Genetic Analysis of the Circadian Clock System of Drosophila melanogaster

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Randall F. Smith

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Abstract

The circadian rhythm phenotypes of eight chromosome aberrations with a breakpoint in the region of the *per* locus (3B1-2) of *Drosophila melanogaster* have been analyzed. Two duplications and five deficiencies with a 3B1-2 breakpoint produce either a wild-type (approx. 24-h period) or an arrhythmic clock phenotype while one translocation with a 3B1-2 breakpoint, *T(1;4)JC43*, produces locomotor-activity rhythms with either very-long periods (31- 39 hr), rhythms that grade into arrhythmicity, or completely arrhythmic phenotypes. The clock phenotypes of 3B1-2 chromosome aberrations suggest that arrhythmicity results from the total lack of *per* function while long-period phenotypes result from a reduction, but not complete elimination, of *per* activity. An extensive complementation analysis of 3B1-2 chromosome aberrations and *per* mutant alleles provided no compelling evidence for genetic complexity at the *per* locus. This is in contrast to the report of Young and Judd (1978). Analysis of both the locomotor-activity and eclosion phenotypes of 3B1-2 chromosome aberrations did not uncover differences in the genetic control of these two rhythms.

The normal 24-h period of the circadian rhythms of locomotor activity and eclosion of *Drosophila* is shown to be altered by changes in *per* gene dosage. Females with only one dose of per^+ or per^s (the 19-h short-period mutant allele) or per^l (the 29-h long-period mutant allele) have periods which are about 1-2 h longer than the corresponding females with 2 doses. Females with 3 doses of per^+ and males with 2 doses of per^+ or per^s have periods which are ½ to 1 h shorter than the corresponding individuals without the extra dose. Males with three per^+ doses have periods which are about 1.5 h shorter than wild-type males; additional per^+ doses do not shorten period further. The observation that decreased *per* dosage lengthens period while increased dosage shortens period suggests that the long- and short-

period mutations alter period by respectively decreasing and increasing *per* gene or gene product activity. The per^+ dosage results and the complementation behavior of per^s indicate that the hypermorphic phenotype of per^s results from increased activity of the per^s gene product rather than an overproduction of per^+ product. This is the first report of such a mutant action in *Drosophila*.

By screening mutagenized sex-linked and autosomal stocks for ones in which the normal period or phase of the circadian rhythm of eclosion (adult emergence) has been altered, a new X-linked clock mutant has been isolated which lengthens the normal 24-h period of both the the eclosion and adult locomotor-activity rhythms to about 25.5 h. This mutant, which we have named Andante (*And*), is not an allele of the *per* locus; recombination and deficiency mapping has placed the Andante locus at a separate site between polytene chromosome bands 10E2 and 10F1 (tentatively at 10E3, just proximal to the *m-dy* complex at 10E2-3). Andante, like all of the *per* mutant alleles, has a semi-dominant effect on period. The eclosion rhythm of Andante, like wild-type, has a low-amplitude (Type 1) phase-resetting response to light pulses, but compared to wild-type the Andante phase-resetting curve (PRC) is lengthened by 1-2 h per cycle.

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Chapter 1

General Introduction:

The Molecular Basis of Circadian Time-keeping

1.1 Introduction.

A wide variety of biological activities, ranging from subcellular processes (e.g. enzyme activities) to behavioral patterns (e.g. sleep/wake activity), are temporally regulated by endogenous time-keeping systems (see Aschoff 1981; Winfree 1980). Those biological clocks which generate rhythms of activity with a period of about one day in the absence of daily environmental cues are termed circadian. Circadian clocks are a ubiquitous feature of all major groups of eucaryotic organisms and have presumably evolved as an adaptive response to an environment which changes in a predictable fashion with each rotation of the earth.

Circadian clocks share the following basic features (see Hastings et al 1976). In the presence of environmental cues (called zeitgebers) such as daily light and temperature cycles, circadian clocks become synchronized to (entrained by) the external cycle and maintain a distinct phase-angle relationship to the entraining zeitgeber. In the absence of daily cues (i.e. under "free-running" conditions) circadian rhythmicity can be phase-shifted (advanced and/or delayed) by single zeitgeber signals. A plot of the phase at which a signal is given vs. direction (advance/delay) and magnitude of the phase shift is known as a phase response curve (PRC). Since the phase-shifting response elicited at a particular phase is constant from cycle to cycle, PRCs provide a measure of the internal state of a clock throughout the course of a cycle.[1] Circadian rhythms also share the basic feature that their free-running period is temperature-compensated, i.e. they exhibit a Q_{10} very close to 1.

This introduction will review the diverse body of literature that pertains to the molecular basis of circadian rhythmicity, with emphasis on the analysis of the central mechanisms involved in circadian time-keeping. In most cases comparison between studies will be limited to those involving the same circadian system since

1. The measurement of the phase-shifting response to various stimuli is currently the the only method of examining the temporal structure of a circadian cycle.

at this time it is not known whether there is a single common molecular basis for all circadian clocks. Other aspects of circadian biology addressed by these studies will not be presented.

Four major approaches are currently being used to study circadian clock mechanisms. One approach involves analyzing the mechanisms by which the expression of a rhythm is temporally regulated in order to trace a pathway of regulation to its source, the clock mechanism. A second approach involves analyzing the molecular basis of entrainment in order to follow a pathway of entrainment into the clock mechanism. A third approach involves the administration of various drugs known to effect specific biochemical/biophysical processes to determine their effect on clock expression. The fourth approach is a genetic analysis of circadian systems. Mutations with known biochemical deficiencies are analyzed for their effects upon clock properties and mutations with altered clock properties are isolated and characterized to determine the nature of the clock defect.

1.2 Tracing Pathways of Circadian Regulation

In order for any type of biological process to be temporally regulated, it must be linked to a pacemaking system via some pathway of control. Thus by identifying the mechanisms by which the expression of a rhythm is temporally controlled at progressively higher levels of regulation, it might be possible to trace a pathway of temporal regulation to its source, the clock mechanism. Control of the expression of rhythmic activity has been best characterized in two systems, the luciferase/luciferin rhythm in *Gonyaulax* and the *N*-acetyltransferase/melatonin rhythm in the rat and chick pineal.

The circadian rhythm of bioluminescence in the marine dinoflagellate *Gonyaulax polyedra* is currently being used as a model system to study the circadian control of enzyme activity (see Dunlap et al 1981; Dunlap and Hastings 1981). Bioluminescence is the product of the oxidation of the substrate luciferin by molecular oxygen, catalyzed by the enzyme luciferase. Luciferase activity in extracts made in the middle of the night phase is 7-10 times greater than in extracts from day phase cells. The specific activity of the night- and day-extracted luciferase, however, is identical and there do not appear to be any physiochemical nor immunological differences between night and day species. Control of the enzyme rhythm via inhibitors or activators has also been ruled out. These results suggest the circadian rhythm in luciferase activity is the result of a change in the concentration of enzyme molecules controlled by either a rhythm in the synthesis or degradation of this protein. Unfortunately, a direct measurement of timed synthesis and destruction of luciferase is impossible because *Gonyaulax* fails to take up exogenously added amino acids.

Another enzyme system in which the circadian control of activity has been examined is the *N*-acetyltransferase (NAT) rhythm in the pineals of mammals and birds.

(see Zatz 1980; Jacklet 1981). The pineals of rats and chickens exhibit a circadian rhythm in the level of melatonin a 10-fold increase in concentration occurring during the night. This rhythm is regulated by a circadian rhythm in NAT activity which exhibits a 30-100 fold increase in enzymatic activity during the night (Klein and Weller 1970; Binkley et al 1973). The circadian rhythm in NAT activity is regulated by cyclic AMP, which both induces the synthesis of this enzyme (Axelrod and Zatz 1977; Deguchi 1979a,b). In mammals, changes in cAMP levels in the pineal are regulated by a circadian rhythm in norepinephrine release (Axelrod and Zatz 1977; Nir et al 1978). Norepinephrine is released from nerve terminals in the pineal and binds to beta-adrenergic receptor, stimulating adenylate cyclase activity. The sympathetic neurons which innervate the pineal originate in the superior cervical ganglia, and the regulation of norepinephrine release is apparently controlled by a central neural clock in the suprachiasmatic nucleus (SCN) of the hypothalamus (Moore and Klein 1974; Rusak and Zucker 1979). The identification of the next highest level of circadian control of NAT activity in mammals would involve an analysis of the circadian control of neuronal activity in the SCN, obviously a formidable task.

In chickens, circadian regulation of NAT activity is under endogenous control of the pineal itself: the NAT rhythm in chicken pineals is not affected when sympathetic input into the pineal is blocked surgically (Binkley 1976) or pharmacologically (Deguchi 1979a) and organ cultures of pineal glands exhibit a circadian rhythm of melatonin release and NAT activity which persists (although damped) for 2-4 cycles under constant conditions (Kasal et al 1979; Takahashi et al 1980). Dispersed cell cultures of pineal glands have been shown to exhibit a rhythm of NAT activity for at least two days in constant conditions and the NAT rhythm in these cells can be entrained to a reverse photocycle (Deguchi 1979c), suggesting that each cell has its own photoreceptor and clock.[2] Circadian fluctuations in cAMP

2. This conclusion is tentative, however, since a histogram of "cell group size" is lacking in this study.

levels are also observed in organ-cultured chick pineal (Wainwright 1980) and pharmacological studies indicate that cAMP is involved in the regulation of NAT activity in this system (Deguchi 1979a,b). Thus the identification of the next highest level of regulation of NAT in chicken pineals would involve an analysis of the circadian regulation of intracellular cAMP levels.[3]

3. As described below (§1.3 and 1.4.4), cAMP has also been implicated in having a role in both the entrainment and central time-keeping mechanisms of other circadian systems.

1.3 Tracing Entrainment Pathways

Another approach that is being used to study clock mechanisms involves tracing entrainment pathways into the clock. The rationale behind this approach is thus similar to the approach described above: since circadian clocks can be entrained by certain types of daily environmental cycles (primarily light or temperature cycles/pulses), there must exist mechanisms which couple clocks with the environment. If an entrainment pathway could be traced from a receptor activity into a clock it might be possible to identify processes involved in the primary clock mechanism. The first step in such an approach is the identification and localization of a zeitgeber receptor. Since entrainment and the phase-shifting action of the zeitgeber would be abolished by surgically, pharmacologically, or genetically deleting the receptor or any part of the entrainment pathway, the pathway of entrainment can be traced by blocking the pathway at increasingly higher levels as it enters the clock system. The clock could then be localized to that level at which a blocking treatment disrupts clock function rather than merely blocking entrainment.

Such a tracing of an entrainment pathway has been successful on an anatomical level in some animal systems where circadian photoreceptors that mediate entrainment and the clock which controls rhythmicity are anatomically distinct (see Menaker et al 1978; Rusak and Zucker 1979). In cockroaches and rats, for example, the eye contains the photoreceptor that mediates light entrainment of the locomotor activity rhythm while the clock controlling rhythmicity resides in the brain. Blinding these animals or interrupting the visual pathway mediating entrainment via surgical lesions blocks light entrainment without abolishing rhythmicity. By tracing entrainment pathways it has been possible to identify specific regions of the brain (the lobula in cockroaches; the suprachiasmatic nucleus in rats) in which

lesions abolish or disrupt rhythmicity.

The tracing of an entrainment pathway on cellular and subcellular levels is currently being conducted using the eye of *Aplysia* (see Eskin 1979; Menaker et al 1978). The *Aplysia* eye cultured *in vitro* shows a circadian rhythm in the frequency of compound action potentials (CAPs) recorded from the optic nerve. The eye consists of receptor cells, pigmented support cells surrounding the receptors, second- and higher-order neurons and glia cells, with gap junctions between receptor cells and between receptor and second-order cells. Spike activity is not observed in the receptor cells, indicating that CAP activity is produced by the nonreceptor cells. Treatments with either high Mg^{+2}, low Ca^{+2} or tetrodotoxin do not block phase shifting by light, suggesting that the entrainment pathway does not involve chemical synaptic transmission or action potentials. However, treatment with low Na^{+}, which reduces the photoreceptor potential, does block phase shifting by light. These results suggest that the clock resides in either the photoreceptor cells or in second-order neurons which are electrically coupled to the photoreceptor cells. Isolated photoreceptor cells cultured *in vitro* do not possess circadian light sensitivity (Strumwasser et al 1979b), suggesting that the clock does not reside in these cells, however this result does not rule out the possibility that the generation of the CAP rhythm requires cell-cell interactions between photoreceptor cells.

Of special interest in regard to the intracellular mechanism of entrainment is the observation that serotonin, a putative neurotransmitter, and a cAMP analog, 8-benzylthio-cAMP, produce similar PRCs when given as pulses to the eye (Eskin et al 1982; see also §1.4.4). The similarity in the PRCs is a particularly intriguing correlation since it has been recently shown that serotonin (or 8-benzylthio-cAMP) induces hyperpolarization of the R15 pacemaker neuron of *Aplysia*. This hyperpolarization is the result of an increase in K^{+} conductance mediated by an increase in intracellular cAMP (Drummond el al 1980). Since serotonin also increases cAMP levels (by

13-fold) in the *Aplysia* eye (Eskin et al 1982), the similarity between the PRCs for serotonin and 8-benzylthio-cAMP indicate that cAMP also mediates the phase-shifting action of serotonin in the eye (Levitan and Benson 1981; Eskin et al 1982).[4] If the phase-shifting action of serotonin is directly mediated by cAMP, it might be possible to identify the next intracellular step involved in entrainment (e.g. protein phosporylation via a cAMP-activated kinase; see Greengard 1978) that would lead to the identification of time-keeping functions. It is also possible that the cAMP system is itself part of the central clock system (see §1.4.4).

4. Strumwasser and Stephens (1981) have recently observed that the *Aplysia* eye has increased levels of cAMP when measured at the phase of the peak in the amplitude of the CAP rhythm, at about ct 2.5 (ct = circadian time, *modulo* 24 h; ct 0 corresponds to the time of light onset in a LD 12:12 cycle), compared to measurements taken at the phase of the rhythm trough (at about ct 14.5) under both LD and DD conditions. Since the peak in cAMP occurs during the day, it is possible that the phase-shifting action of light is also mediated by an increase in cAMP levels in the eye. However, the PRC of light is 180° out of phase with the PRCs of serotonin and 8-benzylthio-cAMP, suggesting that if the phase-shifting action of light and serotonin is mediated by cAMP, these agents should have an opposite effect on cAMP levels in the eye.

