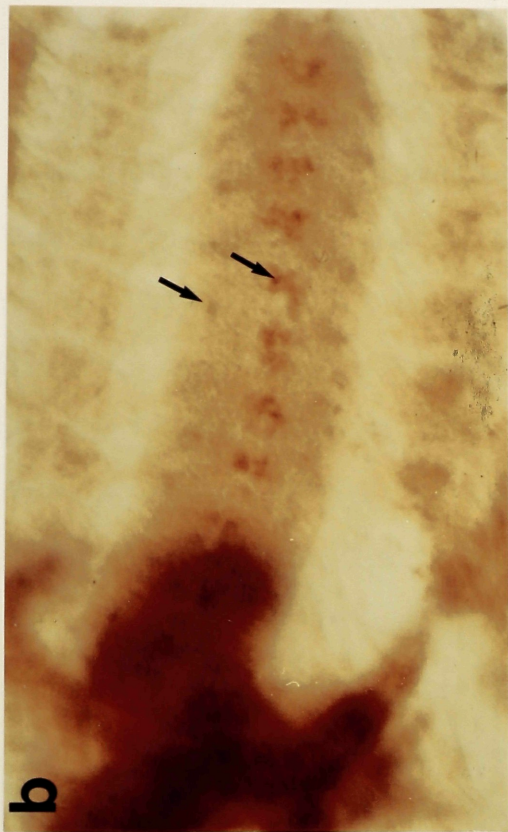
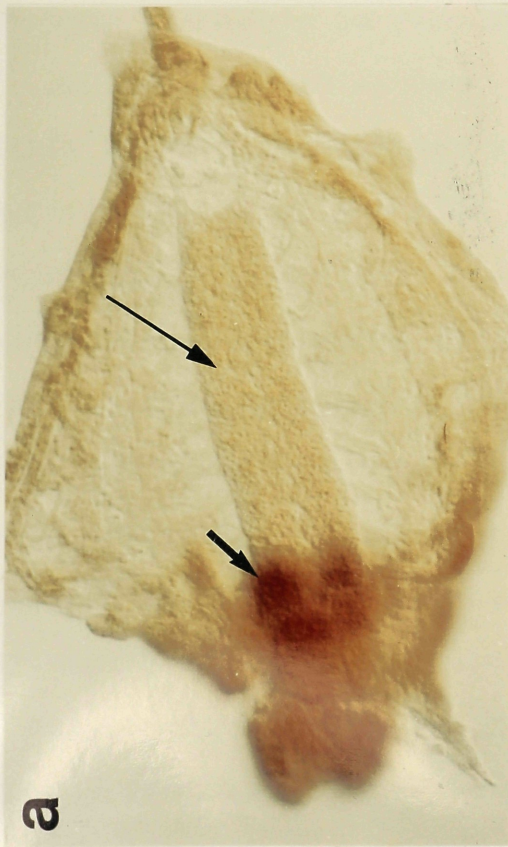
Embryonic stages were based on Campos-Ortega and Hartenstein (1985). Embryo "fillets" are presented from the dorsal view with anterior to the left. Embryo "fillets" were probed with the anti-*per* antibody, 19-47, as described in the experimental procedures.

Panel (a) shows a stage 15, *y, per*⁰; *ry*⁴² embryo which would be deficient for the *per* protein, probed with the anti-*per* antibody. No staining is seen in the vnc (long arrow), although background staining is detected in the head (short arrow). The tissue which is splayed out is the muscles and epidermis.

Panel (b) shows a wild-type (*per*⁺) stage 15 embryo probed with the anti-*per* antibody. Distinct staining is seen in cells along the midline as well as in cells located laterally in the vnc. Background staining in the head precludes any identification of the *per* expressing brain cells.

Panel (c) is a close-up of the vnc (ventral nerve cord) of a stage 13 embryo. Four to six cells label with *per* in each segment. No staining is seen laterally in the vnc.

Panel (d) is a close-up of the vnc of a stage 15 embryo. The lateral *per*-labelling cells are shown as well as the increased number of *per*-expressing cells along the ventral midline.



C) Further clarification of the per-expressing cells of the ventral nerve cord

Studies are in progress to verify the identity of the *per*-expressing cells of the ventral nerve cord. Mutants exist in *Drosophila* which lead to the disruption of all or a subset of the ventral midline cells (Nusslein-Volhard et.al., 1984; Mayer and Nusslein-Volhard, 1988; Thomas et.al, 1988; Rothberg et.al., 1988). Moreover, a recent publication describes markers (antibodies and enhancer traps) which label subsets of the midline cells (Klamt et.al., 1991). Confirming the identity of the *per*-expressing cells would further our understanding of role that *per* would be playing in the embryo.

per RNA expression has been analyzed in two mutants known to disrupt the ventral midline cells, *single-minded (sim)* and *slit (sli)*. The final phenotype in *sim* and *slit* null mutants is the same - - absence of commissures (the "rungs") and collapse of the longitudinal axons into a single, fused bundle at the midline. In *sim* embryos, the midline precursors divide at least once but then die during stage 12. In *slit* embryos, cell death is not observed; however, during stage 12 the midline cells are displaced ventrally and fail to differentiate properly (Nambu et.al, 1990; Klamt et.al., 1991). *sim* and *slit* mRNA and protein are expressed in all

midline cells as early as stage 9. As development proceeds, expression of both *sim* and *slit* concentrates in the three pairs of midline glial (Crews et.al., 1988; Rothberg et.al., 1988). *sim* , however, is still expressed, but at a reduced level, in the median neuroblast and ventral unpaired neurons (Crews et.al., 1988).

per expression is altered in each of these mutants. In the *slit* embryos, *per* expression in the midline cells first appears normal (stages 10-early 12). Upon the completion of germ band retraction (stage 13) and until the end of development, the *per* expression pattern is dramatically altered. The *per*-expressing cells which cluster in each segment at the midline have either disappeared completely or are scattered in the collapsed nerve cord. There are a few remaining *per* labelling cells in each segment (from 12-14 cells per segment in wildtype to 4-6 cells in *slit*); these could be the scattered remains of the midline population and/or the more lateral cells which express *per* at this stage (Figure 20b and 20c). This disrupted pattern continues for the remainder of embryogenesis. The expression of *per* in and around the brain remains unchanged in the *slit* mutants (Figure 20a).

The pattern of *per* expressing cells is also changed in *sim* mutants. The defect in pattern,

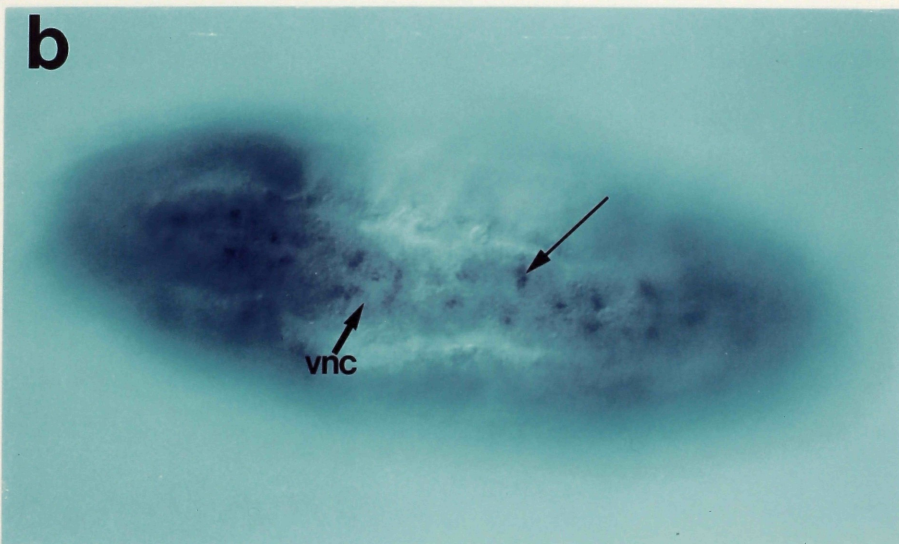
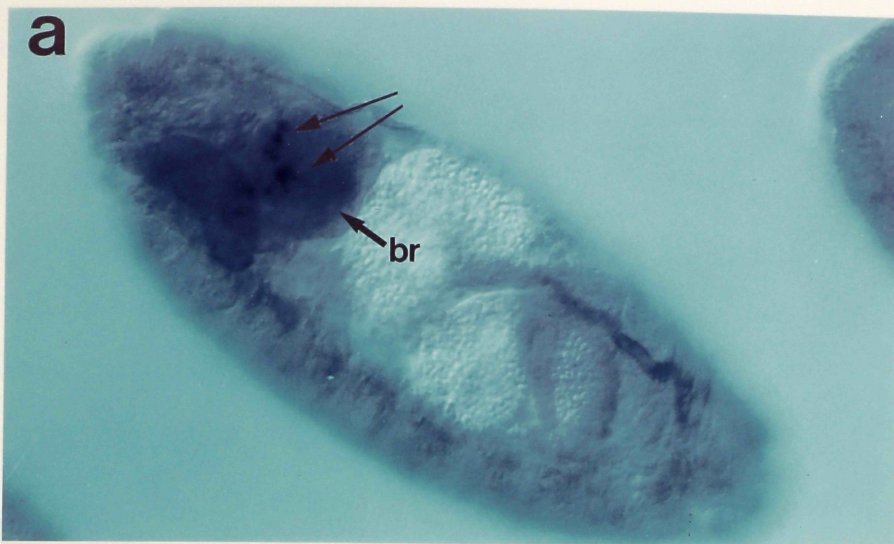
Figure 20. *per* RNA expression in *slit* embryos.