1.4 Drug Studies

A large and growing number of drugs and other chemical agents have been shown to affect the period and/or phase of various circadian rhythms. A major difficulty in interpreting the results of drug studies involves side effects. Even when a treatment is shown to affect a given biochemical/biophysical activity in a manner in which it is thought to act (an analysis very rarely practiced in the studies described below) the clock disruption may be due to a side effect of the drug. One method for determining the action of putative side effects is through the analysis of the effects of several different treatments having the same primary target or by the use of active and inactive analogs. Another method, which has been recently applied for the first time (described below), is the use of mutant strains which are resistant to the primary effect of the treatment.

Since the effect of the treatment may be to perturb a biochemical/biophysical activity which is not a component of the clock but which acts through a series of one or more intermediates to affect clock variables (see Tyson et al 1976), it is not currently possible to distinguish a direct from an indirect mode of action of a treatment on clock function.[5] One class of indirect effects could be those mediated by entrainment pathways. Since it is currently impossible to separate a clock from *all* portions of an entrainment pathway, the primary effect of a treatment could be on some portion of an entrainment pathway, producing a steady-state phase shift.[6] Even period effects could conceivably be the result of an indirect action of a treatment: dim light lengthens period for many circadian systems, thus a chemical treatment which produces a "dim light response" in any portion of the entrainment pathway for light could indirectly alter period.

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5. This caveat also applies to genetic perturbations of clock function (see §1.5).
6. For example, the phase-shifting action of serotonin on the CAP rhythm of the *Aplysia* eye may be the result of an effect on an entrainment pathway (see Corrent et al 1978; Eskin et al 1982).

Only those treatments observed to affect period and/or steady-state phase are described below. Results of treatments which reduce or abolish rhythmicity or which have no observed effect on rhythmicity will not be presented. Treatments which inhibit rhythmic expression without altering period (when applied continuously) or steady-state phase (when pulsed) could do so by merely blocking the output pathway from the clock rather than affecting the time-keeping processes. (Hastings 1960). Negative results are inconclusive; treatments with no observable effect on rhythmicity may or may not affect the presumed target. In most cases comparison between studies will be limited to those involving the same circadian system since a particular treatment might have different effects on clocks of different species due to fundamental differences in clock mechanisms between organisms.

1.4.1 Treatments with unknown/nonspecific targets

A number of chemical treatments with undetermined or nonspecific modes of action have been shown to alter phase or period in a variety of different circadian systems (Table 1). The effects of alcohols on circadian rhythms have been repeatedly used to implicate membrane fluidity and/or permeability in clock mechanisms (e.g. see Sweeney 1976, 1978; Njus et al 1976; Engelmann and Schrempf 1980) however the effectiveness of alcohols of graded chain lengths on phase-shifting the bioluminsescence rhythm of *Gonyaulax* is not correlated or is inversely correlated with their lipid solubility (Sweeney 1978). Acetaldehyde, the immediate metabolite of ethanol, is more effective than ethanol in phase-shifting the bioluminescence rhythm of *Gonyaulax* and the effectiveness of aldehydes in phase shifting (like the alcohols) is also inversely correlated with chain length (Taylor and Hastings 1979). Aldehydes may have multiple cellular sites of action, for example blocking protein synthesis or mitochondrial respiration or acting as a sulfhydryl reagent (see Taylor

and Hastings 1979). D_2O and Li^+ lengthen period in all circadian systems analyzed to date. Both treatments may affect a number of different cellular and subcellular activities (see Sheard 1980; Pittendrigh et al 1973) and their specific effect on clock function is not known.[7] Hormones and neurotransmitters may affect entrainment pathways as well as the clock itself.[8] The specific clock targets of the other treatments in Table 1 are also not known. In sum, the effects listed in Table 1 provide little insight into the molecular basis of circadian rhythmicity at this time although such effects may have significance once more is known about clock mechanisms.

1.4.2 Treatments affecting membrane permeability/depolarization

Treatments that appear to alter clock expression via changes in membrane permeability to ions are shown in Table 2 (listed by organism). High K^+ pulses and as well as pulses of strophanthidin (a Na-K pump inhibitor) and light produce similar PRCs for the the CAP rhythm of the optic nerve of *Aplysia* and these treatments may exert a common clock effect via depolarization (Eskin 1977b, Jacklet and Lotshaw 1981).[9] In *Gonyaulax*, valinomycin (a K^+ ionophore) and vanillic acid[10] produce dissimilar PRCs and both are different from the phase-shifting action of light (Sweeney 1974). In *Neurospora*, nystatin (which permeablizes fungal plasma membranes) and valinomycin produce PRCs similar to that produced by light

7. Li^+ is known to inhibit adenylate cyclase in many systems (see Sheard 1980) and cyclic AMP has been implicated in clock function (see below).

8. The PRC for carbachol, a cholinergic agonist, is similar to that produced by light pulses for the NAT rhythm in the rat pineal (Zatz 1981). In *Aplysia* the serotonin and light PRCs are displaced by 180°, suggesting that serotonin affects a non-ocular entrainment pathway (Eskin 1979). Serotonin and a cAMP analog, 8-benzylthio-cAMP, produce similar PRCs in *Aplysia* (Eskin et al 1982) suggesting that hormones and neurotransmitters might exert indirect clock effects via changes in intracelluar cAMP levels (see below).

9. High K^+ and strophanthidin should have opposite effects on intracellular K^+ concentrations suggesting that the primary effect of these treatments is via a change in transmembrane potential rather than a change in intracellular ion concentrations (Eskin 1979). Changes in the external concentration of other ions (Na^+, Ca^{++}, Mg^{++}) are ineffective in phase-shifting (Eskin 1977a, Eskin and Corrent 1977).

10. A substituted benzoic acid, thought to affect membrane permeability to ions. Produces membrane depolarization in *Gonyaulax* (Kiessig et al 1979).

pulses. However light *hyper*polarizes *Neurospora* membranes (Sargent and Briggs 1967) while the others are believed to be depolarizing agents in *Neurospora* (Koyama and Feldman 1981). In the bean plant, *Phaseolus*, light pulses and valinomycin produce similar PRCs, however the PRC for K^+ pulses is very different. Digitonin, a detergent, shortens period and it is possible that this is the result of changes in membrane permeability. Another compound which is thought to affect ion permeability, abscisic acid (a plant hormone), produces only phase delays in both *Phaseolus* and *Kalanchoe*. Fusaric acid, another compound which appears to have general effects on membrane permeability to ions, phase-shifts the leaf movement rhythm of *Gossypium*. While the above results suggest that membrane permeability/depolarization plays a role in clock function, direct evidence is lacking for such a role.

1.4.3 Inhibitors of oxidative phophorylation/Ca^{2+} uptake

Treatments that appear to affect clock function via inhibition of oxidative phosphorylation and/or Ca^{++} uptake by mitochondria are shown in Table 3. With the exception of one system, the leaf-movement rhythm of *Phaseolus*, these treatments produce phase delays when given as pulses. The energy of electron transport can be used in the accumulation of divalent cations or in the formation of ATP, thus uncoupling agents such as cyanide, CCmP, and dinitrophenol as well as anoxia inhibit both activities. The Ca^{++}/Mg^{++} ionophore, A23187, and Mn^{++} are also thought to inhibit both activities by causing the loss of Ca^{++} out of mitochondria while simultaneously inhibiting ATP formation by stimulating the energy-dependent uptake of this cation (see Eskin and Corrent 1977). The phase delays observed after pulsed treatment of 2-deoxy-*D*-glucose in *Aplysia* may also be the result of inhibition of ATP formation (Strumwasser et al 1979a). Lanthanum (La^{3+}), which blocks Ca^{++} currents by binding to Ca^{++} channel binding sites and which displaces

Ca^{++} from surface bound cellular sites (Weiss 1974; Hajiwara and Byerly 1981), lengthens period in *Aplysia*. In *Phaseolus*, cyanide and azide produce phase advances rather than delays but this appears to be the result of membrane depolarization rather than an inhibition of energy metabolism since the PRCs of these two compounds are out of phase with the time course of the energy requirement of this rhythm (Mayer 1981). While the role of Ca^{++} uptake by mitochondria in clock function is unclear, these studies do suggest that clock function requires an energy source, as might be expected. They also suggest that treatments which only produce phase delays or period lengthening might do so by simply poisoning the clock. These studies do not suggest, however, the means by which energy is utilized in clock function.

1.4.4 Treatments affecting cyclic AMP levels

Treatments that appear to affect circadian clocks via changes in cAMP levels are shown in Table 4. In *Neurospora*, four phosphodiesterase inhibitors, caffeine, aminophylline, theophylline and isobutylmethylxanthine, have been shown to lengthen period while an adenylate cyclase inhibitor, quinidine, shortens period. Since theophylline (from which aminophylline is compounded) and caffeine have been shown to raise intracellular cAMP levels in *Neurospora* while quinidine has been shown to lower cAMP levels (Scott and Solomon 1975), the effect of these drugs on period is correlated with their effect on intracellular cAMP levels. The phase-shifting action of these drugs, however, is not correlated with their effects on cAMP levels: caffeine and aminophylline as well as quinidine have very similar PRCs.[11] One explanation for this discrepancy is that the phase-shifting action of pulses is the result of side

11. Isobutylmethylxanthine has a much smaller effect on period and produces no significant effect on phase; theophylline was not tested for phase effects. Period and phase effects were tested under different culture conditions in these experiments; under conditions similar to those in which the phase effects were tested, theophylline ***lowers*** rather than raises intracellular cAMP levels (the effects of caffeine and quinidine were not altered). It is therefore possible that aminophylline has the same effect as theophylline under these conditions.

effects of these drugs which are masked in longer exposures by the effect on cAMP levels. The known side effects of methyl xanthine phosphodiesterase inhibitors include affects on intracellular Ca^{++} levels (Cardinali 1980) and inhibition RNA and protein synthesis (Costantini et al 1978) and other treatments which are thought to affect these activities are observed to have clock effects (see §1.4.2, 1.4.5).[12]

Phosphodiesterase inhibitors also lengthen period in each of the other rhythms in which an effect on period is observed (Table 4). In *Trifolium*, continuous application of cAMP also lengthens period but produces a PRC very different from that for pulsed applications of theophylline. Imidazole, an activator of phosphodiesterase, produces a PRC similar to that for cAMP pulses, however imidazole would be expected to lower intracellular cAMP levels. Thus for this rhythm also the immediate clock effect of pulsed treatments of one or more of these treatments may be the result of a side effect rather than a direct effect on intracellular cAMP levels.

In addition to the the period lengthening effect of phosphodiesterase inhibitors on the CAP rhythm of the *Aplysia* eye, pulses of papaverine and pulses of the cAMP analog, 8-benzylthio-cAMP, phase-shifts the CAP rhythm with a PRC similar to that for serotonin (Eskin et al 1982). While cAMP may have a central role in the time-keeping system of the *Aplysia* eye, as described above in §1.3, cAMP may be part of an entrainment pathway in the eye such that the clock effects of treaments that alter cAMP levels may have only an indirect effect on this particular clock system. In addition, a wide variety of different cellular activities are known to be regulated by cAMP (see Greengard 1978) such that any disruption of the cAMP system could have numerous direct and indirect effects on clock function.

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12. Since the regulatory actions of cAMP and Ca^{++} are linked in many cases (see Greengard 1979), treatments that affect either Ca^{++} (e.g. those in Table 3) or cAMP (Table 4) could have a common basis of action.

1.4.5 Inhibitors of RNA and protein synthesis

Treatments that appear to affect circadian clocks via inhibition of RNA and/or protein synthesis are shown in Table 5. The only reported case of an RNA synthesis inhibitor having an effect on period or steady-state phase phase-shifting or an alteration in period is the period-lengthening effect of actinomycin D in *Nicotiana*.[13] Inhibitors of protein synthesis on 80 S ribosomes, on the other hand, have been shown to alter steady-state phase or period in a variety of organisms.[14] In some of these studies the effects of treatments on protein synthesis have also been measured. In *Euglena*, the degree of lengthening of period is proportional to the degree of inhibition of protein synthesis by cycloheximide (Feldman 1967). In *Neurospora*, the amount of phase-shifting produced by increasing concentrations of cycloheximide is also proportional to the degree of inhibition, with maximum phase shifting resulting when protein synthesis is inhibited by greater than 80% (Nakishima et al 1981a). The phase-shifting effect of cycloheximide appears to be the direct result of the inhibition of protein synthesis rather than a side effect of this drug since in *Neurospora* mutants whose ribosomes are resistant, the inhibitory effects of cycloheximide on protein synthesis are also resistant to the phase-shifting action of this drug (Nakishima et al 1981b).

In *Aplysia*, continuous application of anisomycin at a concentration which inhibits protein synthesis by about 10% lengthens the period of the CAP rhythm of the eye by about 1 h while higher concentrations suppress rhythmicity without abolishing CAP activity (Jacklet 1980a).[15] Puromycin and anisomycin pulses produce

13. Studies in several other organisms (e.g. *Acetabularia*, *Gonyaulax*, *Neurospora*, *Aplysia*) report that RNA synthesis inhibitors have no consistent effects on period or phase or that rhythmicity is abolished (see Sargent et al 1976). The clock effects of aflatoxin, an inhibitor of both RNA and protein synthesis, is discussed below.

14. With one exception, inhibitors of organelle protein synthesis (i.e. on 70 S ribosomes) have not been observed to affect phase or period (see Sargent et al 1976). Both *D* and *L* isomers of chloramphenicol shorten the period of the conidiation rhythm of *Neurospora*. However, since only the *D* isomer has an effect on organelle protein synthesis, the clock effect appears to result from a side effect of this drug (Frehlinger et al 1976).

similar PRCs at concentrations which reduce protein synthesis in the eye by ≥ 50% (Rothman and Strumwasser 1976,1977; Jacklet 1977). The similarity in the PRCs indicate that these two drugs have a common basis of action on the clock. One indication that this action is a direct result of the inhibition in protein synthesis rather than the result of a side effect is that derivatives of puromycin (pamino nucleoside) and anisomycin (deacetylanisomycin), which have no significant effect on protein synthesis in the eye, lack the ability to phase-shift the CAP rhythm (Rothman and Strumwasser 1976; Jacklet 1980b).[16] Actinomycin D abolished the CAP rhythm at a concentration which reduced RNA synthesis (as measured by uridine incorporation) by about 60%.[17] Aflatoxin, which was shown to inhibit both RNA and protein synthesis in the eye by about 50%, produced phase-delays when applied at one phase point and abolished rhythmicity at other phases (Rothman and Strumwasser 1976, 1977). Presumably the phase-shifting effect of this drug is the result of the inhibition of protein, rather than RNA, synthesis given the clock effects of actinomycin D and the protein synthesis inhibitors in this system.