Whole mount *in situ* hybridization with a digoxigenin labelled *per* probe was completed as in Figure 18 (see experimental procedures). *slit*⁻ embryos were generated from a *sli*^{lG107} *cn bw sp/CyO* stock; hence half of the embryos were *slit*⁻. The remaining *slit*⁺ embryos served as internal controls. Anterior is left in all panels. Photos were taken with a blue filter.

Panel (a): A sagittal view of a *slit*⁻ embryo (~ stage 16). Brain labelling by *per* seems similar to that of wild-type (arrows). Nerve cord labelling (ventral = bottom) is diffuse. Background staining was seen in the trachea (along the dorsal surface) in this preparation.

Panel (b): A ventral view of a ~stage 14 embryo. The ventral nerve cord (vnc) is disrupted and has a snake-like appearance due to the collapse of the commissures in the *slit*⁻ mutant (Rothberg et.al., 1988). Arrows indicate the *per*-expressing cells.

Panel (c): A close-up of a ventral view of the vnc shown in (b). Arrow shows 2-4 cells/ segment which express *per*.



however, is noted earlier than in the *slit* mutants; *per* expression is not detected in the midline at all (stages 10, 11, and 12) (Figure 21a). Late expression patterns reveal few *per*-expressing cells (2-4 cells, arising from those lateral *per*-expressing cells?). Brain and sensory organ *per* expression is unmodified (Figure 21b and 21c).

These results indicate that *per* is expressed in some of the mesectodermally derived midline progeny as thought, since the genetic removal of these cells leads to the disappearance of most of the *per* expression. Nambu et.al. have shown recently that *sim* is required for proper expression of *sl1* in the midline. Our data would indicate that *sim* is also required for proper *per* expression either directly (through transcriptional activation) or indirectly (through initiation of the proper differentiation of those cells). This is contrary to an initial analysis done with a *per*-*Bgal* fusion in a *sim*⁻ background (S.Crews, personal communication). Although the actual data has never been published, reports indicated no difference in *per* expression (Hall and Kyriacou, 1990). The discrepancy in the results could be due to use of the *per* reporter construct instead of direct *in situ* detection of the *per* transcript, a different *sim* mutant analyzed, or

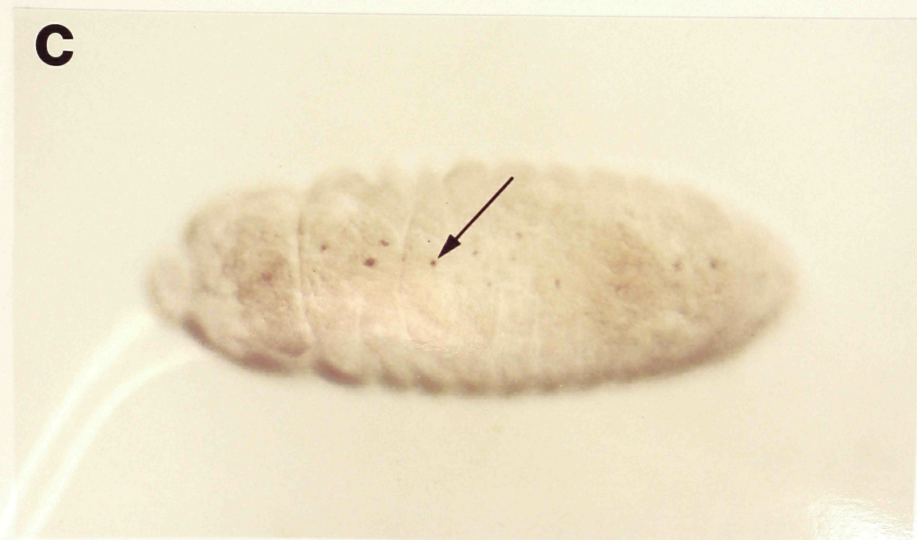
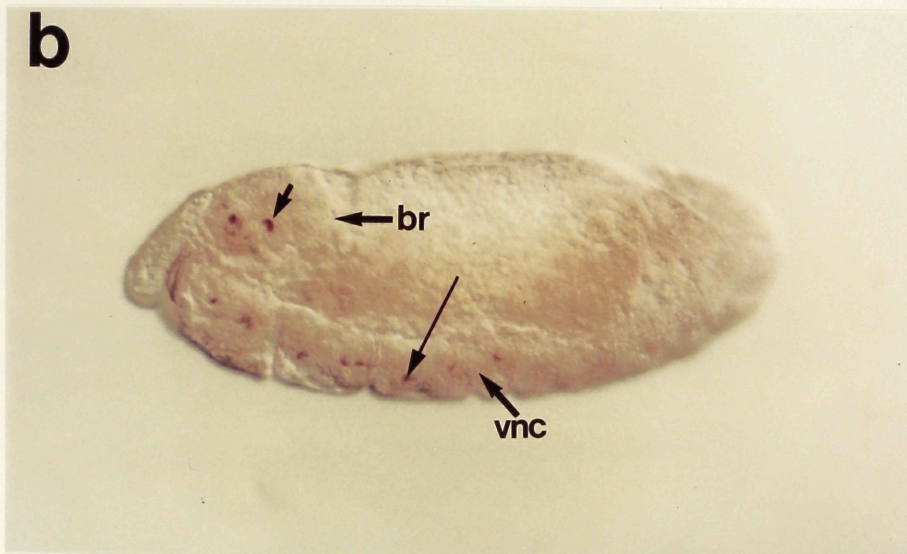
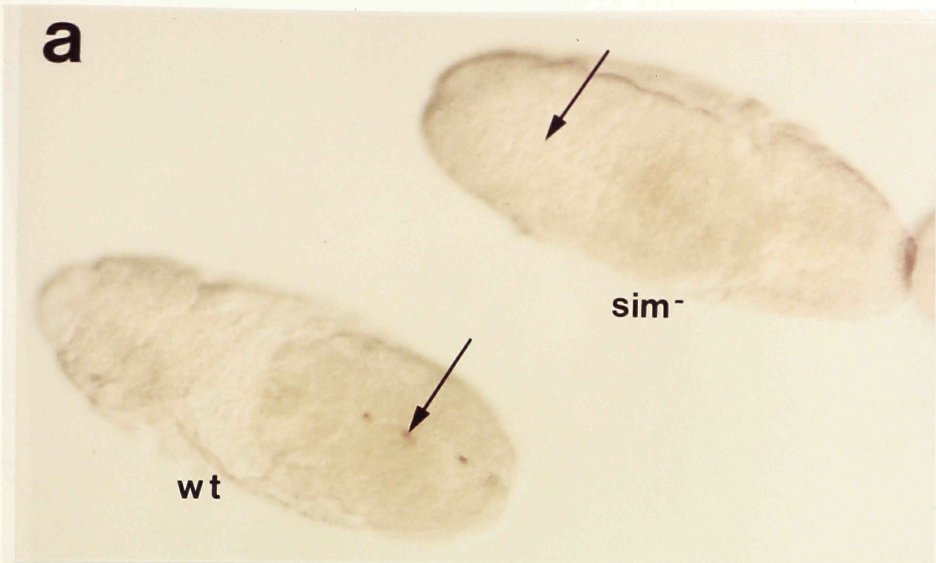
Figure 21. *per* RNA expression in *sim*⁻ embryos.

Whole mount *in situ* hybridization with the digoxigenin labelled *per* probe was done as detailed in the experimental procedures. *sim*⁻ mutants were generated from a *sim*^{H9}, *kar/mkrs* stock; therefore, one half of the progeny was *sim*⁻. The remaining *sim*⁺ embryos served as an internal control.

In panel (a), a wild-type (wt) embryo (stage 12) and a *sim*⁻ embryo (stage 11) are shown from a dorsal view. Clusters of *per*-expressing cells are seen in the wt whereas no staining is seen in the *sim*⁻ embryo. Even though the *sim*⁻ embryo is slightly younger, expression of *per* should be apparent (cf 18b).

In panel (b) a lateral view of a ~ stage 13 *sim*⁻ embryo is depicted. *per* expression in the brain seems normal for this stage of development. However, the expression in the ventral nerve cord is drastically reduced (arrow).

Panel (c) shows the ventral view of a similarly staged *sim*⁻ embryo. Some *per* expression is seen; it is much less than in wild-type (compare figure 18e).



incomplete information about *per* expression pattern available at that time.

With the mRNA detection technique we are unable to reliably see salivary gland staining as reported earlier from our group (Bargiello et.al., 1987). We hypothesize that this may be due to our present manner of fixation and/or hybridization. We are currently testing other methods and tissues (i.e. third instar larvae salivary gland tissue) to clarify the variability which we see with this technique.

DISCUSSION

In this study we analysed *per* expression in the nervous system of the *Drosophila* embryo. Previous reports give no detailed information about cell type or time course of the pattern of *per* expression. We attempt to ameliorate this situation by employing more sensitive techniques as well as genetic ablation to characterize the *per*-expressing cells.

Our results indicate a complex pattern of *per* expression in the nervous system of the embryo. We see not only a number of cells along the midline of the ventral nerve cord (vnc) which express *per* but also discrete clusters in and around the brain of the embryo. These clusters in the brain region have

not been previously described. The overall pattern of *per* expression develops over time, indicative of the cell divisions and further differentiation occurring in the nervous system.

The identity of the *per*-expressing cells in the brain region are unknown. The clusters of cells in the brain could correspond to the region which would give rise to the optic lobe. A comparison of the *per*-expressing cells with the published localizations of *disconnected* and *glass* mRNA, two genetic mutants which cause degeneration of a part of the optic lobe, suggests that *per* is expressed in a different subset of brain cells than *disco* and *glass* (Moses and Rubin, 1991; Lee et.al., 1991). A further description of the *per* labelling in the brain is planned by analyzing *per* expression in these two mutants as well as others.