The results from the *Neurospora* and *Aplysia* systems provide good evidence that the clock effects of protein synthesis inhibitors in these systems are not the result of non-specific side effects. The role of protein synthesis in clock function remains unknown, however. Since the phase-shifting produced by protein synthesis inhibitors varies with the phase at which pulses are given, presumably the presence or activity of certain proteins are required during specific phases of the circadian cycle and that at least part of the phase-specific regulation of these activities is via

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15. X-irradiation of the eye also suppresses rhythmicity without abolishing CAP activity while at lower doses the amplitude of the rhythm is decreased without a significant effect on period (Woolum and Strumwasser 1980). This treatment presumably causes a general inhibition of gene expression as a result of its mutational effect.

16. Three other inactive derivatives of anisomycin (not specifically tested for their effects on protein synthesis in the eye) also failed to produce phase shifting (Jacklet 1980b).

17. Protein synthesis (as measured by leucine incorporation) was also reduced by about 40% but this effect was delayed until 49 h after the drug pulse. In comparison, close to maximum inhibition of RNA synthesis was observed 1 h after treatment.

the *de novo* synthesis of such proteins at specific phases. Unfortunately, the identification of clock components synthesized at particular phases might prove to be a difficult task if 1) the number of clock proteins synthesized at a particular phase is small compared to overall protein synthesis rates, and 2) a large number of non-clock proteins are synthesized at specific phases as a result of protein synthesis that is regulated by the clock.

1.5 Genetic Studies

Two complementary approaches have been used in the genetic analysis of circadian clocks. In the first, mutants with known biochemical lesions have been examined for altered clock properties to determine the roles of specific biochemical activities in clock function. In the second approach, mutants with altered clock properties have been isolated and characterized genetically, physiologically, and biochemically to identify the affected clock functions.

The genetic approach to the identification of clock components is subject to some of the same drawbacks as the drug studies. As with a drug treatment, a genetic lesion may affect an activity which acts through a series of one or more intermediate steps to indirectly perturb clock function. For example, if one or more clock functions require ATP as an energy source (§1.4.3), mutations that either directly or indirectly alter ATP production/utilization could indirectly disrupt circadian time-keeping. The period alterations produced by the oligomycin-resistant mutants of *Neurospora*, which effect ATP synthetase, may exert such an indirect effect on clock function (§1.5.1). Since it is currently impossible to distinguish a direct from an indirect mode of mutant action, one must invoke an operational definition that clock components/activities are those that alter period or steady-state phase if perturbed.[18] Whereas a drug treatment may have multiple primary sites of action (e.g. see §1.4.4), only the function(s) encoded by a single transcriptional unit are subject to the primary (i.e. initial) effect of a single mutation. However, both drug and genetic lesions could have multiple secondary (pleiotropic) effects, with one or more of these effects contributing to a disruption of clock function. As an example of a pleiotropic mutational effect, the period alterations produced by the *cel* mutant of *Neurospora* may result from an altera-

18. As with the drug studies, the simple elimination of rhythmicity may be the result of a disruption of an output pathway rather than the result of an effect on a time-keeping function.

tion in a subunit common to two different enzymes (see §1.5.1). In addition, mutations that affect regulatory systems (e.g. the cAMP system) could have numerous direct and indirect pleiotropic effects on clock function. The primary advantages of the genetic approach are that 1) it provides the only method that is currently available for *systematically* identifying clock components, 2) genetic mosaics and chimeras can be used for identifying the anatomical location of circadian pacemakers and their entrainment and output pathways (e.g. see Konopka and Benzer 1971; Konopka 1972; Handler and Konopka 1979), and 3) it provides a method in which clock genes and gene products can be biochemically marked for isolation and characterization via molecular genetic and biochemical techniques.

1.5.1 Biochemical Mutants

The identification of biochemical mutants that affect clock function has been conducted using the *Neurospora* system for which a large collection of previously characterized biochemical, morphological, and developmental mutants is available for the analysis of clock effects. Biochemical mutants of *Neurospora* that have been shown to affect the circadian clock system are described in Table 6 (see Feldman 1981). Two mutants have been shown to affect the clock photoreceptor system. The respiratory mutant *pokey*, which has reduced levels of both mitochondrial and non-mitochondrial cytochrome, and two riboflavin auxotrophs, which have reduced levels of FAD and FAM, exhibit very reduced light sensitivity when assayed for the threshold intensity required for inhibition of banding in constant light or the dose of light required to reach maximum phase-shifting. The genetic results corroborate other physiological evidence that indicates that a plasma membrane flavin/b-type cytochrome complex similar to that found in "blue light" photoreceptors of other procaryotic and eucaryotic systems (Ninnemann 1979) is involved in clock photoreception in *Neurospora*.

One class of biochemical mutations that have been observed to alter circadian period consist of mutants that confer resistance to the drug oligomycin (Dieckmann and Brody 1980). Oligomycin inhibits mitochondrial ATP synthetase. Oligomycin-resistant mutations (*oli*^{r}), which all alter the primary structure of one particular subunit of this enzyme, have period lengths that are about 3 h shorter than wild-type. However it is not known how the *oli*^{r} mutants affect the mechanism of circadian time-keeping. One possibility is that the clock effect is the result of an alteration in ATP production, which would support the evidence obtained from the drug studies described above that ATP synthesis either drives clock processes or is itself a part of the clock mechanism.

Mutations that affect the cysteine biosynthetic pathway comprise another class of biochemical mutants that have been shown to affect circadian periodicity (Feldman et al 1979). When grown on limiting levels of cysteine, cysteine auxotrophic mutations at three separate loci exhibit periods which are shortened depending upon the extent of cysteine limitation. The mechanism of this clock effect is unknown. Methionine and arginine auxotrophs are not observed to affect period on limiting medium.[19]

One other auxotrophic mutation, *cel*, which has a defective fatty acid synthetase, has also been shown to have an effect on period that is dependent on nutritional supplement (Brody and Martins 1979; Mattern and Brody 1979). When supplemented with short-chain saturated fatty acids (e.g. 8:0, 9:0) or longer-chain unsaturated fatty acids (e.g. 18:2, 18:3), this mutant strain, but not wild-type, exhibited a striking increase in period, with period lengths as long as 40 h. Long-chain saturated fatty acids (e.g. 16:0, 18:0) did not affect period. The biochemical basis of the effect

19. A number of auxotrophic mutations affecting other pathways of intermediary metabolism have also been examined and have not been observed to produce clock defects (Brody and Martins 1973). These include auxotrophs for nicotinic acid, nicotinic-tryptophan, histidine, choline, inositol, tyrosine, trytophan, and pyridoxine. Three mutants that slow growth (*spco-6*, *spco-9*, *and pile*) and three mutants with altered morphology of conidiation (*fluffy*, *crisp-3*, *al-2*) also had no observable clock effect.

of this mutant on periodicity is not known. Incorporation of fatty acids is not observed to be significantly different between wild-type and the *cel* strain. Interestingly enough, other results suggest that the *cel* and *oli*^{r} mutants may have a commom basis of action on the clock system. The *cel* mutant is about three-fold more sensitive to oligomycin inhibition than wild-type (Dieckmann and Brody 1980) and a *cel oli*^{r} double mutant strain does not exhibit the sensitivity to the unsaturated fatty acid 18:2 that is observed for *cel* alone (Brody and Forman 1980). In addition, the *cel* mutant has been observed to affect the binding of the prosthetic group 4-phosphopantetheine to a subunit of fatty acid synthetase and 4-phosphopantetheine is also reported to be bound to a subunit of mitochondrial ATP synthetase in yeast (see Dieckmann and Brody 1980). If *cel* also affects the binding of 4-phosphopantetheine to mitochondrial ATP synthetase, then both this mutation and the *oli*^{r} mutations may have a common basis of action on circadian rhythmicity via an effect on ATP synthetase.

One class of biochemical mutants that is of interest as a result of the absence, rather than presence, of an observable clock effect include two derivatives of the *crisp-1* mutant strain that have significantly reduced levels of adenylate cyclase (Feldman et al 1979). Both strains have < 1% of the wild-type level of adenylate cyclase and 9-15% of the wild-type level of cAMP and exhibit wild-type periods. As noted above (Table 4), quinidine, an adenylate cyclase inhibitor, produced only a very slight change in period when applied continuously to *Neurospora* cultures. Thus both the genetic and pharmacological results suggest that a reduction in intracellular cAMP does not affect the expression of normal periodicity in *Neurospora*. The phase-shifting effect of quinidine and the phase-shifting and period-lengthening effects of phosphodiesterase inhibitors in *Neurospora* remain unexplained, however.

1.5.2 Clock Mutants

By screening mutagenized strains for those that exhibit altered period or phase, a number of clock mutants have been isolated in *Chlamydomonas*, *Neurospora*, *Drosophila pseudoobscura* and *D. melanogaster* (Table 7).

In *Chlamydomonas reinhardi*, a photosynthetic flagellate which expresses a circadian rhythm of phototaxis, four long-period mutants have been isolated by screening mutagenized strains for those exhibiting altered period lengths (see Bruce 1976; Feldman 1981). Recombination experiments between the four long-period mutants have demonstrated 1) that all four mutants are unlinked, and 2) that the period lengthening effect of combinations of these mutants is additive, i.e., the periods of double, triple, and quadruple mutant combinations are lengthened by the sum of the lengthening of the single mutants. The additive effects of these mutants on period suggest that these genes act independently of each other. When heterozygous with a wild-type allele in diploids, *per-1* has a dominant phenotype, *per-2* is recessive, while *per-3* is incompletely dominant. One wild-type strain that expresses a short-period phenotype and a spontaneous short-period mutant have also been identified but have not been characterized.

In *Neurospora crassa*, twelve mutants have been isolated which alter the period of the conidiation rhythm (see Feldman et al 1979; Feldman 1981). Seven of these mutants, with periods ranging from 16.5 to 29 h, map to a single locus (*frq*). Five other mutants (four with long-period phenotypes and one with a short-period phenotype) each map to a unique locus.

The effects of altered gene dosage of the *Neurospora* mutants have been examined using heterocaryons which contain both mutant and wild-type nuclei. All of the *frq* alleles expressed intermediate periods when tested in heterocaryons containing equal numbers of mutant and wild-type nuclei. These alleles can therefore

be described as incomplete dominants. When the ratio of mutant/wild-type nuclei was varied, the period length of the three *frq* alleles tested (*frq-1,7,8*) was altered in proportion to the fraction of mutant nuclei present. Two of the mutants at other loci (*chr*, *prd-4*) are also incomplete dominants in 1:1 mutant:wild-type heterocaryons while three other mutants (*prd-1,2,3*) have recessive phenotypes.

Interaction between the *Neurospora* mutants was examined by combining mutants at two different loci. Such double mutant combinations had periods ranging from 13.7 to 34 h and displayed a totally additive effect on period in nearly all of the cases. Some of the combinations of long-period mutants had periods longer than the sum of the single mutant lengthening effects, but this did not appear to be the result of the effect of any one particular mutant. Triple and quadruple mutant combinations also expressed an additive effect. One quadruple mutant combination has the longest period of any mutant circadian clock: 58 h.

As described above (§1.1), phase-response curves (PRCs) provide a measure of the internal state of a clock throughout the course of a cycle. In order to determine the effects of mutant action on the temporal structure of the *Neurospora* circadian clock, the phase-shifting response to short-duration light pulses was measured in the *frq* mutants. Compared to the light PRC of wild-type, the duration of one specific portion of the cycle is altered in the PRCs of all of the *frq* mutants. This portion, which corresponds to a 7-h part of the wild-type cycle in the early subjective night, is shortened by 2 h in *frq-1* and by 4.5 h in *frq-2,4,61* and is lengthened by 9.5 h in *frq-3* and 14.5 h in *frq-7,8*. From these results it would appear that the *frq* gene functions in only one part of the circadian cycle.

In *Drosophila pseudoobscura*, five X-linked mutants with arrhythmic eclosion phenotypes have been isolated (see Bruce 1976; Konopka 1979, 1981). Recombination and complementation results have shown that these mutants comprise two

groups. The three mutants of group 1 are totally arrhythmic in constant darkness (DD) and weakly rhythmic in a light-dark (LD) cycle while the two mutants of group 2 are arrhythmic in both LD and DD conditions. Mutants of either group 1 or 2 fail to complement each other but incomplete complementation occurs when group 1 mutants are combined with group 2 mutants as *trans*-heterozygotes. These later combinations have a long-period phenotype in DD. All of the mutants are incomplete dominants; in mutant/wild-type heterozygotes, period is normal in DD but the phase of the peak of eclosion is altered in LD.

In *Drosophila melanogaster*, eleven mutants have been isolated which alter the period and/or phase of the the circadian rhythms of locomotor activity and eclosion. Six of these mutants have been mapped to a single locus, called *per* in the 3B1-2 region of the distal X-chromosome (see Konopka 1979, 1981). Two mutant alleles, per^0 and per^{02}, completely abolish rhythmicity of both the eclosion and locomotor-activity rhythms, per^l lengthens the period of both rhythms to about 29 h, while per^s shortens period to about 19 h. The locomotor-activity phenotype of a new long-period mutant (per^{l2}) is very dependent on temperature (see below).

In addition to the EMS-induced mutations at the *per* locus, several different chromosome aberrations that affect the 3B1-2 region have mutant *per* phenotypes (described in Chapter 2). Deficiencies of the entire *per* region and four deficiencies and one duplication with 3B1-2 breakpoints have arrhythmic clock phenotypes. The only available translocation with a 3B1-2 breakpoint, *T(1;4)JC43*, produces activity-rhythm records that are totally arrhythmic, very long-period (31-39 h), or transiently long-period before becoming arrhythmic.

Five other clock mutants of *D. melanogaster* have also been isolated. Two X-linked mutants, *And* (described in Chapter 4) and Clk^{KO6}, respectively lengthen and shorten period by about 1.5 h. Two autosomal mutants, *psi-2* and *psi-3*, advance

the phase of eclosion in an LD cycle and lengthen period in DD, while a third autosomal mutant, *gat*, causes the eclosion rhythm to become arrhythmic within three days after a transition from LD to DD.

All eleven of the clock mutants of *D. melanogaster* can be characterized as incomplete dominants since each of these strains has a partial effect on period length or phase of entrainment when heterozygous with their respective wild-type allele. While the genetic and functional significance of dominance/recessiveness of the *Chlamydomonas* and *Neurospora* clock mutants decribed above is unclear, in *Drosophila melanogaster*, the availability of chromosomal deficiencies and duplications allows the analysis of gene dosage effects which does allow the characterization of mutant gene action. As described in detail in Chapter 3, dosage and complementation analysis of wild-type and mutant alleles of the *per* locus suggests that the short- and long-period mutants alter period by respectively increasing and decreasing *per* gene or gene-product activity and that arrhythmic mutant phenotypes result from very large reductions or the total loss of *per* activity.

As described above (§1.1), temperature compensation of period is a basic feature of all circadian clock systems. Several of the *D. melanogaster* clock mutants have been examined for their effects on the temperature compensation mechanism (Orr 1982). Between 17° and 25° C the period of wild-type is shortened by 0.3 h (from 24.1 to 23.8 h), the period of per^s is shortened by about 1 h (from 19.5 to 18.7 h), while the period of per^l is lengthened by about 3 h (from 27.8 h to 30.5 h). The period phenotype of per^{l2} has even a greater dependence on temperature. At 17° C period is lengthened to about 28 h; at 22° C the period of most individuals is lengthened to about 29 h while other individuals are arrhythmic; at 25° C rhythmicity is abolished in all but a few individuals (the average period of the rhythmic individuals is about 30 h). The long- and short-period mutations apparently have an inverse effect on the temperature compensation mechanism of the *Drosophila* clock

but it is unclear whether this is a direct or an indirect effect of the clock defects producing period alterations in these mutants. In contrast to the large effects of temperature on the periods of the *per* mutant alleles, between 17° and 25° C, the period of Clk^{K06} is shortened by about the same amount as wild-type (0.3 h) while the period of *And* is shortened by less than 0.01 h. Apparently the Clk^{K06} and *And* mutations do not affect the temperature compensation mechanism.

The phase-resetting response of the locomotor-activity rhythm to short-duration light pulses has also been determined for several of the *D. melanogaster* clock mutants (Orr 1982). A comparison of the PRCs wild-type, per^s and per^l suggests that the short- and long-period mutations alter the period of the circadian clock by differentially shortening and lengthening one portion of the circadian cycle, that corresponding to the light insensitive phase (the subjective day).[20] While the period alterations produced by *And* and Clk^{K06} could not be localized to one portion of the cycle, the 6 h phase delay portion of the circadian cycle does not appear to be affected by these mutations or by the per^s and per^l mutations.

In addition to their effects on circadian periodicity, the *per* mutants have been shown to alter the period of a very short-term rhythm in the male courtship song of *Drosophila* in a fashion parallel to that observed for circadian rhythmicity: the per^s allele shortens the normal 54 s period of the song oscillation to 42 s, per^l lengthens period to 82 s, and per^0 abolishes rhythmicity (Kyriacou and Hall 1980). While not all aspects of the genetic behavior of the *per* mutants are identical for both rhythms (see §3.5), the observation that the *per* mutants alter both circadian and ultradian periodicities suggest that the *per* gene may encode a fundamental clock function that is commmon to all time-keeping activities in this organism.

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20. The *frq* mutants of *Neurospora*, which also have both long- and short-period phenotypes, alter a different portion of the circadian cycle (the early subjective night; see above) compared to the *per* mutants, but direct comparison of clock functions coded by these two loci would be premature without knowing the degree of homology between the clock mechanisms of these two circadian systems.

Recombinant DNA techniques are currently be used in an attempt to identify and characterize the sequence organization of the wild-type and mutant *per* alleles (J. Perlman, personal communication). The molecular localization of the DNA sequence comprising the *per* locus will be very much aided by the availability and phenotypic characterization of several chromosome aberrations with a breakpoint in the region of the *per* locus (described in Chapter 2).

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1.7 Tables

Table 1. Chemical treatments that affect circadian clocks: unknown/nonspecific clock targets

Treatment	Organism[1]	Effect		Reference
		phase[2]	period[3]	
ethanol	protists,plants, animals[4]	A+D[5]	S[6]/L	see Engelmann and Schremph 1980
Aldehydes[7]	*Gonyaulax*	none	D[8]	Taylor and Hastings 1979
D_2O	protists,plants, animals[9]	D[10]	L	see Engelmann and Schremph 1980
Li^+	plants, animals[11]	D[12]	L	see Engelmann and Schremph 1980
hormones:				
estradiol	*Mesocricetus*		S[15]	Takahashi & Menaker '80
testosterone	*Mus*		S[15]	Daan et al 1975
melatonin	*Passer*		S[16]	Turek et al 1976
neurotransmtrs:				
serotonin	*Aplysia*	A+D[19]		Corrent et al 1978
carbachol[17]	rat pineal[20]	A+D[20]		Zatz 1981
pentobarbital	*Rattus*	D		Ehret et al 1975
sulfhydral reagents:				
PCMB[13]	*Gonyaulax*	A		Hastings 1960
arsenite	"	A+D		"
acetoazolamide[14]	*Gonyaulax*	A+D		Sweeney 1976

1 Rhythms:
protists: *Gonyaulax* = photosynthesis or stimulated bioluminescence; *Euglena* = phototaxis
plants: *Phaseolus*, *Kalanchoe*, *Desmonium* = leaf or petal movement
animals: *Drosophila*, *Passer* (sparrow), *Mus* (mouse), *Mesocricetus* (hamster), *Peromycus* (deer mouse) = locomotor activity
Rattus = body T°
Aplysia = compound action potential (CAP) activity of optic nerve
2 pulsed treatment; A = advances, D = delays
3 continuous application; S = shortening, L = lengthening
4 e.g. *Gonyaulax*, *Euglena*, *Phaseolus*, *Kalanchoe*, *Desmonium*, *Peromyscus*
5 in *Gonyaulax*; no phase shifts observed in *Euglena*
6 in *Gonyaulax* and *Kalanchoe*
7 formaldehyde, acetaldehyde, butraladehyde and propionaldehyde
8 possibly advances as well
9 e.g. *Gonyaulax*, *Phaseolus*, *Kalanchoe*, *Aplysia*, *Excirolana*, *Drosophila*, *Peromyscus*
10 in *Phaseolus*, *Kalanchoe*
11 e.g. *Phaseolus*, *Kalanchoe*, (*Aplysia*: Strumwasser and Viele 1980), *Leucophea*
12 in *Aplysia* (Eskin 1977,1979)
13 *p*-chloromercuribenzoate
14 inhibits cabonic anhydrase (changes CO_2 availability ?)
15 only by 0.1-0.3 h
16 by ?
17 chlolinergic agonist
18 pineal serotonin N-acetyltransferase activity
19 PRC is similar to that for 8-benzylthio-cAMP (Eskin et al 1982; see Table 4)
20 PRC is similar to PRC for light pulses

Table 2. Chemical treatments that affect circadian clocks: Membrane permeability/depolarization effects

Organism[1]	Treatment	Effect		Reference
		phase[2]	period[3]	
Gonyaulax	valinomycin	A+D		Sweeney 1976
	vanillic acid	D		Kiessig et al 1979
Neurospora	valinomycin	A+D[5]		Koyama and Feldman 1981
	nystatin	A+D[5]		Koyama and Feldman 1981
Gossypium	fusaric acid	A+D		Sundararajan et al 1978
Kalanchoe	abscisic acid	D	none	Schremph 1980
Phaseolus	K^+	A		Bunning and Moser 1973
	valinomycin	A+D		Bunning and Moser 1972
	digitonin		S	Keller 1960
	abscisic acid	D	none	Schremph 1980
Aplysia	High K^+	A+D[4]		Eskin '72, Jacklett & Lotshaw '81
	strophanthidin[7]	A+D[4,6]		Eskin 1977b

1 *Neurospora* (fungus) = conidiation;
Gossypium (cotten) = leaf movement
(see footnote 1 of Table 1 for others)
2 pulsed treatment; A = advances, D = delays
3 continuous application; S = shortening
4 similar PRC's (also similar to PRC for light pulses)
5 similar PRC's (also similar to PRC for light pulses)
6 phase-shifting abolished in low Na^+
7 Na-K pump inhibitor

Table 3. Chemical treatments that affect circadian clocks: Inhibitors of oxidative phosphorylation and/or Ca^{++}/Mg^{++} uptake by mitochondria.

Organism[1]	Treatment	Effect		Reference
		phase[2]	period[3]	
Gonyaulax	CCmP[4]	D		Sweeney 1976
Kalanchoe	cyanide	D		Steinheil 1970
Phaseolus	cyanide	A[5]		Mayer 1981
	azide	A[5]		"
Aplysia	cyanide	D[7]		Eskin and Corrent 1977
	dinitrophenol	D[7]		"
	A23187[(6)]	D[7]		"
	Mn^{++}	D		"
	2-deoxy-*D*-glucose	D**		Strumwasser et al 1979a
	La^{3+}		L	Woolum and Strumwasser 1981
Drosophila	anoxia (N_2)	D[8]		Pittendrigh 1974

1 see footnote 1 of Table 1
2 pulsed treatment; D = delays
3 continuous application; L = lengthening
4 carabonyl cyanide m-chloro phenylhydrazone
5 similar PRCs; membrane depolarization effect? (see text)
6 Ca^{++}/Mg^{++} ionophore
7 similar PRCs
8 possibly advances as well
** only 2 phase-points tested

Table 4. Chemical treatments that affect circadian clocks: Putative effects on cAMP levels

Organism[1]	Treatment[2]	Effect		Reference
		phase[3]	period[4]	
Chlamydomonas	caffeine		L	Goodenough & Bruce '81
	theophylline		L	"
Neurospora	caffeine	A+D[5]	L	Feldman '75; Perlman '81
	theophylline		L	Feldman '75
	aminophylline	A+D[5]	L	Feldman '75; Perlman '81
	isobutylmethylxanthine	none	L	Perlman '81
	quinidine	A+D[5]	S[6]	Perlman '81
Phaseolus	caffeine	A+D[9]		Mayer and Scherer 1975
	theophylline	A+D[9]	L	Keller '60; Mayer et al '75
	papaverin		L	Keller 1960
Trifolium	cAMP	D[7]	L	Bollig et al 1978
	theophylline	A+D	L	"
	imidazole	D[7]	none	"
Aplysia	8-benzylthio-cAMP	A+D[8]		Eskin et al 1982
	caffeine		L	Woolum & Strumwasser '81
	theophylline	none	L	Eskin et al '82 Woolum & Strumwasser '81
	isobutylmethylxanthine	none	L	Eskin et al 1982 Woolum & Strumwasser '81
	papaverine	A+D[8]		Eskin et al 1982
	Ro-20-1724	A		"
Rattus	theophylline	A+D		Ehret et al 1975

1 *Trifolium* = leaf movement rhythm
(see footnote 1 of Tables 1,2 for others)
2 caffeine, theophylline, aminophylline, isobutylmethylxanthine, papavarin, and Ro-20-1724 (an imidazolidinone) are phophodiesterase inhibitors; quinidine is an adenylate cyclase inhibitor; imidazole is an activator of phosphodiesterase
3 pulsed treatment; A = advances, D = delays
4 continuous application; S = shortening, L = lengthening
5 similar PRCs
6 by < 1 h
7 very similar PRC's
8 similar to the PRC for serotonin (Table 1)
9 similar PRCs ???

Table 5. Chemical treatments that affect circadian clocks: Inhibitors of RNA and protein synthesis

Organism[1]	Treatment[2]	Effect		Reference
		phase[3]	period[4]	
Gonyaulax	cycloheximide	A+D		Walz and Sweeney 1979 Dunlap et al 1980
	streptimidone	A+D		Hastings et al 1981 Karakashian & Hastings '63
	puromycin	D		Hastings et al 1981
	anisomycin	D*		Hastings et al 1981
Acetabularia	cycloheximide	D		Karakashian & Schweiger '76a,b
	puromycin	D**		Karakashian & Schwieger '76a
Euglena	cycloheximide		L	Feldman 1967
Neurospora	cycloheximide	A[6]		Nakashima et al 1981a,b
Phaseolus	cycloheximide	A+D		Mayer and Knoll 1981
Nicotiana	actinomycin D		L	MacDowell 1964
Aplysia	aflatoxin	D[5,10]		Rothman & Strumwasser '76,'77
	cycloheximide	D*,[7]		Rothman & Strumwasser '76,'77
	puromycin	A+D[7,8]		Rothman & Strumwasser '76,'77
	anisomycin	A+D[8,9]	L	Jacklet 1977, 1980a,b

1 *Nicotiana* = sap exudation rhythm in roots
Acetabularia (protist) = photosynthesis rhythm
(see footnote 1 of Tables 1,2 for others)
2 cycloheximide, stepaimidone, puromycin, and anisomycin are inhibitors of protein synthesis on 80 S ribosomes;
actinomycin D is an inhibitor of RNA synthesis;
aflatoxin is an inhibitor of both protein (on 80 S ribiosomes) and RNA synthesis
3 pulsed treatment; A = advances, D = delays
4 continuous application; L = lengthening
5 uridine incorp. inhibited by 50-75%; leucine incorp. inhib. by 40-70%
6 maximum phase-shifting when leucine incorp. inhibited by > 80%
7 leucine incorp. inhibited by about 50%
8 similar PRC's
9 leucine incorp. inhibited by 80-90%
10 at only 1 phase-point; rhythmicity abolished at other phases
* only 1 phase-point tested
** only 2 phase-points tested

Table 6. Biochemical mutants of *Neurospora* that affect clock or clock-related functions

allele	Biochemical effect	Clock Phenotype	Reference
pokey	respiratory mutant[1]	decrease in light sensitivity[2]	Brain et al 1977
rib-1	riboflavin auxotroph[3]	"[4]	Paietta & Sargent '81
rib-2		"[5]	
		period (h)	
wild-type		21.5	
oli^r (16-1)	oligomycin resistant[6]	18.5	Dieckmann & Brody '80
oli^r (16-3)	"	18.5	
oli^r (16-14)	"	18.5	
oli^r (16-16)	"	19.1	
oli^s (16-16R45)	oli-sensit. revertant	22.2	
		medium / period	
cys-x	cysteine auxotroph[7]	low meth. 18.8 high meth. 22.0	Feldman et al 1979
		fatty acid supplement / period	
		none 21.5	
		8:0 29.1	
cel	fatty acid auxotroph[8]	9:0 35.5	Brody & Martins '79;
		16:0 21.6	Mattern & Brody '79
		18:0 21.7	
		18:1 26.0	
		18:2 40.5	
		18:3 33.0	
cel oli^r	double mutant	18:2 ≃ w.t.	Brody & Forman '80