The other clusters surrounding the brain may coincide with sensory organs. These components of the peripheral nervous system are important for relaying information about the environment to the brain of the embryo/larva. In particular, during stage 13, some of these *per*-expressing clusters anterior to the brain could be Bolwig's organ (see Figure 18c and inset) (Campos-Ortega and Hartenstein, 1985). Bolwig's organ is a cluster of photoreceptor cells from which a nerve (Bolwig's)

extends and establishes the path between the light-sensitive cells and the embryonic brain. The organ can first be identified at this stage (13, ~9hr) (Compos-Ortega and Hartenstein, 1985; Heilig et.al., 1991). The *per* RNA expression in these cells is transient, however, as it disappears later in embryonic development. Further characterization (using enhancer traps, for example) is necessary for the positive identification of the cells and for the determination of the significance of *per*'s expression there.

The location and pattern of development of the *per*-expressing cells in the vnc has given us more clues to their identity. The majority of *per*-expressing cells in the vnc are progeny of the mesectodermal or midline precursors. The initial results from the genetic ablation work with the midline mutants, *single-minded* and *slit*, corroborates this finding. The midline cells play an important role in the pioneering of the commissures and longitudinals of the ventral nerve cord (Thomas et.al., 1984; Jacobs and Goodman, 1989a,b; Klamt et.al., 1991). It is unlikely that *per* is involved in this activity; axon pathfinding appears normal in the *per*⁰ mutant. Later roles for the midline cells are not known at this time. In grasshoppers, however, an homologous subset of these midline cells

produce the neurotransmitter, octopamine (Goodman and Bate, 1981). Interestingly, octopamine synthesis is reduced in *per*⁰ mutants (Livingstone and Tempel, 1983). Lastly, the neuroblast lineage and role of the lateral cells of the vnc which express *per* RNA transiently are unexplored. Hence, it is difficult to speculate on *per*'s function in all these cells.

Further genetic ablation experiments are currently underway to confirm the particular identity of the *per*-expressing cells in the midline. For example, the mutant *orthodenticle* (*otd*) has been shown to selectively eliminate the ventral unpaired neurons (VUMs) (Klambt et.al., 1991). It is our expectation that, if *per* is expressed in these cells, most of the *per* expression along the midline would be absent in the *otd* mutant. Moreover, other loci, *Star*, *rhomboid*, and *spitz* are known to remove a subset of the midline cells (Klambt et.al., 1991). We believe that *per* is not expressed in this particular subset (the three pairs of midline glia). Thus, although a disruption in the *per* pattern would be expected in *Star*, *rhomboid* and *spitz*, all the *per*-expressing cells would be present if our hypothesis is correct. Through a series of complementary genetic ablation experiments such as those mentioned above, a more exact description of the *per*-expressing cells will be produced.

Sehgal and coworkers (manuscript submitted) have shown a synchronization of activity rhythms in an adult population by a light cue given during embryonic development. This clock activity displayed by the embryos may be related to the presence of *per* gene products in the embryonic nervous system. Which *per*-expressing cells (brain, sensory organ, midline, all or some of these cells) are responsible for the setting and memory of the light phase is unknown. However, with Sehgal's et.al. elegant demonstration of clock activity in the embryo and now this restricted pattern of *per* embryonic expression, it will become possible to correlate *per* function with a small number of cells.

Overall Discussion / Summary

Steps have been taken to understand the role of *per* in the production of biological rhythms. The amount of the *per* product is integral to the pace of the clock. Absence of *per* product leads to the abolishment of circadian rhythmicity. More *per* product shortens period length; less *per* product lengthens period length. Single amino acid changes in the *per* product can mimic these results. The loss of circadian rhythmicity in *per*⁰ is due to a truncated *per* protein. A valine-to-aspartic acid change in the *per*¹ mutant lengthens period without changing the overall titre of the RNA. Likewise, the serine-to-asparagine substitution in *per*^S flies shortens period length without any change in RNA titre. Although these data did not explain the biochemical function of the *per* protein, the determination of the altered amino acids in the *per*¹ and *per*^S mutants has given clues to the functionally significant regions of the *per* protein.

Further mutagenesis of the *per* protein has identified regions important for *per* activity. Alterations in the area around the *per*^S substitution indicate a domain of common function. This portion of the wild-type protein serves to restrain *per* activity, and by doing so sets the periodicity of

the clock to 24 hours. Various models of how this region may be modulating *per* activity or stability - by post-translational modifications and/or by protein-protein interactions -- have been proposed and remain to be tested.

A cell level assay has given some insight into *per* function. Intercellular communication mediated by gap junctions has been altered in the *per* mutant series. *per*⁰ salivary glands show little "coupling" whereas *per*^S salivary glands show much greater "coupling". Possibly, the effect on intercellular communication which is present in the salivary glands is reproduced in the nervous system as well as in other tissues where *per* is present. By modulating intercellular communication in these tissues, a synchronization of function might be obtained, whether in the follicle cells of the ovary or in the brain of the adult. The regulation of intercellular communication thus provides a convenient model for *per*'s role in the manifestation of rhythmicity.

Pavlidis (1973) has shown theoretically that in multicellular organisms a circadian period could arise from the mutual coupling of a number of non-circadian oscillators. In this model, the overt period length would be a function of the strength of the "coupling" as well as the number of oscillators

recruited. In the wild-type case, the mutual coupling would yield close to a 24h period. Dowse and his colleagues have analyzed the circadian locomotor records of the *per* alleles and report the existence of non-circadian, ultradian rhythms in the weakly coupled *per*⁰ mutants (Dowse and Ringo, 1987; Dowse, et.al 1987). Moreover, they reveal that ultradian rhythms can be observed, but to a lesser extent, in the remaining *per* alleles. The frequency in occurrence of ultradian rhythms in the locomotor records of the *per* alleles is as follows: *per*⁰ > *per*¹ > *per*⁺ > *per*^S. Dowse and Ringo suggest that the *per* gene product is responsible for determining the "tightness" of coupling of a population of ultradian oscillators, such that the period would be lengthened as the coupling afforded by *per* loosens (Dowse and Ringo, 1987). Indeed, the effect on gap junctions by the *per* mutant series might provide the biochemical basis for Dowse and Ringo's observations.

However, we are still left with the question of how *per* actually modulates gap junctions. We know from electron microscopy that the number of gap junctions do not seem to be different in the *per* mutants (T. Bargiello, unpublished). The regulation or organization of gap junctions are other possibilities by which *per* may be affecting

intercellular communication. In theory, the localization of *per* could give clues for this investigation. However, the *per* protein is located in a number of subcellular localizations: the nucleus in some cell types and the cytoplasm (and possibly the nucleus) in others. *per* could be active in both or only one compartment. Knowing the site of its activity would narrow the choices of the biochemical role of *per*. If *per* was active in the cytoplasm, it could be, for example, modulating ion (calcium?) concentrations or, by analogy to *arnt*, shuttling molecules to the nuclear membrane which in turn could be affecting the physiology or structure of the junctions. However, if the site of *per*'s action is in the nucleus, it may regulate gene transcription or be involved with nuclear import/export with the downstream effect of modulating gap junctions. The *per* protein does not seem to bind DNA itself in assays in which known DNA binding proteins do (unpublished observations, Treisman and Baylies). However, preliminary *in situ* chromosome squashes from third instar salivary glands probed with the *per* antibody show chromosomal sites of *per* immunoreactivity (Saez, unpublished observations; Hall et.al., unpublished observations). This could indicate that *per* could be involved in transcriptional regulation through an

adaptor molecule (Berger et.al., 1990; Ptashne and Gann, 1990). Studies are currently underway to assess the site of *per* activity -- nuclear, cytoplasmic or both. Better grounded hypotheses may then be put forth as to *per*'s biochemical role in the cell.

Sehgal and her colleagues (manuscript submitted) have shown that the clock can fully assemble and function in the absence of environmental cues. Moreover, a light pulse given during late embryonic/early larval development can synchronize the population, producing the same phase relationship in the majority of the population. The detailed localization studies provide precise regions of the embryo to further explore the effect of *per* on clock function, perhaps ultimately uncovering the progenitors of the adult pacemaker.

In conclusion, the three main areas of ongoing research -- localization, cell level examination, and structure/function analysis -- should further define the nature and role of the *per* gene in clock function.

EXPERIMENTAL PROCEDURES

I. Regulation and Structure/Function Analysis of the *per* Gene product

A) Fly strains

Flies are cultured on standard yeast-cornmeal-glucose-tegosept-agar medium at 25°C. The Canton S strain is used for the wildtype *per* stock. Mutant alleles of *per* utilized in these studies are *y per*⁰; *ry*⁴², *per*^S, and *per*¹ (generated from a *per*¹/*attached^X ywf* stock). Derivative lines of the above mutant strains were created to include additional markers (Lindsley and Grell, 1968): *y per*⁰; *CyO/Sco*; *ry*⁵⁰⁶/*TM3*, *y per*⁰; *CyO/Sco*, *y per*⁰; *Ser/TM3*, *per*^S; *CyO/Sco*, *per*^S; *Ser/TM3*; and *per*^S/*FM7* and *per*¹/*FM7*. Balancers and marked chromosomes had no effect on the locomotor activity rhythms generated by the individual *per* alleles (A. Sehgal and L. Vosshall, personal communication) with the exception of *per*^S, *CyO/Sco*. This lengthened the period length to 23.5hr. The basis of this lengthening is under investigation. The *per*⁻ deletions employed in these experiments are *Df(1) TEM202* and *Df(1) 64j4*. Each of these deletions alone is recessive lethal; however, females carrying both *Df(1) TEM202* and *Df(1) 64j4* are viable and homozygously deficient for a 10kb interval including

the *per* gene and are arrhythmic (Bargiello and Young, 1984; Smith and Konopka, 1981; Young and Judd, 1978). The deletions can be maintained in males, covered by a w^+Y duplication. This duplication rescues both the recessive lethality of the single deletion and the arrhythmia (Smith and Konopka, 1981; Young and Judd, 1978). *Df(1) 64j4* is marked with w^a and *Df(1) TEM202* is marked with w^- (Young and Judd, 1978). The generation and maintenance of the transformant lines will be described below.