1 also non-mitochondrial cytochrome reduced by 0.84
2 1/50th as sensitive (assayed as threshold intensity for inhibition of banding in constant light)
3 on riboflavin-limiting medium
4 1/80th as sensitve in damping response (see #2 above); 1/16th as sensitive in phase-shifting response - (assayed as dose required to reach maximum phase-delays)
5 1/4th as sensitive in phase-shifting response (see #4 above)
6 all alleles affect one of the subunits of mitochondrial ATP synthetase
7 *cyc-4* and *cys-12* mutants show similar effects
8 defective fatty acid synthetase

Table 7. Circadian clock mutants

Organism	allele[1]	mutagen[2]	map loc.	period[3]	dominance	Reference
Chlamydomonas (phototaxis)	*w.t.*			23.5-25[4]		
	90-	-[5]	?	21	?	Bruce 1972
	$w^{-}c_1$	spont.	?	21.5	?	"
	per-1	NG	?[17]	27	domin.	Bruce 1974;
	per-2	NG	?	26.5	recess.	Bruce & Bruce '78
	per-3	NG	?	26.5	?	"
	per-4	NG	?	28	incompl.	"
Neurospora (conidiation)	*w.t.*			21.6		
	frq-1	NG	VII R	16.5	incompl.	Feldman & Hoyle '73
	frq-2	"	"	19.3	incompl.	"
	frq-3	"	"	24.0	incompl.	"
	frq-4	"	"	19.3	incompl.	"
	frq-6	"	"	19.2	incompl.	Gardner & Feldman '80
	frq-7	"	"	29.0	incompl.	"
	frq-8	"	"	29.0	incompl.	"
	chr	NG	VI L	23.5	incompl.	Feldman et al 1979
	prd-1	NG	III C	25.8	recess.	Feldman & Atkinson '78
	prd-2	UV	V R	25.5	recess.	Feldman et al 1979
	prd-3	UV	I C	25.1	recess.	"
	prd-4	UV	?	18.0	incompl.	"
Drosophila pseudoobsura (eclosion)	*w.t.*			24		
	group 1	EMS	X	arrhy.	incompl.	Pittendrigh 1974[6]
	group 2	EMS	X	arrhy.	incompl.	"
Drosophila melanogaster (eclosion & locom. act.)	*w.t.*			24		
	per^{0}	EMS	X:3B1-2	arrhy.	incompl.[7]	Konopka & Benzer '71
	per^{02}	"	"	arrhy.	incompl.[7]	Smith & Konopka '82[11]
	per^{s}	"	"	19	incompl.	Konopka & Benzer '71
	per^{l}	"	"	29[6]	incompl.	"
	per^{l2}	"	"	30/arr.[6,16]	incompl.	Orr 1982
	per^{JC43}	X-ray[9]	"	see # 10	incompl.[7]	Smith & Konopka '81[12]
	And	EMS	X:10E2-F1	25.5	incompl.	see Chapt. 4
	Clk^{K06}	EMS	X:distal	22.5[6]	incompl.	Orr 1982
	psi-2	EMS	II	see # 13	incompl.	Jackson 1981
	psi-3	EMS	III	see # 14	incompl.	"
	gat	EMS	II	see # 15	incompl.	"

1 w.t.= wild-type
2 NG = nitrosoguanidine; EMS = ethyl methane sulfonate
3 in h
4 for 5 different w.t. strains
5 wild-type strain (one of 6 tested)
6 see also Bruce 1976, Konopka 1979
7 see Smith and Konopka 1982 = chapter 3

8 eclosion rhythm not tested
9 from *T(1;4)JC43*, however this *per* mutant may have an orgin independent of the X-ray induced 3B1-2 breakpoint
10 31-39 h and/or arrhythmic activity records (as 1 dose in a female); eclosion is completely arrhythmic
11 Chapter 3
12 Chapter 2
13 eclosion peak is phase advanced by 2-3 h in LD 12:12; 25-26 h eclosion rhythm period
14 eclosion peak is phase advanced by 3-4 h in LD 12:12; 24-25 h eclosion rhythm period
15 eclosion is arrhythmic in DD after 2 d of transient rhythmicity
16 period is T° dependent; at 25° C, period = 30.3 ± 2.3 (n=8) + n=19 are arrhythmic
17 *per 1-4* are unlinked

Chapter 2

Circadian Clock Phenotypes of Chromosome Aberrations with a Breakpoint at the per Locus *

* Smith RF, Konopka RJ (1981) Mol Gen Genet 183:243-251

2.1 Summary

The circadian rhythm phenotypes of eight chromosome aberrations with a breakpoint in the region of the *per* locus (3B1-2) were analyzed. Two duplications and five deficiencies with a 3B1-2 breakpoint produce either a wild-type or an arrhythmic clock phenotype while one translocation with a 3B1-2 breakpoint, *T(1;4)JC43*, produces locomotor-activity rhythms with either very-long periods (31-39 hr), rhythms that grade into arrhythmicity, or completely arrhythmic phenotypes. This is a unique phenotype that had not previously been observed for mutants at the *per* locus. An extensive complementation analysis of 3B1-2 chromosome aberrations and *per* mutant alleles provided no compelling evidence for genetic complexity at the *per* locus. This is in contrast to the report of Young and Judd (1978). Analysis of both the locomotor-activity and eclosion phenotypes of 3B1-2 chromosome aberrations did not uncover differences in the genetic control of these two rhythms. The clock phenotypes of 3B1-2 chromosome aberrations, the three *per* mutant alleles, and per^{+} duplications suggest that mutations at the *per* locus shorten, lengthen, or eliminate periodicity by respectively increasing, decreasing, or eliminating *per* activity.

2.2 Introduction

Daily rhythmicity in behavioral and physiological activity has evolved as an adaptive response to the 24-hour fluctuations in the environment. When these rhythms persist in the absence of environmental cues and have a period of around one day, they are termed circadian. Genetic analysis of the circadian "clock" mechanism can be performed by the isolation and characterization of mutations that alter period or phase. Circadian clock mutants have been isolated in *Drosophila melanogaster* (Konopka, 1979), *Drosophila pseudoobscura* (Pittendrigh, 1974), Neurospora (Feldman et al., 1979) and Chlamydomonas (Bruce,1976). Of these, only in *Drosophila* is it possible to conduct a genetic analysis of the neural mechanisms underlying circadian behavior.

In *Drosophila melanogaster*, three mutant alleles of the *per* locus have been isolated that drastically alter the period of the adult emergence (eclosion) rhythm and the adult locomotor activity rhythm (Konopka and Benzer, 1971). The per^s allele shortens the normal 24-hr period of both rhythms to about 19 hr, the per^l allele lengthens the period to about 29 hr, while the per^o allele completely abolishes rhythmicity. The *per* locus maps within one of the most extensively investigated regions of the *Drosophila* genome, between zeste and white on the distal X-chromosome (Konopka, 1972, 1979). Its location, between a lethal complementation group assigned to band 3B1 and another lethal group assigned to band 3B2 (Young and Judd, 1978) is within one of the few known regions of the *Drosophila* genome that contains more than one gene per polytene chromosome band (see Lefevre, 1974; Judd, 1977).

The existence of several chromosomal aberrations with a breakpoint in the 3B1-2 region (see Young and Judd, 1978) provided us with an opportunity to study the locomotor activity and eclosion rhythm phenotypes of additional mutational events

at the *per* locus and to compare these phenotypes to those of the 3 previously isolated *per* mutants. An analysis of the clock phenotypes of 3B1-2 chromosome aberrations seemed particularly intriguing since these chromosomes have been observed to exhibit complexity in their complemenation behavior (Young and Judd, 1978). We report here that an extensive complementation analysis of 3B1-2 chromosome aberrations provided no compelling evidence for genetic complexity at the *per* locus.

2.3 Materials and Methods

The cytogenetic extent of the chromosome aberrations used in this study are shown in Fig. 1. A description of these chromosome aberrations and other mutants of *D. melanogaster* used in this study can be found in Lindsley and Grell (1968), Judd *et al.* (1972), Young (1975), Young and Judd (1978) and Liu and Lim (1975). *T(1;4)JC43* also carries an inversion, In(1)3B1-2; 3E3-4 (G. Lefevre, personal communication). *Df(1)TEM-202* was kindly provided by Dr. J.K. Lim.. All other chromosome aberration stocks and the two *zw* lethals were kindly supplied by Dr. Burke Judd. The per^0 chromosomes were marked with *y* or *w spl* or sn^3 *m*. The per^s chromosomes carried *w spl* or *y* sn^3 *m*. The per^l chromosomes were unmarked or marked with *y* sn^3 *m*. The w^+Y chromosome was obtained from a *Df(1)64j4/* w^+Y*/ C(1)DX* stock. In the activity-rhythm studies, per^+ X-chromosomes were obtained from a Canton-S (*C-S*) wild-type strain. In the eclosion studies, per^+ chromosomes were either *C-S* or *FM7*.

Locomotor activity of individual adults was monitored at constant temperature (± 0.5°C) in the range 23-25°C and in infrared light using a device similar to that described previously (Konopka and Benzer,1971). The wing-tips of most flies used in locomotor-activity studies were clipped to facilitate movement in the activity monitor chamber. This operation had no effect upon rhythm phenotype. The number of activity events per hour was electronically counted and computer-plotted. The periods were determined as the average interval between successive offsets for each cycle. The activity offset was defined as the time at which the falling edge of the activity peak drops to a point halfway between its maximum and minimum amplitude, estimated to the nearest half-hour. For all except the very long-period rhythms, period estimates were obtained from those records showing at least 6 cycles of rhythmicity. Since the phase of activity offsets of the very long-

period rhythms were often difficult to assess, the periods of these rhythms were obtained by periodogram analysis (Enright, 1965) for those records showing at least 4 cycles of rhythmicity.

For the determination of activity and rest durations, periodogram analysis was used to determine period and form estimates were used to determine the phase of the activity peaks. The activity onset and offset of each cycle were defined as that time at which 20% and 95% of the total summed counts of each cycle had occurred, measured to the nearest hour (Konopka and Orr, 1980). This yields a measure of alpha (the duration of the active portion of each cycle) which is similar to that obtained from visual analysis of activity profiles recorded using an Esterline-Angus event recorder. Some single hourly data points of the raw activity records were found to have a very large amplitude (larger than twice the maximum amplitude of the other data points) apparently as the result of continuous blocking of the light beam by the fly. Since the periodogram and activity-rest duration analyses are very sensitive to such large amplitude spikes in the data, such points were replaced by the average amplitude of the two data points immediately preceding and following this point. No more than 4 such points were averaged in any record.

Eclosion rhythms were monitored in constant darkness and constant temperature using "bang-boxes", as described previously (Konopka and Benzer, 1971). Some of the bang-boxes were generously provided by Dr. Colin Pittendrigh. The bang-boxes automatically collected flies that emerged each hour; these flies were subsequently scored and counted. Crosses were reared at the same temperature at which the eclosion rhythms were monitored (18, 22 or 25°C). Between 18 and 25°C the variability in the period of the eclosion rhythm of wild-type and the *per* mutants is on the order of an hour (Konopka and Benzer, 1971). Most eclosion runs were conducted at 18°C since this temperature maximized run duration. This allowed a more accurate estimate of period than at higher temperatures without

seriously decreasing the amplitude of the eclosion peaks. The period of the eclosion rhythm was determined as the average interval between medians of each eclosion peak, with each median estimated to the nearest half-hour.

2.4 Results

2.4.1 Activity Rhythm Phenotypes of 3B1-2 Chromosome Aberrations

Since the chromosome aberrations studied are lethal when homozygous, the clock phenotypes of 3B1-2 chromosome aberrations were first monitored in combination with per^0. Since per^0 acts as a recessive null allele of the per locus (Konopka and Benzer, 1971), the period phenotype of each of the 3B1-2 chromosome aberrations should be fully expressed when combined with per^0.

A wild-type phenotype (i.e. 24- 25 hr period) was displayed by *Df(1)K95*, $Df(1)w^{-64d}$ (Table 1) and $Dp(1;4)w^{m65g}$ (Table 3). Figs. 2a and 2b show the locomotor activity records of individuals of the genotype per^0/per^+ and $per^0/Df(1)K95$. To the right of each record is a periodogram, which is a statistical analysis of the strength of a record's periodicity. Peaks in the periodogram indicate periodicity in the data at the designated trial period. The periodograms of both records show a large peak centered at 24-25 hr and at 48 hr. A large peak at 48 hr and smaller peaks at 12 and 36 hr which appear in many wild-type periodograms (e.g. Fig. 4a) represent harmonics of the 24-hr rhythm (Enright, 1965).

Arrhythmic activity phenotypes were produced by deficiencies for the entire 3B1-2 region, $Df(1)w^{rJ1}$ and *Df(1)64f1*, and by the 3B1-2 chromosome aberrations *Df(1)64j4*, *Df(1)62d18*, *Df(1)TEM-202* (Table 2) and $Dp(1;3)w^{m49a}$ (Table 3). Figs. 2c and 2d show activity records of individuals of the genotype $per^0/Df(1)w^{rJ1}$ and $per^0/Df(1)62d18$. The periodograms of these records show no prominent peaks. In the case of $Dp(1;3)w^{m49a}$, arrhythmicity could be the result of a wild-type *per* gene that is inactivated by position-effect variegation rather than of a break within the *per* locus since the white, roughest, and Notch loci within the duplicated segment are variegated (Lefevre, 1951).

A unique phenotypic class is produced by a translocation with a 3B1-2 breakpoint, *T(1;4)JC43* (Table 2). Six of the fourteen *T(1;4)JC43/per^0* individuals monitored displayed 4-6 cycles of very long-period rhythmicity (average period 35 hr). The variation in cycle length of these individuals was greater than that observed for other rhythmic phenotypes. The other *T(1;4)JC43/per^0* individuals showed either transient long-period rhythmicity that graded into arrhythmicity (designated "long-arr" in Table 2) or were arrhythmic from the onset of the run.

Very long-period and arrhythmic phenotypes were also observed when *T(1;4)JC43* was combined with any of the deficiencies with an arrhythmic phenotype (Table 2). Fig. 3 shows examples of an arrhythmic, a long-arrhythmic and a very long-period activity record obtained from *Df(1)64j4/T(1;4)JC43* siblings that were monitored simultaneously. Fig. 3a is a completely arrhythmic record with a virtually flat periodogram. Fig. 3b shows a record which is weakly rhythmic for 1-2 cycles before becoming arrhythmic. The periodogram shows a broad hump between 28-37 hr. Fig. 3c shows a very long-period record which is rhythmic up to the end of the activity run. The periodogram shows a higher and narrower peak centered at 36 hr.