B) Behavioral Assays

1. Locomotor Activity Assay

The experimental procedures and hardware and software for the locomotor activity collection and analysis have been described in detail elsewhere (Hamblen et.al., 1986; Ewer et.al., 1990). For our particular set-up, the procedure briefly is as follows.

Flies selected for the assay were entrained to a 12L:12D cycle in food vials for a minimum of three days. Usually, 1-3 day old males were chosen for the assay. If females were used, care was taken to insure that they were virgins; hatched larvae could churn up the food and lead to artifacts. In the experiments with the transformants, males hemizygous

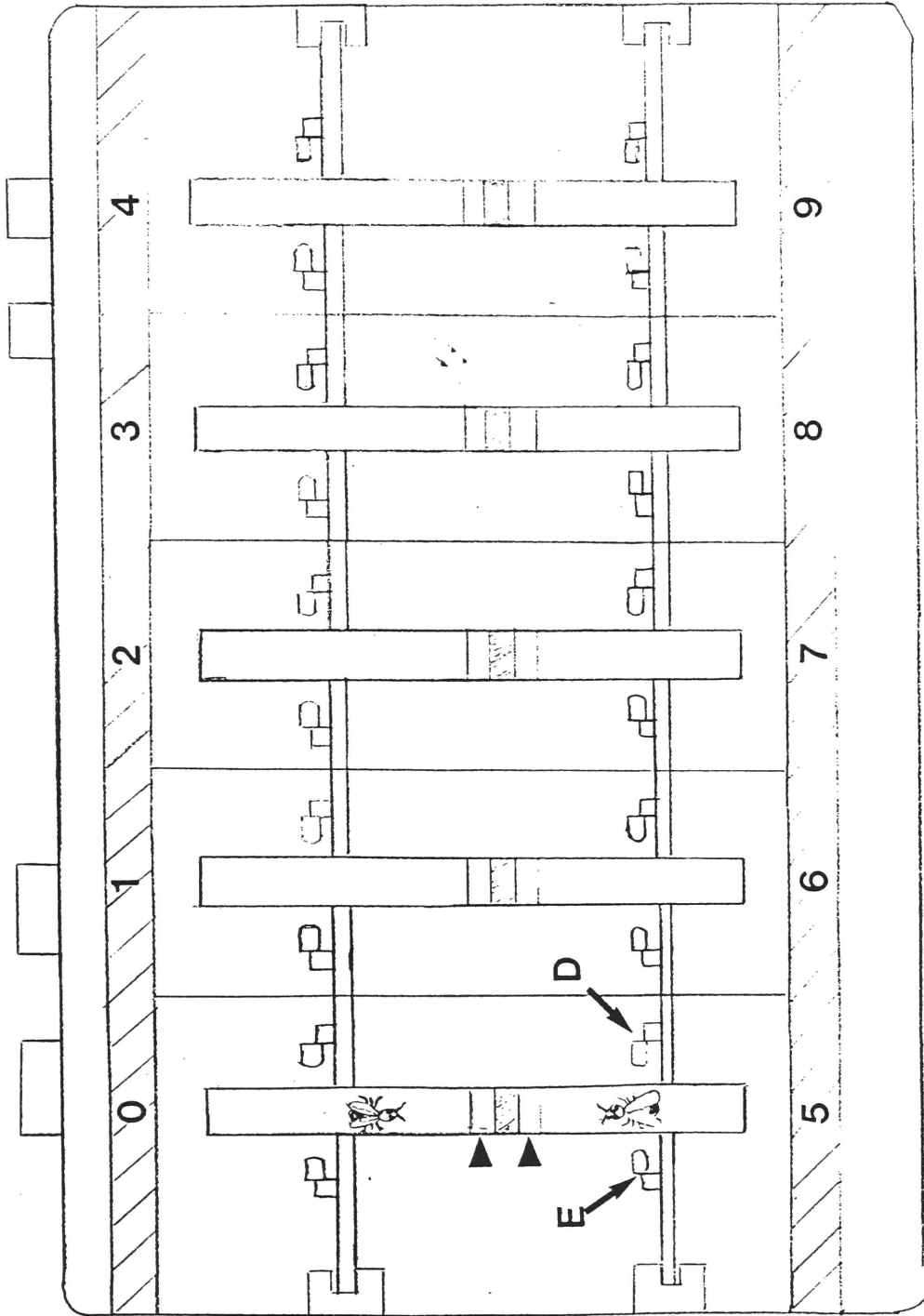
for *per*⁰ and carrying one copy (or, in later experiments, two copies) of the autosome-linked construct were tested.

The chamber used to test an individual fly consists of one half of a borosilicate glass tube (Anderson Glass, Fitzwilliam, NH). The glass tube or cuvette is separated into two halves by a dampened bead of cotton, on either side of which is placed a small block of fly food (see diagram, Figure 22). Flies are anesthetized with ether or CO₂ and individually placed into the test chambers (two flies/glass tube). Cotton is used to plug the ends of the chambers.

The glass tubes are placed into monitoring boxes (see Figure 22) with 5 tubes or 10 flies per box. Each chamber is flanked by an infrared emitter and detector pair (arrows) located just beyond the surface of the food. The infrared wavelengths (850-950nm) do not affect the *Drosophila* visual and circadian systems (Frank and Zimmerman, 1969). The monitoring boxes are placed in a temperature and light controlled incubator (~10 boxes/incubator). The temperature is kept constant (25°C). The flies are given one more 12L:12D entrainment cycle in these surroundings before being placed into constant darkness. The conditions of "constant darkness"

Figure 22. Apparatus for monitoring *Drosophila* locomotor activity.

This drawing shows the apparatus used in the locomotor assay. This box contains five glass cuvettes, each of which is separated by fly food (arrow heads, for example) into two chambers. A total of ten independent chambers, labelled 0 - 9, is shown for this monitor. Each chamber would contain a single fly as depicted in cartoon form for chambers 0 and 5. The chamber is closed at the end by a cotton plug to prevent the fly's escape. Each chamber is flanked by an infrared emitter (E) and detector (D) pair. When the fly breaks the beam of infrared light, it creates a signal which is immediately digitized and stored via software (Sulzman, 1982) on an Apple IIe computer (see text). Approximately ten such monitors containing a total of 100 chambers are monitored in any one experiment.



allow measurement of the free running activity rhythm.

Data collection begins when the flies are placed in the incubators (day 1). The locomotor activity can be measured by monitoring the events in which the fly breaks the infrared beam. When the fly does break the beam, a signal is created which is digitized and stored on an Apple IIe computer. Data are collected in this digital form every 30 minutes (Sulzman, 1982; Hamblen et.al., 1986). The free-running locomotor activity was monitored in constant darkness for, on average, seven days. An example of the raw data collected from such an experiment for the transformant G593S is shown in Figure 23.

The raw activity data for an individual fly can be manipulated for period information. Locomotor activity rhythms under free running conditions are assessed by inspection of actograms and by performing Chi-square periodogram analyses (Hamblen et.al., 1986; Sokolove and Bushell, 1978). Figure 24 shows examples of actograms from a wild-type fly, a *per*⁰ fly, and a transformant, G593S fly. An actogram is a direct plot of the raw data. Each horizontal line represents one day in the life of the fly under the test conditions. The small vertical ticks represent activity "events" during an interval of time. The first panel in Figure 24 is an example of

Figure 23. Locomotor Activity Assay --- Raw Data

This figure shows an example of the raw data collected for an individual *per*⁰ male fly heterozygous for the construct p[G593S]. Listed vertically in half hour intervals is the time. The number of days in which the experiment has been performed is shown horizontally across the top. The numbers listed in the columns (for example, 488 at 0800, day 2) indicate the number of times the infrared beam was broken by the fly during the set interval of one half hour. 0 indicates no activity. This fly shows distinct bouts of activity which begin earlier and earlier each successive day. See text for more experimental details.

Raw Data

G593S

CH29

TITLE = CH29 06/25/90*16.0

TIME

DAY

	1	2	3	4	5	6	7	8
0800		488	121	18	303	4	0	0
0830		0	66	20	261	0	3	0
0900		264	90	72	181	3	0	0
0930		205	78	238	82	0	0	7
1000		0	68	244	0	3	0	
1030		160	3	249	0	0	0	
1100		143	131	235	0	11	0	
1130		108	182	219	0	3	3	
1200		391	175	249	0	0	0	
1230		552	292	132	4	0	0	
1300		387	263	4	0	0	0	
1330		355	245	0	0	0	0	
1400		276	294	0	16	7	0	
1430		27	237	0	6	0	0	
1500		23	214	0	3	0	0	
1530		731	193	0	4	0	5	
1600	43	311	10	4	0	0	0	
1630	353	121	146	6	0	0	0	
1700	270	20	74	3	4	4	3	
1730	168	98	0	2	0	0	13	
1800	107	2	3	13	0	0	17	
1830	268	4	0	10	4	17	16	
1900	22	13	0	0	0	23	3	
1930	132	7	0	6	17	5	23	
2000	157	0	0	0	2	2	216	
2030	8	6	9	0	0	28	223	
2100	0	0	5	0	0	24	236	
2130	69	0	0	0	12	190	161	
2200	37	0	6	2	15	264	134	
2230	55	0	0	0	8	270	52	
2300	7	4	0	3	232	208	0	
2330	7	0	2	0	111	246	0	
2400	353	0	0	12	259	160	64	
2430	76	0	14	7	66	153	211	
0100	81	2	10	14	137	103	196	
0130	141	0	27	182	213	214	140	
0200	0	0	4	271	208	109	126	
0230	9	0	12	247	245	95	133	
0300	106	4	6	163	146	0	14	
0330	85	0	14	163	152	0	0	
0400	5	18	178	150	140	0	0	
0430	243	0	253	199	166	0	0	
0500	11	38	274	167	109	0	0	
0530	471	301	216	45	9	14	0	
0600	252	243	194	12	0	2	0	
0630	0	187	215	28	0	0	0	
0700	243	148	156	289	3	3	4	
0730	236	192	0	226	0	0	0	

Figure 24. Locomotor Activity Assay --- Actograms

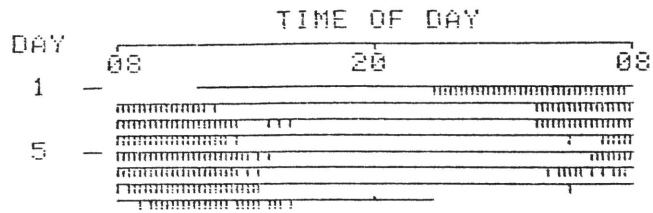
Examples of actograms from *per*⁺, *per*⁰, and G593S individuals are shown. The raw data for this particular G593S fly was displayed in Figure 23. The actogram is plotted directly from this raw data. Each horizontal line in the plot represents one day in the experiment. The number of horizontal lines indicates the number of days in which the fly was tested in this experiment. The small vertical ticks represent the activity of the fly in the set interval.

In the top panel, an example of a Canton S, *per*⁺ fly is given. Note that bouts of activity are separated by intervals of rest. The onset of activity is approximately 24h from the onset the previous day. For a *per*⁰ fly (middle panel) no discrete bouts of activity are discerned. For the *per*⁰ fly carrying the p[G593S] construct, distinct bouts of activity and rest are seen; however, the onset of activity occurs earlier each day.

Actograms

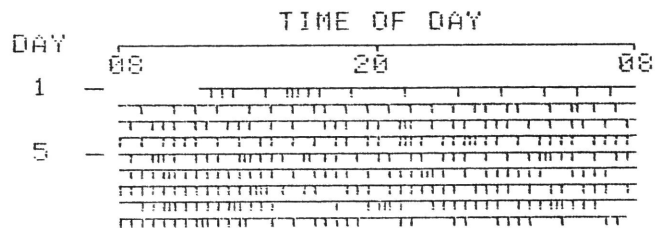
CH9

per+



CH33

per0



CH29

G593S

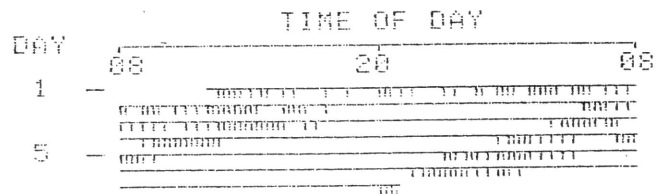


Figure 25. Locomotor Activity Assay --- Periodograms

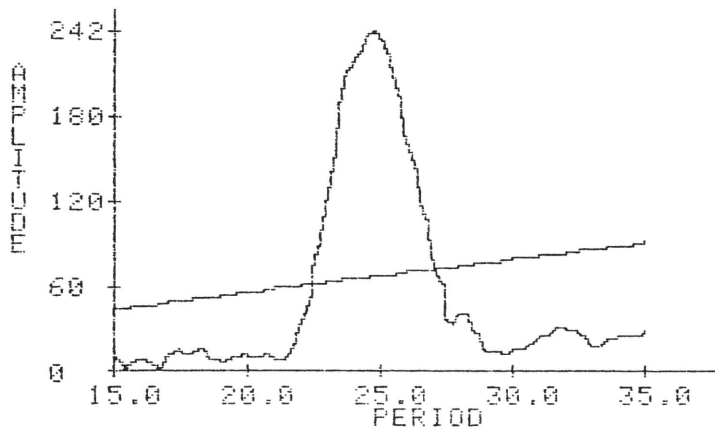
The raw data (example, Figure23) is subjected to Chi-square analysis (Hamblen et.al., 1986, Sokolove and Bushell, 1978) on the Apple IIe. In this analysis, the raw data is sampled to find the best period fit for the range selected. For the periodograms in this figure, the range was set to 15-35h. The greater the amplitude (on the Y-axis), the better the period fit. The peak amplitude, therefore, corresponds to the best period fit. A single peak indicates a single strong period fit. Amplitudes above the horizontal line are statistically significant (level set to 5%).

In the top panel, the periodogram for the *per*⁺ fly shows a peak period length of 24.3h. In the middle panel, no statistically significant peak emerges for the *per*⁰ individual. The periodogram for the G593S fly (bottom panel) reveals a period length of 21.8h.

Periodograms

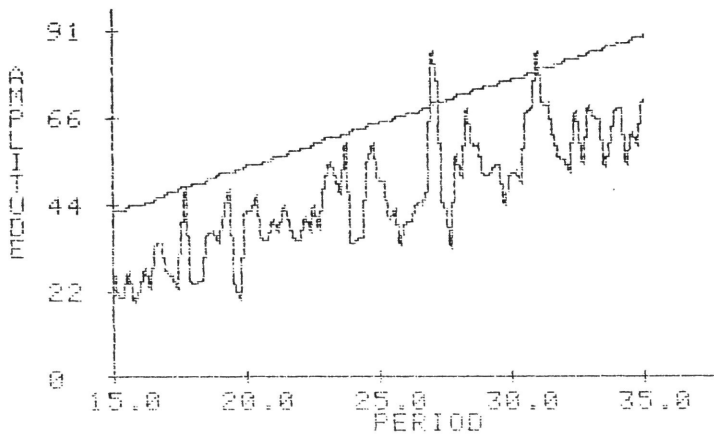
CH9.C

per+



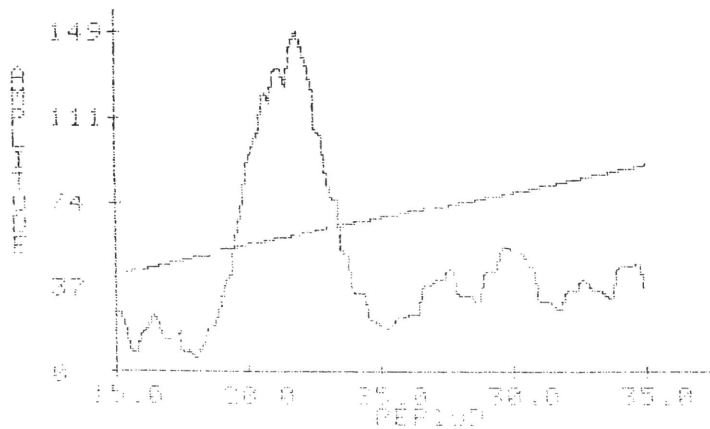
CH33.C

per0



CH29.C

G593S



a wild-type actogram. Heavy periods of activity are separated by periods of rest. The onset of activity occurs approximately 24h from the last onset. With the *per*⁰ mutant shown in the second panel of Figure 24, no discernable bouts of activity versus rest are seen. In the case of the transformant G593S (the third panel), the onset of activity occurs earlier and earlier each day indicative of a short period mutant.

The period length can be assessed from the raw data of each of the tested flies by utilizing the Chi-square analysis (Hamblen, et.al., Sokolove and Bushell, 1978). This type of statistical analysis samples intervals from the raw data and looks to find the best period fit in the range set (for example, 15-35h). Figure 25 displays the periodograms for the same wild-type, *per*⁰, and G593S flies shown in Figure 24. The greater the amplitude (as plotted on the Y axis), the better that particular test period fits the data. Therefore, the peak amplitude would be the best period fit for the data. For the wild-type example in Figure 25, this corresponds to approximately 24h. A single peak indicates a strong single period fit. The sloping line represents the confidence level for the analysis which is set at 5%. Amplitudes above this line are statistically significant. For the *per*⁰

fly, no single peak emerges as significant. For the transformant G593S, a 21.8h peak results.

Statistically significant period lengths were assessed and recorded for all constructs created. A mean period length \pm the standard deviation was calculated for each individual line of each construct. To generate an overall period length for a construct, a mean period length was again calculated from the period lengths of the individual lines and the standard error assessed according to Stickberger (1968).

2. Two point Eclosion Assay

For each genotype being tested, flies are placed into 2-4 bottles containing fly media. The controls (per^0 ; ry^{42} , per^S , per^L , and Canton S) are also prepared at this same time. Generally, per^S flies develop faster than the other controls, therefore it is usually prudent to set up more bottles of this genotype one to two days after the bottles of the other genotypes have been started. The adult flies are allowed to deposit embryos for 2-4 days upon which time the adults are removed. The bottles are transferred into an 18°C incubator and exposed to a 12L:12D cycle for the duration of the experiment.

When the majority of developing flies have reached the late pupal stages, the bottles are cleared 2-3h before lights go off to remove any recently emerged adults. Subsequently, two collections of newly emerged flies are made each day: the first, 45 minutes before lights are turned on, the second, two or three hours before lights are turned off. Flies collected at each point are counted and the data is recorded. The collections continue for five to eight days (Konopka and Benzer, 1971; Jackson, 1983)

The data is recorded and then plotted using *CricketGraph*^C on the Macintosh computer. The eclosion profiles from the test genotypes can be compared to those of the controls and the phenotype determined.