The X-distal, 4-proximal element of *T(1;4)JC43* can be combined with the X-proximal, 2-distal element of *T(1;2)RC45* to form a synthetic X-chromosome deficiency extending proximally from the 3B1-2 breakpoint of *T(1;4)JC43* to the 3C2 breakpoint of *T(1;2)RC45* (Fig. 1; Table 2). Since this synthetic deficiency also exhibits the very long-period and long-arrhythmic phenotypes when combined with *per^0* (Table 2), that portion of the per locus which is distal to the 3B1-2 breakpoint of *T(1;4)JC43* is sufficient for the production of the very long and long-arrhythmic phenotypes. Although *T(1;4)JC43* is inviable when homozygous as a result of at least one lethal mutation proximal to white, the combination *X^D4^PJC43;*

$X^P2^DRC45/T(1;4)JC43$ is viable and exhibits much stronger rhythmicity than *$per^0/T(1;4)JC43$* (Table 2; Fig. 3d). Almost all the individuals of this genotype displayed very long-period rhythms and none were completely arrhythmic. We have not been able to construct a synthetic deficiency using the X-distal element of T(1;4)JC43 that would extend distally from the 3B1-2 breakpoint of *T(1;4)JC43*.

Of all of the *T(1;4)JC43* and *$X^D4^PJC43; X^P2^DRC45$* genotypes that produced very long-period and long-arrhythmic phenotypes, only one individual of each of the genotypes *Df(1)TEM-202/T(1;4)JC43* and *$Df(1)64j4/X^D4^PJC43; X^P2^DRC45$* was observed to have a near wild-type phenotype. However, rhythmicity was weak for both individuals. The periodograms displayed only a small peak at 25 hr and 23 hr, respectively (Table 2 and 5a).

Since Young and Judd (1978) observed several examples of complex complementation behavior for 3B1-2 chromosome aberrations, these chromosomes might show a different pattern of complementation when combined with the other *per* alleles. Complementation tests were therefore performed using the *per^s* and *per^l* mutants. With the exception of T(1;4)JC43, 3B1-2 chromosome aberrations were found to exhibit the same pattern of complementation with respect to all three mutant *per* alleles. Those 3B1-2 chromosome aberrations that behave as *per^+* when combined with *per^0* (*Df(1)K95* and *$Df(1)w^{-64d}$*) also behave as *per^+* when combined with *per^s* or *per^l* (Table 1). Likewise, those 3B1-2 chromosome aberrations that behave as *per^0* when combined with *per^0* (*Df(1)64j4*, *Df(1)62d18* and *Df(1)TEM-202*) also behave as *per^0* when combined with *per^s* or *per^l* (Table 2). The alleles of the 2 lethal complementation groups flanking the *per* locus, *l(1)zw3* and *l(1)zw6*, also behaved as *per^+* in all of the combinations tested (Table 1).

T(1;4)JC43, however, does exhibit a difference in complementation pattern when combined with the different *per* alleles (Table 2). When combined with *per^0*,

T(1;4)JC43 exhibits weak rhythmicity in its expression of very long-period phenotypes and thus acts as a hypomorph. When combined with either per^s or per^l, on the other hand, *T(1;4)JC43* behaves as a deficiency for the entire 3B1-2 region, and thus acts as an amorph.

3B1-2 chromosome aberrations were also combined with per^+ to test for dominant mutant effects. All such combinations exhibit a wild-type (24-25 hr period) phenotype (Tables 1 and 2).

In order to test for complex complementation behavior between two different 3B1-2 chromosome aberrations, complementation tests were performed 1) between *T(1;4)JC43* and all of the deficiencies with a 3B1-2 breakpoint, and 2) for most of the viable combinations of two deficiencies with a 3B1-2 breakpoint. No examples of complex complementation behavior were observed for any of these combinations. Those chromosomes that behaved as per^+ when combined with the *per* mutant alleles also behaved as per^+ when combined with *T(1;4)JC43* (Table 1) while those chromosomes that behaved as per^o also behaved as per^o when combined with *T(1;4)JC43* (Table 2). Similarly, those combinations which included at least one of the two deficiencies with a wild-type activity phenotype, $Df(1)w^{-64d}$ and *Df(1)K95*, also exhibited a wild-type phenotype (Table 4a; Fig.4a) while those combinations which included only deficiencies with an arrhythmic phenotype, *Df(1)64j4*, *Df(1)62d18*, and *Df(1)TEM-202*, also produced arrhythmic phenotypes (Table 4a; Fig. 4b).

2.4.2 Eclosion Rhythm Phenotypes of 3B1-2 Chromosome Aberrations

The eclosion phenotypes of various 3B1-2 chromosome aberration genotypes were monitored tc detect differences in the genetic control of the eclosion and activity rhythms. Tables 4b and 5 show that with the exception of those *T(1;4)JC43* geno-

types that showed the long-arrhythmic phenotypes, all of the genotypes monitored displayed an eclosion phenotype which corresponds to its activity rhythm phenotype. Figs. 5a-d show examples of wild-type and arrhythmic eclosion profiles produced by the 3B1-2 chromosome aberrations, *Df(1)w^{-} ^{64d}* and *Df(1)64j4* and by the combinations *Df(1)64j4/Df(1)w^{-} ^{64d}* and *Df(1)64j4/Df(1)TEM-202*. Young and Judd (1978) reported that the combination *Df(1)w^{-} ^{64d}/Df(1)64j4* produced a long-period (28-hr) eclosion profile. We found that this genotype produces a wild-type phenotype for both the activity and the eclosion rhythm (Table 4b; Figs. 4a and 5c).

T(1;4)JC43 was the only chromosome that did not produce similar eclosion and activity rhythm phenotypes. Those *T(1;4)JC43* genotypes that exhibited very long-period and long-arrhythmic activity phenotypes exhibited completely arrhythmic eclosion profiles (Table 5). For example, the genotype *Df(1)64j4/T(1;4)JC43* showed no evidence of long periodicity in its eclosion profile (Fig. 6a), while the internal control population which eclosed simultaneously in the same bang-box (*Df(1)64j4/per^{+}* siblings) showed 4 cycles of wild-type rhythmicity (Fig. 6b).

The eclosion rhythms of most of the genotypes shown in Tables 4b and 5 were also monitored at 25°C in order to determine if there were any significant differences between the eclosion and activity phenotypes as a result of differences in temperature at which the rhythms were monitored (activity at 24°C; eclosion at 18° or 22°C). Since only 2-3 cycles per eclosion run can be monitored at this temperature, period could not be accurately assessed and thus the eclosion rhythms at 25°C were only characterized as rhythmic or arrhythmic. No difference in the eclosion rhythm phenotypes at the different temperatures were detected on this basis.

2.4.3 Dosage Senitivity of the per *Locus*

Young and Judd (1978) reported that per^+ males carrying one of several different duplications of the entire 3B1-2 region showed an altered locomotor activity phenotype in which the duration of the active portion of each 24-hr cycle (called alpha) was lengthened from the normal 12 hr to 19-20 hr of activity. These per^+ duplications were not reported to have any effect on period length. We have re-examined this dosage-sensitive phenotype and found no consistent effect of per^+ duplications on alpha. In order to control for the effects of differences in genetic backgrounds we have studied two different per^+ stocks, *C-S* and $w\ sn^3\ m$, and two different per^+ duplications, w^+Y and $Dp(1;3)w^{67k27}$ (Table 6). Both of the per^+ duplications produced a wild-type phenotype when combined with per^0 (Table 3). When w^+Y was combined with a *C-S* per^+ chromosome, we observed that alpha was shortened ($p < .001$) rather than lengthened. However, when w^+Y was combined with a $per^+\ w\ sn^3\ m$ chromosome, we observed no significant effect on alpha. There was also no significant effect of the per^+ duplication $Dp(1;3)w^{67k27}$ on alpha. Young and Judd (1978) also reported that w^+Y produces an arrhythmic phenotype when combined with *Df(1)64f1* or *Df(1)K95*. However, we find that both of these genotypes have a wild-type phenotype for both the locomotor-activity and eclosion rhythms (Table 7 and Figs. 4c,d).

Although we found no consistent effects of per^+ duplications on alpha, we found that both per^+ duplications tested shortened period by approximately 1-hr in all combinations tested (Table 6).

2.5 Discussion

We have shown that two duplications and five deficiencies with a 3B1-2 breakpoint produced either a wild-type or an arrhythmic clock phenotype while one translocation with a 3B1-2 breakpoint, *T(1;4)JC43*, exhibited very long-period and arrhythmic activity phenotypes. The wild-type clock phenotypes of *Df(1)K95*, $Df(1)w^{-64d}$ and $Dp(1;4)w^{m65g}$ appear to result from a breakpoint within the 3B1-2 interval but outside of the *per* locus. Since arrhythmic phenotypes are produced by deficiencies for the entire 3B1-2 region, the arrhythmic phenotypes of per^{o}, *Df(1)64j4*, *Df(1)62d18*, *Df(1)TEM-202* and $Dp(1;3)w^{m49a}$ would appear to be the result of genetic alterations that completely block *per* function.

If arrhythmicity results from the total lack of *per* function, the weakly rhythmic and arrhythmic phenotypes produced by *T(1;4)JC43* would appear to be the result of drastic reduction of *per* activity. Such an effect of *T(1;4)JC43* on *per* activity is consistent with the observation that two doses of $X^{D}4^{P}JC43$ (in the combination $X^{D}4^{P}JC43$; $X^{P}2^{D}RC45/T(1;4)JC43$) produced stronger rhythmicity than one dose of $X^{D}4^{P}JC43$ or *T(1;4)JC43*. If the reduction in *per* activity of *T(1;4)JC43* is the result of the 3B1-2 breakpoint rather than a separate (e.g. co-induced) point mutation within the *per* locus, it would be one of the very few known cases in *Drosophila* of a chromosome aberration producing a reduction, rather than complete elimination, of gene activity (see Lefevre, 1973, 1974; Spradling, 1980).

Greatly reduced levels of *per* activity for *T(1;4)JC43* could account for the differences in patterns of complementation when this chromosome was combined with the 3 mutant *per* alleles. *T(1;4)JC43* behaved as a extreme long-period allele when combined with per^{o} or the arrhythmic deficiencies, but behaved as a null allele when combined with either per^{s} or per^{l}. It is possible that a greatly reduced level of *per* activity is sufficient to produce weak rhythmicity when *T(1;4)JC43* is

combined with a null *per* allele but is not sufficient to influence the activity levels of the other *per* alleles.

In their mapping study of the per locus, Young and Judd (1978) examined the eclosion rhythm phenotypes of five 3B1-2 chromosome aberrations (*T(1;4)JC43*, *Df(1)K95*, *Df(1)w^{-} ^{64d}*, *Df(1)64j4*, and *Df(1)62d18*) when in combination with one another and with *per^{0}*. Since we found that the period phenotypes of several of the genotypes observed in our locomotor activity study did not correspond to the eclosion phenotype reported by Young and Judd, we re-examined the eclosion phenotypes of 3B1-2 chromosome aberrations using methods that would more accurately estimate period (see Methods section). Of those genotypes in common, our eclosion results are comparable to those of Young and Judd except for the one case in which they observed unusual complementation behavior. Young and Judd report that the combination *Df(1)w^{-} ^{64d}/Df(1)64j4* displayed a long-period (28 hr) eclosion rhythm while we observed that this genotype displayed a wild-type phenotype for both the eclosion and activity rhythms (Table 2B). In fact we found no significant differences between activity and eclosion rhythms for any of the genotypes in which both rhythms were monitored, except for *T(1;4)JC43*. In the case of *T(1;4)JC43*, the apparent difference in phenotype between rhythms (very long-period and arrhythmic activity phenotypes vs. completely arrhythmic eclosion phenotypes) is most likely the result of two properties of the very long-period and long-arrhythmic phenotypes which could mask its expression in the eclosion rhythm. First, most of the individuals of this phenotypic class are either arrhythmic from the onset of the run or become arrhythmic shortly thereafter. Since an eclosion profile is obtained from a population, an arrhythmic majority could mask the expression of a rhythmic minority. Second, in order to show a rhythmic eclosion pattern, the individuals of the population must not only be rhythmic, but the individuals must have similar period and phase. Since those individuals which exhibit the very long period

phenotype are variable in both period and phase, the rhythm of a population of such individuals would be less rhythmic than each individual rhythm.

We could not confirm Young and Judd's (1978) report that the addition of a per^+ duplication to a wild-type male significantly increased the duration of the active portion of each cycle. Since we found no consistent effects of per^+ duplications on the alpha of wild-type strains using our method of analysis , the complex complementation pattern for dosage sensitivity observed by Young and Judd for 3B1-2 chromosome aberrations may not be a reflection of genetic complexity at the *per* locus. In contrast to Young and Judd (see also Young, 1975; Judd, 1977), we observed no cases of inconsistent complementation behavior in any of our studies and thus found no compelling evidence for genetic complexity at the *per* locus.

Although we found no consistent effects of per^+ duplications on alpha, we did find that an additional per^+ dose in wild-type males did shorten period length by about 1 hr. This result, along with the observation that arrhythmicity is the null phenotype and our suggestion that weak rhythmicity of *T(1;4)JC43* is the result of a drastic reduction in *per* activity, suggests that the *per* alleles per^s, per^l and per^o alter period by respectively increasing, decreasing, or totally eliminating the quantity or activity of the *per* gene product. A detailed dosage analysis of the *per* locus supports this hypothesis (manuscript in preparation).

In summary, we find that *T(1;4)JC43* produces a unique activity rhythm phenotype that had not previously been observed for mutants at the *per* locus. An extensive complementation analysis of 3B1-2 chromosome aberrations provides no compelling evidence for genetic complexity at the *per* locus. Analysis of both the activity and eclosion phenotypes of 3B1-2 chromosome aberrations did not uncover differences in the genetic control of these two rhythms. Thus either a single clock system controls both rhythms or the action of the *per* gene is similar for both clock

systems. The clock phenotypes of 3B1-2 chromosome aberrations, the three *per* mutant alleles, and per^{+} duplications suggest that mutations at the *per* may alter period by increasing as well as decreasing *per* gene or product activity.

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2.7 Figures

Fig. 1. Schematic representation of the zeste-white region of the distal X-chromosome (based on Lefevre 1976) showing the cytological extents of the chromosome aberrations used in this study.