C) Determination of the molecular lesions responsible for per^1 , per^0 , and per^S .

The 5.7kb Eco RI/Hind III genomic *per* DNA fragment was isolated for the per^1 , per^0 , and per^S mutations from genomic libraries made from the respective mutant flies. Each mutant fragment was subcloned in m13mp18 and mp19, and ordered sets of overlapping deletions were generated by the method of Dale et.al.(1985). The following alterations were made in procedure : 1. The annealing reaction was

done at 65°C for 1h. 2. The reaction was scaled up five-fold (i.e. 5ug of single-stranded DNA in a 100ul volume instead of 1ug single-stranded DNA in 20ul) 3. Digestion time for EcoRI and Hind III was increased to 4h. 4. Timepoints for the exonuclease digestion were taken every 10 minutes, usually over the course of 60 minutes. The efficiency of the exonuclease reaction depended often on the supplier of the T₄ DNA Polymerase. The most consistent results were obtained with IBI T₄ DNA Polymerase. 5. For the tailing reaction, 3.0ul 1mM dGTP for mp19 or 3.0ul 1mM dATP for mp18 were added to the reaction. The tailing reaction was carried out at 37°C for 10 minutes. 6. Ligations were usually carried out for 2-3h at room temperature. Two to three uls of this ligation mix was used to transform JM109 according to published protocols (Maniatis et.al., 1982).

DNA sequencing of the deletions was completed as described (Jackson et.al., 1986; Sanger, 1981). Compressions were resolved by either by replacing dGTP with dITP (2mM) (Gough and Murray, 1983) or adding formamide (50%) to the sequencing gels (IBI). Resolution of these problems also occurred by sequencing the other strand. Sequence data was directly compared to the wild-type sequence.

D) P-element Germline transformation

1. Preparation and maintenance of transformed *Drosophila* lines.

P-element constructs containing wild-type or modified *per* genes and the selectable marker *ry*⁺ were coinjected at a concentration of 300ug/ml with the helper plasmid pii25.7wc (Karess and Rubin, 1984) at a concentration of 100ug/ml into *yper*⁰; *ry*⁴² embryos as described (Spradling and Rubin, 1982; Rubin and Spradling, 1982; Rubin, 1985; Spradling, 1986;). Survivors from the injection procedure were pair-mated with *yper*⁰; *ry*⁴² flies and G₁ progeny were selected for wild-type (*ry*⁺) eye color, which indicates successful germ-line integration of the construct. Transformed individuals were maintained as heterozygous lines by backcrossing to *yper*⁰; *ry*⁴² or mated *inter se* until homozygous. Alternatively, some of the transformed lines were mated to *yper*⁰; *CyO/Sco*; *ry*⁵⁰⁶/*TM3* to a) determine the chromosome upon which the construct was inserted and b) select for homozygous lines.

Plasmid DNAs for injection were prepared initially by cesium chloride banding (Rubin and Spradling, 1982). Subsequently, a modified alkaline plasmid preparation utilizing Proteinase K and RNase A was used (Victoria Corbin, personal communication,

Maniatis et.al., 1982). This method gave high quality DNA quickly (one day), yet this DNA could be difficult to inject due to carbohydrates which were not removed in the procedure. An alternative procedure which utilized an alkaline plasmid preparation followed by passage of the DNA over Quiagen columns (Diagen GmbH, 1990) has yielded high quality, easily injected DNA in a fraction of the time necessary for the other method (< 1day). Transformation efficiencies appear to be approximately the same in comparison to the other methods of DNA preparation.

2. Transformation Constructs

a) 7.1kb *per*⁺ construct -- RNA dosage experiments

The preparation of this construct and the subsequent injection in *yper*⁰; *ry*⁴² embryos is detailed elsewhere (Bargiello et.al., 1984). For comparison to the other constructs detailed in this study, however, a brief description of the preparation of the 7.1kb *per*⁺ plasmid (pCp1) is included.

All *per* fragments originate from the phage ZW106 (Bargiello and Young, 1984; Jackson et.al., 1986). A 7.1kb fragment containing all the *per* coding sequences is generated from a Hind III digest

of this phage. This fragment was subsequently cloned into the 10.8kb Carnegie 20 transformation vector (Rubin and Spradling, 1982; Spradling, 1986) which was partially digested with Hind III. Restriction mapping confirmed the insertion of the *per* fragment into Carnegie 20. The orientation of the *per* transcription unit to that of the *rosy* marker's was "head to head" (3'-5', 5'-3') to eliminate any possibility of read-through from one gene to the other.

b) Controls -- the 12.3kb construct design

To guard against the position effects seen with the 7.1kb construct, more flanking DNA was necessary (Spradling and Rubin, 1982; Goldberg et.al., 1983). The construction of the subsequent vectors was done to add more 5' and 3' flanking regions; however, this cloning was planned to insure rapid, efficient cloning and to provide the maximum flexibility for the preparation of modified *per* genes.

An approximately 14-15kb SphI DNA fragment isolated for the phage ZW106 was subcloned into the 4.2kb pEMBL20 vector (Dente et.al., 1983) and was designated p20SphI106. This fragment contains all of the *per* coding sequences as well as 5' and 3' flanking sequences. For all the cloning steps, the DNA fragments were separated on agarose gels

(Maniatis et.al., 1982) and were then purified by electroelution (IBI). Restriction mapping and/or sequencing confirmed the identity of the fragments. In addition for each cloning step, linearized vectors were treated with calf intestinal alkaline phosphatase (Boehringer Mannheim) to increase cloning efficiency (Maniatis et.al., 1982).

To increase the amount of 3' flanking sequence, a ~0.7kb Hind III- BamHI fragment was isolated from p20SphI106. The overhanging ends generated by the restriction enzymes were filled in with dNTPs and the Klenow fragment of DNA polymerase (Maniatis et.al., 1982). This "blunt-ended" fragment was subcloned into a SphI digested and blunt-ended (with T₄ DNA Polymerase, Maniatis et.al., 1982) pEMBL20 vector. After the selection of the properly oriented fragment through a diagnostic set of restriction enzymes (Eco RV, for example), the fragment was subsequently removed from this vector by digestion with HindIII and BamHI. These were located in the polylinker of the vector pEMBL20. This seemingly redundant cloning step was required such that the unique sites included in the pEMBL20 polylinker between the HindIII and BamHI sites, particularly XhoI, could be included in this per fragment. These sites will later facilitate cloning of the reconstructed per gene into the Carnegie 20

transformation vector. This modified HindIII - BamHI fragment was subcloned into the -7zf(+) vector (Promega) linearized by HindIII and BamHI digestion. This vector plus insert was designated -7zfgpa.

The main body of the *per* gene was isolated as a 4.8kb XbaI - HindIII fragment. -7zfgpa was linearized with XbaI and HindIII and subsequently, the XbaI - HindIII *per* fragment was subcloned into this vector. This new vector with combined inserts (all of which are correctly oriented for proper transcription) was labelled -7zfper⁺ XbaI/XhoI.

The promoter and 5' flanking region of the *per* gene was isolated as an approximately 6.5kb SphI - XbaI fragment from p20SphI106. This fragment was cloned into -7zf(+) linearized with SphI and XbaI and was designated -7zfSphI/XbaI promoter. To complete the construction of the entire *per* gene, the SphI - XbaI "promoter" fragment was subcloned into the SphI and XbaI linearized -7zfper⁺ XbaI/XhoI vector. This new construct is designated -7zfper⁺ SphI/XhoI.

For germline transformation, the reconstructed *per* gene is cloned into the transformation vector, Carnegie 20 (Rubin and Spradling, 1982; Spradling, 1986), This was accomplished first by digesting Carnegie 20 with the restriction enzyme, SalI. -7zfper⁺ SphI/XhoI was digested with XhoI to liberate

the entire *per* fragment (~12.3kb). XhoI and SalI restriction sites have compatible "sticky ends" which facilitates the cloning of this large *per* XhoI fragment into the large Carnegie 20 vector. Correctly cloned fragments are ascertained by diagnostic restriction endonuclease digestion with HindIII and EcoRI. The "correct" inserts, in addition, have the *per* transcription unit in opposite orientation to the *rosy* transcription unit. For constructs containing point mutations (see below), the reconstructed *per* gene in the Carnegie 20 vector was checked by double-stranded sequence analysis (detailed later in experimental procedures) to confirm the mutation prior to injection into embryos. This overall construct scheme is used for all the constructs subsequently built.

The p[s] control was constructed as follows. A 2.504kb SmaI - HindIII fragment containing the *per*^S mutation was isolated from a pSp64 vector containing the 5.7kb EcoRI - HindIII fragment from the *per*^S gene. This fragment has been shown to contain the nucleotide altered in the *per*^S gene. The -7zf*per*⁺ XbaI/XhoI vector was digested with SmaI and HindIII to remove the homologous *per*⁺ fragment. The 2.504kb SmaI - HindIII *per*^S fragment was cloned into this vector, thereby replacing the wild-type *per* fragment with that from *per*^S. The new -7zf *per*^S XbaI/XhoI

vector subsequently had the promoter region added and was cloned into the transformation vector as described above.