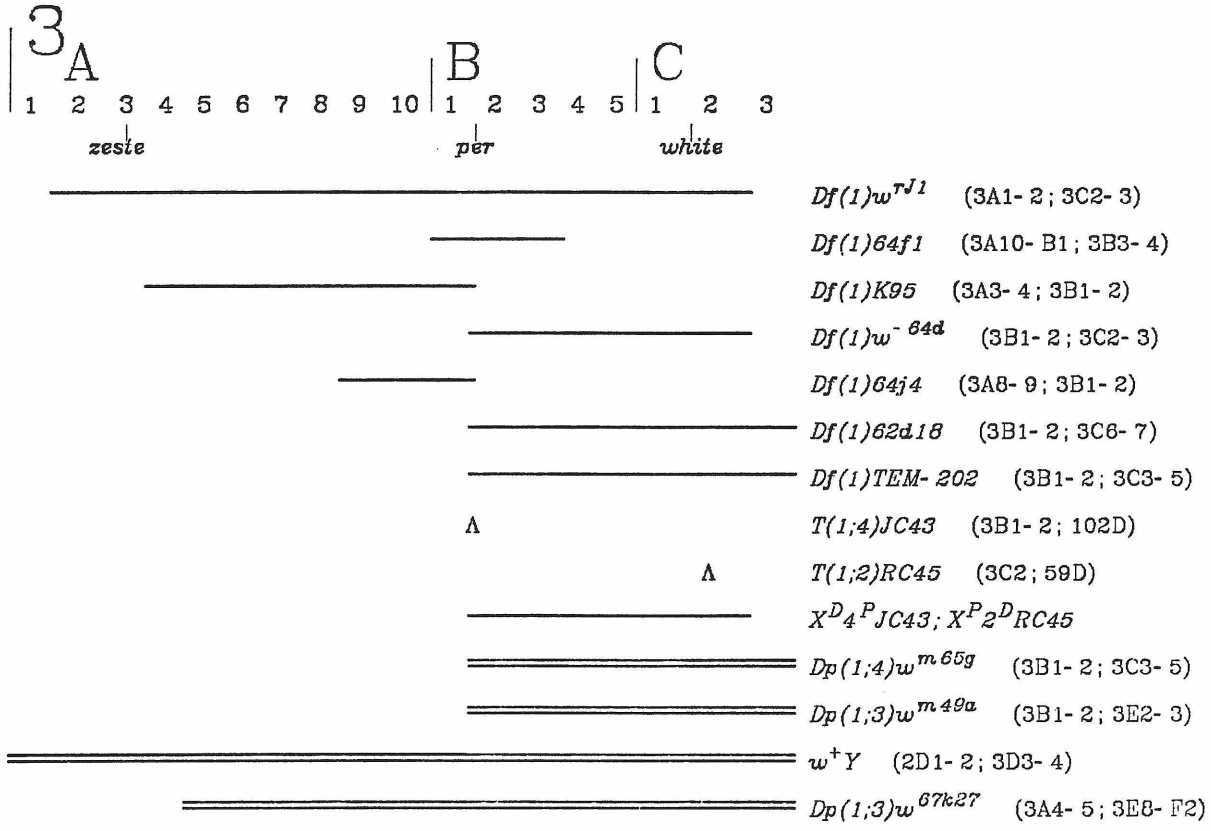

3A
1 2 3 4 5 6 7 8 9 10
B
1 2 3 4 5
C
1 2 3
zeste
per
white
Df(1)w^rJ1 (3A1-2; 3C2-3)
Df(1)64f1 (3A10-B1; 3B3-4)
Df(1)K95 (3A3-4; 3B1-2)
Df(1)w^-64d (3B1-2; 3C2-3)
Df(1)64j4 (3A8-9; 3B1-2)
Df(1)62d18 (3B1-2; 3C6-7)
Df(1)TEM-202 (3B1-2; 3C3-5)
T(1;4)JC43 (3B1-2; 102D)
T(1;2)RC45 (3C2; 59D)
X^D4^PJC43; X^P2^DRC45
Dp(1;4)w^m65g (3B1-2; 3C3-5)
Dp(1;3)w^m49a (3B1-2; 3E2-3)
w^+Y (2D1-2; 3D3-4)
Dp(1;3)w^67k27 (3A4-5; 3E8-F2)

Fig. 2. Locomotor activity phenotypes of the following X-chromosomes (assayed when heterozygous with per^{o}): a) per^{+}, b) *Df(1)K95*, c) $Df(1)w^{rJ1}$, d) *Df(1)62d18*.

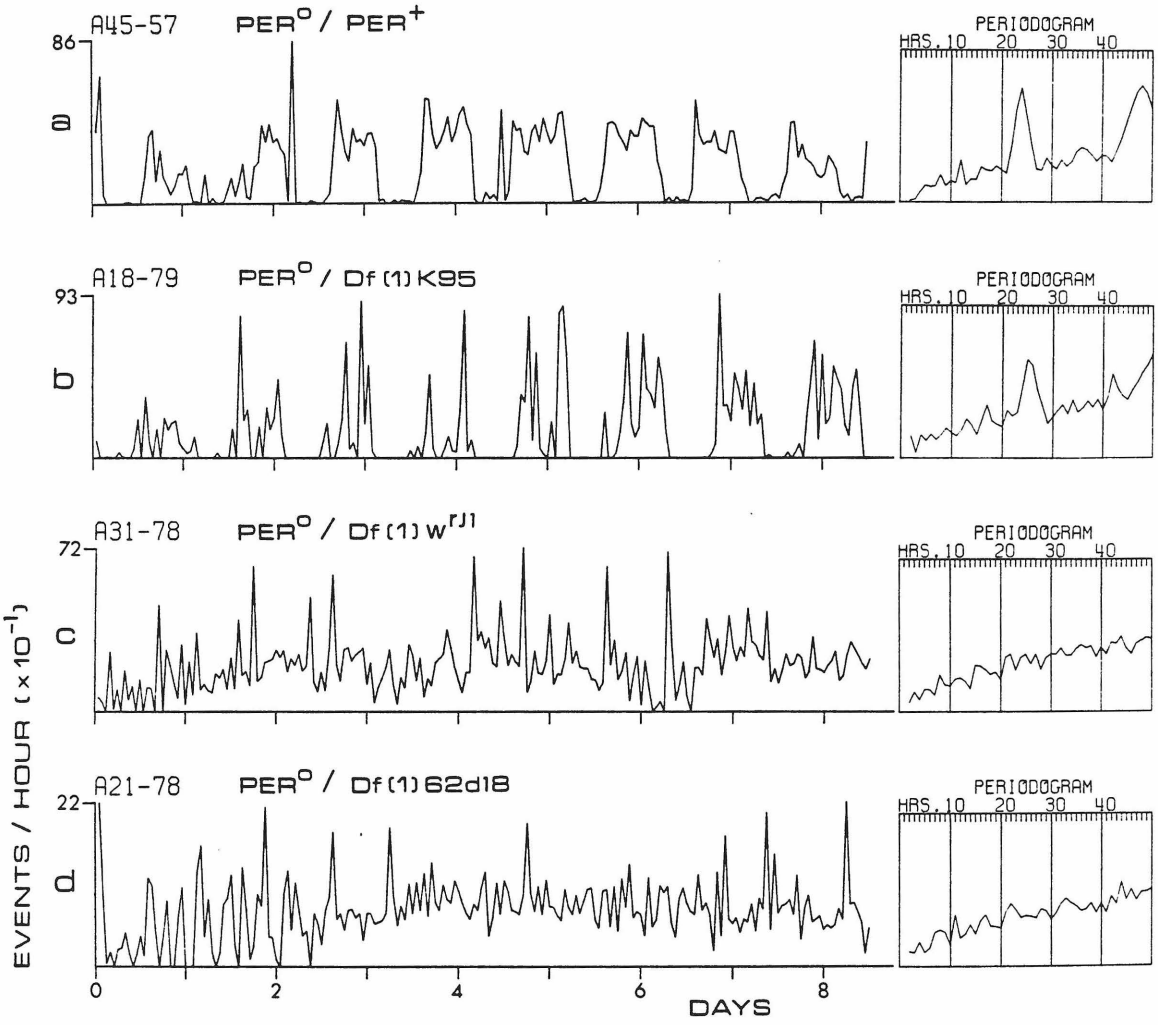

A45-57
PER^{O} / PER^{+}
86
a
PERIODOGRAM
HRS. 10 20 30 40
A18-79
PER^{O} / Df(1)K95
93
b
PERIODOGRAM
HRS. 10 20 30 40
A31-78
PER^{O} / Df(1)w^{rJ1}
72
c
PERIODOGRAM
HRS. 10 20 30 40
A21-78
PER^{O} / Df(1)62d18
22
d
PERIODOGRAM
HRS. 10 20 30 40
EVENTS / HOUR (x10^{-1})
0
2
4
6
8
DAYS

Fig. 3. Activity rhythm phenotypes of *T(1;4)JC43*: a-c) activity phenotypes of siblings of the genotype *Df(1)64j4 / T(1;4)JC43* ranging from totally arrhythmic (a) to ≥ 6 very long-period cycles (c); d) very-long period phenotype of a $X^{D}4^{P}JC43$; $X^{P}2^{D}RC45 / T(1;4)JC43$ individual.

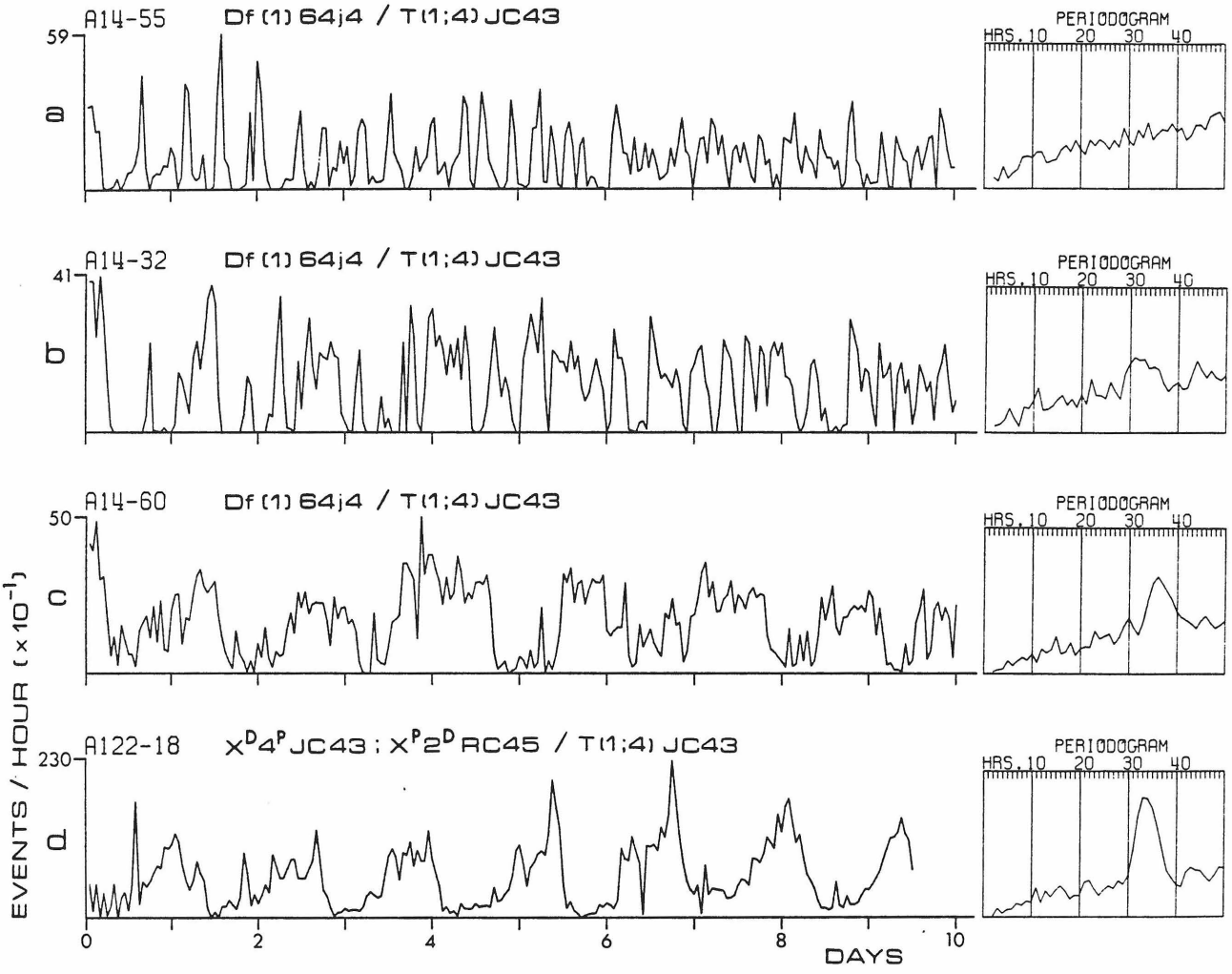

A14-55
Df (1) 64j4 / T(1;4) JC43
59
a
PERIODOGRAM
HRS. 10 20 30 40
A14-32
Df (1) 64j4 / T(1;4) JC43
41
b
PERIODOGRAM
HRS. 10 20 30 40
A14-60
Df (1) 64j4 / T(1;4) JC43
50
c
PERIODOGRAM
HRS. 10 20 30 40
A122-18
X^D4^P JC43 ; X^P2^D RC45 / T(1;4) JC43
230
d
PERIODOGRAM
HRS. 10 20 30 40
EVENTS / HOUR (x10^-1)
0
2
4
6
8
10
DAYS

Fig. 4. Activity rhythm phenotypes: a,b) deficiency combinations *Df(1)64j4/Df(1)w*$^{-}$ *64d* and *Df(1)64j4/Df(1)62d18*; c,d) *Df(1)64f1/w*$^{+}$*Y* and *Df(1)K95/w*$^{+}$*Y*.