The construction of the p[l] control utilized the following restriction fragments. A 2.281kb XbaI - SmaI fragment from pSp64 EcoRI/HindIII *per*^L was isolated. This particular XbaI - SmaI fragment contains the *per*^L mutation. As with the p[s] cloning, the homologous *per*⁺ fragment from -7zf*per*⁺ XbaI/XhoI was removed by XbaI and SmaI restriction enzyme digestion. The *per*^L XbaI - SmaI fragment was then cloned into this vector as a replacement. Addition of the promoter region to this -7zf *per*^L XbaI/XhoI construct and the subsequent cloning into Carnegie 20 was as described above.

c) p[l/s] construct

The -7zf*per*^S XbaI/XhoI construct was digested with XbaI and SmaI restriction endonucleases to remove a 2.28kb fragment. This fragment does not contain the *per*^S mutation. The homologous 2.28kb XbaI - SmaI fragment carrying the *per*^L mutation (see above) was then cloned into this *per*^S containing, XbaI and SmaI digested, -7zf *per*^S XbaI/XhoI vector. This cloning step generates a *per* fragment (XbaI - XhoI) containing both the *per*^L mutation and the *per*^S mutation. The promoter region was added and the

newly constructed per gene cloned into Carnegie 20 as detailed above.

d) p[3'P*site]

Alteration of the cAMP-dependent phosphorylation site was completed by changing the potential targets of the kinase -- the serines and threonines (amino acids 1202 - 1205). A 1.5kb BamHI - HindIII fragment containing this phosphorylation site was subcloned into -7zf(+) (Promega) from the -7zf *per*⁺ XbaI/XhoI construct.

The resultant -7zf 1.5kb BamHI - HindIII construct was partialled with DdeI restriction endonuclease for 5 minutes at 37°C. The overhanging ends of the restriction site were filled in using dNTPs and the Klenow fragment (Maniatis et.al., 1982). Subsequently, the correct DdeI partial fragment (as determined by size on an agarose gel) was purified and SalI linkers (10mer, NEB) were ligated into this site. After an overnight incubation at 22°C, the ligation was digested first with SnaBI restriction endonuclease for 4h at 37°C. This digestion was followed by an increase in salt to 150mM and the subsequent addition of SalI. Digestion was continued for 2 more hours, and the restriction site ends filled in with dNTPs and Klenow as above. Ligation of these new ends was completed

overnight at room temperature. This manipulation resulted in the removal of ~68bp without changing the reading frame of the *per* gene. The recircularized plasmids were transformed into HB101F+. Correct minipreps were selected initially by restriction endonuclease digestion with Sall, SnaBI, and DdeI. The final confirmation of the correct minipreps was completed by DNA sequencing (Jackson et.al., 1986; Sanger, 1981). Single stranded DNA was generated from the -7zf 3'P* 1.5 kb BamHI - HindIII vector using the helper phage R408 (Russell et.al, 1986). DNA sequencing was performed with Sequenase Version 2.0 according to the manufacturer's protocol (USB). The new amino acid sequence created in this area is shown in Part I results, Table 6.

e) *per*^S regional mutagenesis

1) Construct scheme

A 531bp SmaI - XmnI fragment (nucleotides 4730 - 5261) which contains the location of the *per*^S change (nucleotide 4793) was chosen for further mutagenesis. This size fragment was selected for ease in manipulation for the mutagenesis procedure as well as efficient assessment of mutagenesis by DNA sequencing. For mutagenesis, the wild-type SmaI

- XmnI fragment was cloned into -7zfX1. The -7zfX1 vector was created by first eliminating the XmnI site in the ampicillin resistance gene (Sutcliffe, 1978) of -7zf(+) (Promega) by changing one nucleotide. Since it was a third base of the codon, this nucleotide substitution had no effect on the reading frame nor the amino acid; therefore, it did not destroy the ampicillin resistance selection conferred by the plasmid. The removal of the XmnI site was accomplished by oligonucleotide-directed *in vitro* mutagenesis (Zoller and Smith, 1982). The oligonucleotide was prepared by the Rockefeller University protein sequencing facility. Enrichment of the number of mutagenized plasmids was obtained by using the Amersham oligonucleotide-directed *in vitro* mutagenesis kit (RPN.1523) which is based on the methods of Eckstein and his colleagues (Taylor et.al., 1985). Final selection of the mutated vector was digestion with XmnI and the correct vector labelled -7zfX.

A new XmnI site was created in the polylinker of -7zfX by digesting this vector with EcoRI, filling in the ends with dNTPs and the Klenow fragment of DNA polymerase, and afterward, religating. This vector, -7zfX1, then, has the necessary sites available in its polylinker for cloning the SmaI - XmnI per fragment.

After mutagenesis of the SmaI - XmnI *per*⁺ fragment, which will be described below, the SmaI - XmnI fragment containing a mutated sequence was subcloned back into -7zf*per*⁺ XbaI/XhoI construct which had the wild-type, homologous region previously removed. The addition of the promoter region and the subsequent cloning into the Carnegie 20 transformation vector was as described previously.

2) Oligonucleotide-directed mutagenesis of the SmaI - XmnI *per*⁺ fragment

Random mutagenesis (Ner et.al., 1988; Hubner et.al., 1988) was selected in order to maximize the number and type of changes in this region. After preparation of a variety of alterations, I could select from this pool particular types of mutations for further study.

The oligonucleotides employed for this random mutagenesis were created in either of two ways. Both methods of preparation were novel for the Rockefeller protein sequencing facility; however, this group was willing to work with me in creating these oligonucleotides. Both methods employed contamination or "spiking" of the phosphoramidite mixes used in the preparation of the oligonucleotides such that degeneracy in a base or

bases of the oligomer occurred. The major difference in the methods lay in the number of positions which have the possibility of being changed.

Method I was a slight variation on the standard operation of the oligonucleotide synthesizer in the Rockefeller facility. A 29mer oligonucleotide which extends from nucleotide 4783 to 4812 was initially selected as it includes the amino acid altered in the *per^S* gene. Based on the relative frequency of occurrence in the first and second positions of the codons in the 29bp region, two bases were chosen for mutagenesis (in this example, C and T). Therefore, a total number of 10 positions in this 29bp region had a chance of being substituted. Whenever the sequence required a C or a T during the preparation of the oligomer, a "spiked" or degenerate mix of nucleotides were supplied instead of 100% C or 100% T, respectively. For example, the machine would add phosphoramites from three channels when a "C" was required in the sequence: two channels were 100% C and 1 channel was a 1:1:1:1 mix of A:G:C:T. The level of degeneracy at a particular C or T in the sequence using this method was ~24%. In this way, theoretically, a population of different oligonucleotides were created, each having at least one particular base changed. With this level of degeneracy, it was not unusual to have 2 or 3

changes in an oligonucleotide. Only two bases (C and T in this example) could be selected due to the set up of the oligonucleotide synthesizer (Seven channels are available -- The four nucleotides A,G,C,T, the two extra doses necessary to adjust for the correct level of contamination -- C and T, and the contaminated, 1:1:1:1 mix of A:G:C:T).

The second method was based on the random mutagenesis scheme performed by Knowles and his colleagues (Hermes et.al., 1989). With this procedure, every position in the chosen sequence has a chance to be mutated. Each phosphoramidite mix used in the preparation of the oligomer is contaminated with an equimolar mix of the four bases. The level of contamination was set at 9.37% which would be expected to give a 1 in 10.66 chance of error for each base in a 32 base oligonucleotide. A lower percentage of contamination was tried (2.78%); however, the efficiency for mutagenesis was significantly reduced (almost all wild-type sequences were derived). This method, like that of Method I, would yield a family of oligonucleotides each having on average, 1-3 errors/oligo. It should be noted that with Method II, a) third base or silent changes can be as prevalent as first base changes, b) stop codons can be generated as well, and c) more frame shifting, deletions, and additions

of bases are seen. However, in the final analysis, both methods gave approximately the same type and number of mutations. For example, the number of single changes were approximately the same.

Using the oligonucleotides generated by methods I and II, mutagenesis of a portion of the SmaI - XmnI fragment was eventually completed. In particular, a 40 amino acid stretch around the the amino acid position which I found was altered in the *per*^S mutant was covered. The actual procedure followed for mutagenesis was that supplied by the Amersham oligo-nucleotide-directed in vitro mutagenesis system (kit RPN.1523). The following changes, however, were instituted. 1) The single stranded template DNA was produced from the -7zfx1 *per*⁺ SmaI/XmnI construct with the use of R408 as previously described (Russell et.al., 1986). 2) The ratio of oligonucleotide:single-stranded template was varied: ratios of 2:1 as well as 5:1 and 10:1 were tried. 3) Annealing of the oligonucleotide to the template was performed by boiling the preparation for 5 minutes, transferring it to 37°C for 30 minutes, then placing it on ice. 4) The optional ammonium acetate precipitation step was included. Twenty microliters of this resuspended DNA (total volume = 100ul) was used to transform AG1

competent cells. The AG1 strain was from Statagene and were made competent using standard procedures (Maniatis et.al., 1982).

Analysis of the mutant progeny was performed directly by double stranded sequencing of miniprep DNA from individual colonies from the above bacterial transformation. The double stranded sequencing was done with Sequenase version 2.0 as directed by the manufacturer. The conditions for the denaturation of the double stranded plasmids are as follows: 2-4 ug of plasmid DNA were denatured for sequencing by the addition of 6ul of 1N NaOH in a volume of 30ul for 30minutes at 37°C. 73ul of 3:70 mix of 3M acetate pH 5.0: ethanol was added to neutralize and then precipitate DNA. After a 70% ethanol wash, the DNA was resuspended in 7ul ddH₂O. Addition of the 1ul of the sequencing primer (2.5ng/ul) and 2ul of the 5x Annealing buffer then followed. This mix was then heated to 65°C for 5 minutes and allowed to cool slowly (> 30 minutes). The remainder of the protocol was that provided by USB.

Mutants with alterations which resulted in single, double or triple amino acid changes were selected for further sequence analysis (the remainder of the 531bp in each case) to insure that these were the only alterations. Mutant progeny

which had the desired changes could then be digested with SmaI and XmnI restriction enzymes to begin the reconstruction of the new *per* gene as detailed in the previous sections. The changes created in this area are listed in Part I results, Table 10.

E) Northern Analysis

Poly A⁺ RNA from whole adult flies was isolated as previously described (Bargiello and Young, 1984). Five micrograms of the RNA was electrophoresed through 1% agarose/2.2M formaldehyde gels and transferred to Biodyne A membrane (Pall) according to the manufacture's specifications. The 1.5kb HindIII - EcoRI *per* fragment containing the first exon of the *per* gene was subcloned into the riboprobe vector pSp65 (Promega). Single-stranded ³²P labelled RNA probes can be produced from the cloned insert in this vector as described (Promega, Maniatis et.al., 1982). Subsequently, the labelled RNA probe was hybridized to the membrane bound *Drosophila* RNA in the following solution : 50% formamide, 50mMNaPO₄ (pH 7), 0.8M NaCl, 1mM EDTA, 0.1% SDS, 1x Denhart's solution, 250ug/ml denatured salmon sperm DNA, 500ug/ml yeast RNA, and 10ug/ml poly(A) at 60°C. Prehybridization was carried out with the same solution minus the probe for 2-3h at

60°C. After hybridization overnight, the membrane was washed in 5mM NaCl, 2mMNaPO₄ (pH 7), 1mM EDTA, 0.1% SDS. The filter was exposed for 1-2 days at -70°C. The ³²P-labelled actin DNA probe (pDmA2) was also hybridized as described (Bargiello and Young, 1984; Fryberg et.al., 1983).

Quantification of the amount of the per poly A+ RNA was completed by Dr. M. Young. Autoradiographs were analyzed by densitometry. Peaks were cut and weighed, and the relative levels of the 4.5kb per RNA were determined after normalization with the actin mRNA. *In situ* hybridizations to polytene chromosomes was also carried out by M.Young as described (Pardue, 1986).

II. Cell Level Analysis of per Function

Preparation of the salivary glands from third instar larvae

Flies were grown on standard media as described in Part I at room temperature (variation 20 - 29°C). No particular L:D cycle was imposed on these flies initially. However, at a later date, the flies were placed into light and temperature controlled incubators (~12L:12D cycle, 24-25°C). Flies were

transported from the Young laboratory to our collaborators' laboratories (Dr. David Spray initially, Dr. Vito Verselis and Dr. Ted Bargiello, recently) at Albert Einstein College of Medicine.

Using a dissection microscope, the salivary glands from third instar larvae were dissected in *Drosophila* Ringer's solution (Roberts, 1986) or MDM solution. MDM consists of the following: 2mM CaCl_2 , 21.5mM KCl, 15.0mM MgCl_2 , 35.9mM NaCl, 4.76mM NaHCO_3 , 5.29mM trehalose, 11.0mM glucose, 10.0mM Hepes pH 7.0 (start with free acid), and add 1.8g/L glutamine for non-EM applications (Ted Bargiello, personal communication). The dissected glands were freed of extraneous tissues such as fat body. The tissue was then transferred to a clean dish of the same media and carefully stuck to the bottom of the plastic dish. Usually, five to six pairs of glands were placed at the bottom of a dish. The dish was then given to the electrophysiologist, Dr. David Spray (early experiments) or Dr. Vito Verselis (recent experiments) for either Lucifer yellow injections (5% w/v in 150mM LiCl) or electrophysiological recordings. Further details for each of these assays are described elsewhere (Verselis et.al., 1991; Bargiello et.al., 1987; Spray et.al, 1984; Bennett, 1966). In all cases, the "health" of the preparation was determined by

recording the resting membrane potential of the cells of the glands. Glands with cells having a resting membrane potential which fell outside of the -35 to -55mV range were not assayed any further. Individual salivary gland cell pairs were made by a brief treatment of the isolated salivary glands with 0.1% pancreatin followed hand dissection.

For the heat shock experiments, third instar larvae of a particular genotype were placed in a glass vial containing a dampened cotton wad at the bottom of the vial. A dry cotton ball was used to stopper the vial. The vials were then emersed to the level of the cotton stopper in a 36.5°C water bath for 30 minutes. The larvae were allowed to recover at room temperature for a variety of times, from no recovery to 4-6h. The glands were then dissected as described above.

Generally, the experiments are performed with the electrophysiologist being "blind" to the genotype of the salivary gland. Given the resting membrane potentials and results from the assay (for example, photographs of the Lucifer Yellow injections), the genotype of gland was assigned, and then checked against the key for the experiments performed that day.

III. The embryonic localization of per

A) Fly strains

Flies were cultured on the standard media described in the first section of the Experimental Procedures. The fly strains utilized in these experiments were also specified in that section with the addition of *sim^{H9} kar/ mkrs* (Nambu et.al., 1990) and *sli^{1G107} cn bw sp/ CyO* (Rothberg et.al, 1988).

B) RNA localization

In situ hybridization to embryo whole mounts using digoxigenin labelled probes (Boehringer Mannheim protocol, Tautz and Pfeifle, 1989) were carried out using a set of protocols compiled by Rick Garber (personal communication). Briefly, dechorionated embryos were fixed and devitellinized using Protocol 90 from *Drosophila -- A Laboratory Manual* (Ashburner, 1989). All procedures were carried out at room temperature except where noted. The devitellenized embryos were pre-cleared as follows: 2 X 5 minute wash with 100% ethanol, 1 X 5 minute wash with a 1:1 ethanol:xylene mix, and then an incubation over a period of several hours with xylene. After the xylene incubation, the embryos were washed with a 1:1 ethanol:xylene solution 1 X 5 minutes, and then 2 X 5 minutes with 100% ethanol (Bruce Edgar, personal communication). Thereafter,

the embryos are stored at -20°C in 100% ethanol until the hybridization.

The 515bp *per* EcoRI - BglII fragment was isolated from a *per* cDNA clone and contains the first and part of the second exons. This cDNA clone was isolated by F. Rob Jackson. After subcloning it into pEMBL20, I characterized the phage insert by restriction mapping and DNA sequencing. It is almost a full length cDNA: it is missing the first 125bp from the first exon. This exon, however, contains 5' noncoding information. The *per* AUG is in exon 2 (Jackson et.al., 1986; Citri et.al., 1987; Baylies et.al., 1987), thus all the coding regions of *per* are intact in this cDNA. After purification, the 515bp EcoRI - BglII fragment was resuspended in DEPC-ddH₂O at a concentration of ~100ng/ul and stored at -20°C until use.

Probes were labelled by random priming (Feinberg and Vogelstein, 1983) using high concentration of primers (5mg/ml final concentration) and low temperatures (15°C) to generate short probes (Charlie Oh, personal communication). The probes were ethanol precipitated, washed with 70% ethanol, and then resuspended in hybridization solution (5XSSC, 50% formamide, 100ug/ml yeast RNA, 100ug/ml denatured, sonicated salmon sperm DNA, 10mM NaPO₄ pH 7.2, 1X

Denharts, 50ug/ml heparin, 0.1% Tween-20 and 5% dextran sulfate). Embryos were prepared for hybridization essentially as reported by Tautz and Pfieffe (1989). Embryos were hybridized overnight at 52°C using approximately 1ug/ml probe.

The embryos were then washed at 52°C as described by Tautz and Pfieffe (1989). The final wash was in 1X PBS, 0.1% Tween-20 (PBT) for 20 minutes. Afterward, the embryos were washed again 4 X 5 minutes in PBT. Subsequent detection of the hybridized probe was essentially as detailed by Tautz and Pfieffe (1989) using alkaline phosphatase conjugated sheep anti-digoxigenin antibody (Boehringer Mannheim). Alkaline phosphatase activity was then detected as specified in the Genius Kit manual (Boehringer Mannheim). Embryos were later mounted in 80-90% glycerol in PBS. The photographs were taken using Normarski optics on the Zeiss Axiophot microscope.

C) per immunocytochemistry

The preparation of the *per* antibody, 19-47, has been reported in detail elsewhere (Saez and Young, 1988). The "flat prep" or "fillet" protocol was kindly provided by Dr. Michael Bate. Embryos from Stage 13 (9.5h) to early stage 16 (~15h) were selected for dissection. At this point of

development, the central nervous system has a more defined and characterized morphology (Jacobs and Goodman, 1989a, 1989b; Thomas et.al., 1984; Klammt et.al., 1991).

Embryos were first dechorionated with 50% bleach, then washed well with ddH₂O. Correctly staged embryos were selected under the dissection microscope and then dissected flat (ventral side stuck down) on poly-lysine coated microscope slides. These slides had been first prepared with aquarium sealant to form an "aqueous-tight" well within which the embryonic dissections would occur. After treatment with poly-lysine (Sigma L-1524), the slides would be ready for the dissection. On average, four to six flat preps were done on each slide.

The immunocytochemistry was performed essentially as described in Kidd et.al., 1989. The following alterations were made to tailor the procedure for per:

- 1) The embryo fillets were fixed for twenty minutes in PLP fix (PLP = 2% paraformaldehyde in 1X PBS, 0.075M lysine, and 2.5mg/ml sodium m-periodate (McLean and Nakane, 1974)). This fix particularly cross links carbohydrates and has lead to reduced background staining with the per antibody.

2) A 1:100 dilution of the 19:47 antibody was generally used for the *per* immunocytochemistry. The antibody was incubated with the tissue overnight at room temperature.

3) The Vectastain ABC "Elite" kit was used instead of the standard kit. This allowed for easier visualization of the *per* signal.

All other steps were followed as detailed elsewhere (Kidd et.al., 1989). After clearing in methylsalicylate overnight, the flat preps were mounted in cedarwood oil. Photographs were taken with Normarski optics on the Zeiss Axiophot microscope.

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