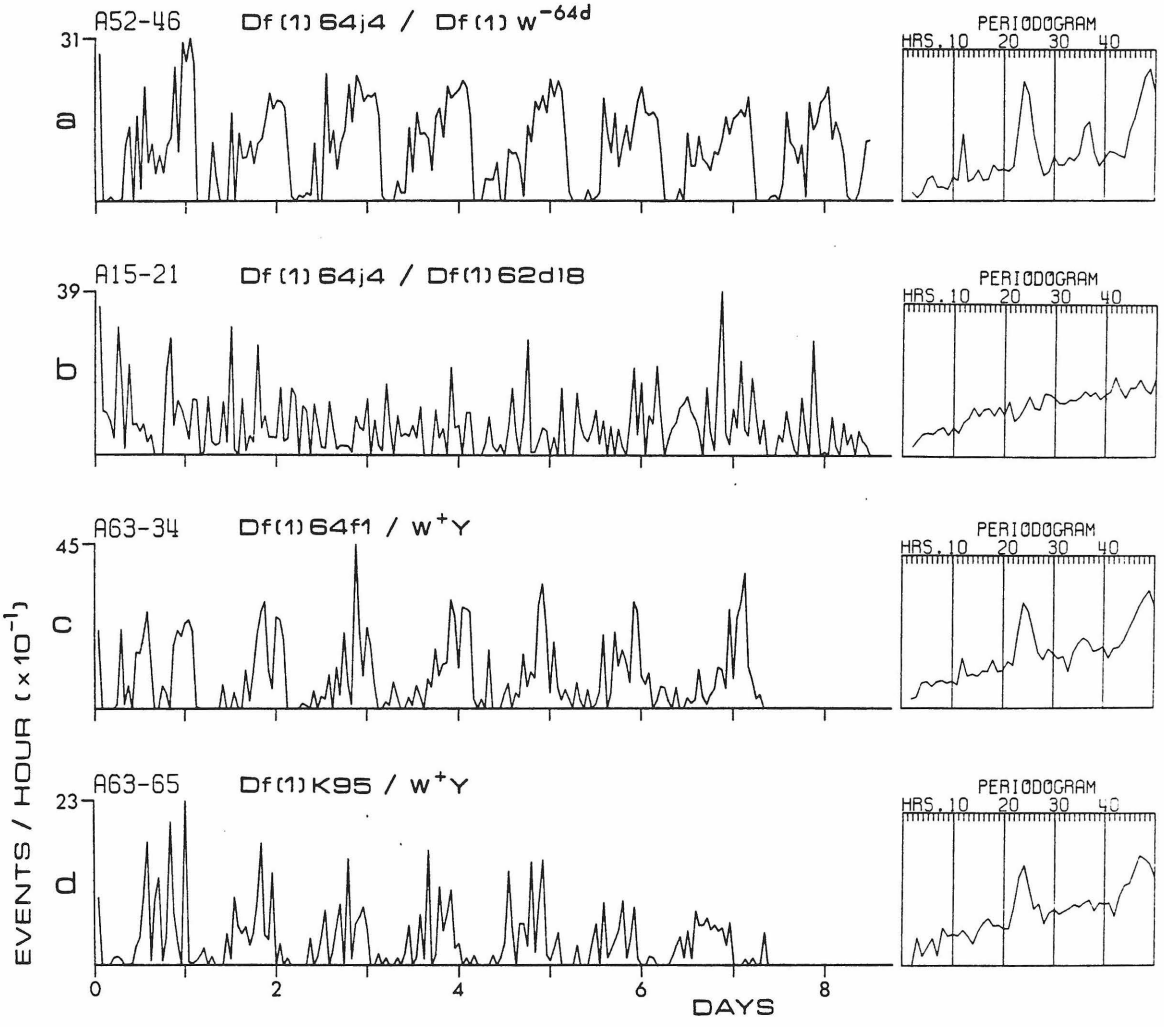

A52-46
Df(1)64j4 / Df(1)w-64d
31
a
PERIODOGRAM
HRS. 10 20 30 40
A15-21
Df(1)64j4 / Df(1)62d18
39
b
PERIODOGRAM
HRS. 10 20 30 40
A63-34
Df(1)64f1 / w+Y
45
c
PERIODOGRAM
HRS. 10 20 30 40
A63-65
Df(1)K95 / w+Y
23
d
PERIODOGRAM
HRS. 10 20 30 40
EVENTS / HOUR (x10-1)
0
2
4
6
8
DAYS

Fig. 5. Eclosion rhythm phenotypes: a,b) *Df(1)w*$^{-64d}$ and *Df(1)64j4* (assayed when heterozygous with *per*^{o}); c,d) deficiency combinations *Df(1)64j4/Df(1)w*$^{-64d}$ and *Df(1)64j4/Df(1)TEM-202*.

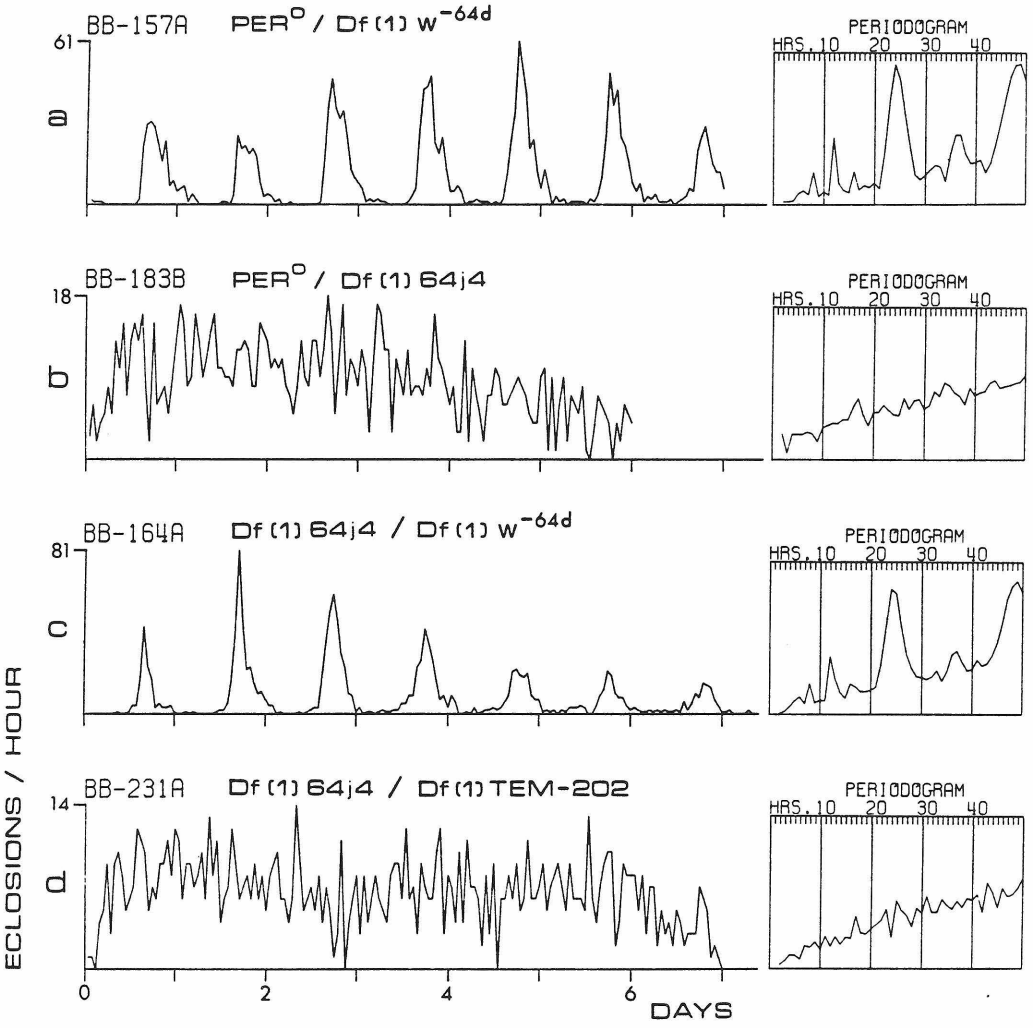
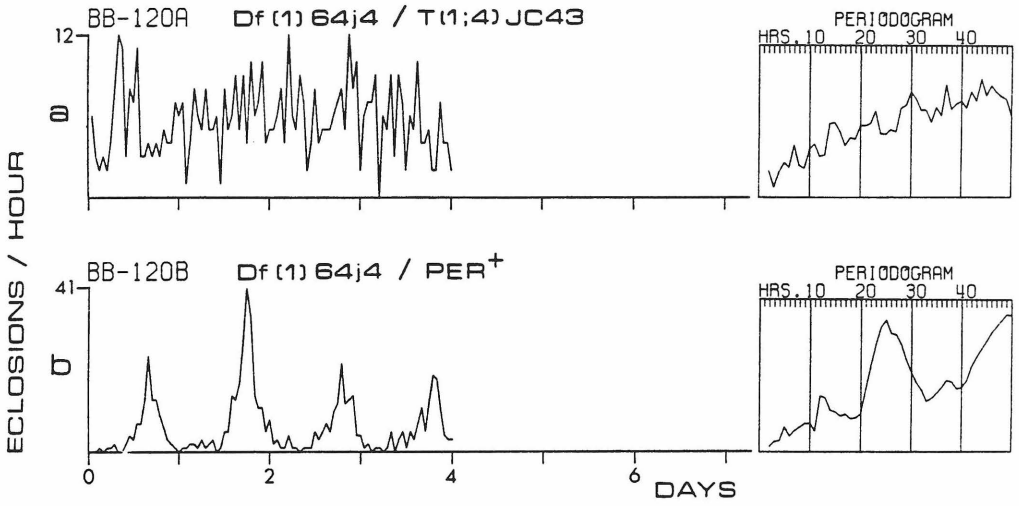

BB-157A PER^{0} / Df(1) w^{-64d}
61
a
PERIODOGRAM
HRS. 10 20 30 40
BB-183B PER^{0} / Df(1) 64j4
18
b
PERIODOGRAM
HRS. 10 20 30 40
BB-164A Df(1) 64j4 / Df(1) w^{-64d}
81
c
PERIODOGRAM
HRS. 10 20 30 40
BB-231A Df(1) 64j4 / Df(1) TEM-202
14
d
PERIODOGRAM
HRS. 10 20 30 40
ECLOSIONS / HOUR
0
2
4
6
DAYS

Fig. 6. Eclosion phenotype of *T(1;4)JC43*: a) arrhythmic profile of *Df(1)64j4/T(1;4)JC43* (cf. Fig. 3); b) rhythmic eclosion profile of the internal control, siblings of the genotype *Df(1)64j4/per*$^{+}$.


BB-120A
Df(1)64j4 / T(1;4)JC43
PERIODOGRAM
HRS. 10 20 30 40
12
a
BB-120B
Df(1)64j4 / PER+
PERIODOGRAM
HRS. 10 20 30 40
41
b
ECLOSIONS / HOUR
0
2
4
6
DAYS

2.8 Tables

Table 1. Activity rhythm phenotypes: complementation behavior of X-chromosomes that behave as per^+ when combined with per^0. (Period in hr)

	per^0		$T(1;4)JC43$		per^s		per^l		per^+	
	period ± s.d.	n	period	n	period	n	period	n	period	n
per^+	24.6 ± 0.2	9	25.0 ± 0.3	9	21.5 ± 0.2	10	24.6 ± 0.8	6	24.2 ± 0.2	13
$Df(1)K95$	25.2 ± 0.3	6	25.1 ± 0.2	6	21.7 ± 0.4	7	25.7 ± 0.5	5	24.5 ± 0.2	6
$Df(1)w^{-64d}$	24.9 ± 0.4	16	24.4 ± 0.3	5	21.4 ± 0.4	12	25.2 ± 0.3	9	23.9 ± 0.2	6
$l(1)zw3^{h2}$	25.5 ± 0.2	6	25.7 ± 0.3	8	22.1 ± 0.4	6	--		24.8 ± 0.4	7
$l(1)zw6^{h15}$	25.6 ± 0.4	8	25.5 ± 0.2	4	21.9 ± 0.5	5	--		24.9 ± 0.2	6

Table 2. Activity rhythm phenotypes: complementation behavior of X-chromosomes that exhibit a mutant phenotype when combined with *per*^{0}

	per^{0}		*T(1;4)JC43*		*per*^{s}		*per*^{l}		*per*$^{+}$	
	period ± s.d.	n	period	n	period	n	period	n	period	n
per^{0}	arrhythmic	18	35.2 ± 3.2 long-arr[a] arrhythmic	6 5 3	19.2 ± 0.4	4	30.6 ± 1.3	5[b]	24.6 ± 0.2	9
Df(1)w^{rJ1}	arrhythmic	16	long-arr arrhythmic	6 11	20.2 ± 0.2	8	30.3 ± 0.6	7	25.1 ± 0.1	5
Df(1)64f1	arrhythmic	13	36.0 long-arr arrhythmic	1 6 12	20.3 ± 0.2	6	30.4 ± 0.4	4	25.4 ± 0.3	10
Df(1)64j4	arrhythmic	19	34.1 ± 2.8 long-arr arrhythmic	6 7 4	19.8 ± 0.3	6	30.1 ± 1.2	6	25.1 ± 0.3	7
Df(1)62d18	arrhythmic	10	32.5 ± 2.1 long-arr arrhythmic	2 7 3	19.5 ± 0.3	10	--		24.5 ± 0.2	7
Df(1)TEM-202	arrhythmic	10	25.0 33.9 ± 2.3 long-arr arrhythmic	1 4 5 6	19.7 ± 0.2	7	--		24.6 ± 0.3	6
T(1;4)JC43	35.2 ± 3.2 long-arr[a] arrhythmic	6 5 3	(lethal)		20.3 ± 0.2	7	29.5 ± 0.9	7	25.0 ± 0.3	9
X^{D}*4*^{P}*JC43;* *X*^{P}*2*^{D}*RC45*	33.5 long-arr arrhythmic	2 2 6	32.5 ± 0.9 long-arr	10 3	--		--		24.4 ± 0.4	8

a Transient long-period rhythmicity (1-3 cycles) that graded into arrhythmicity
b From Konopka and Benzer (1971)

Table 3. Activity rhythm phenotypes of X-chromosome duplications when present in per^0 males

Genotype	period ± s.d.	n
per^0/Y	arrhythmic	25
per^0/w^+Y	23.7 ± 0.3	12
per^0/Y ; $Dp(1;3)w^{67k27}$	23.4 ± 0.2	11
per^0/Y ; $Dp(1;4)w^{m65g}$	23.5 ± 0.2	8
per^0/Y ; $Dp(1;3)w^{m49a}$	arrhythmic	10

Table 4. Complementation behavior of combinations of deficiencies with a 3B1-2 breakpoint

	Activity Rhythm				Eclosion Rhythm[a]			
	Df(1)64j4		*Df(1)K95*		*Df(1)64j4*		*Df(1)K95*	
	period ± s.d.	n	period	n	period ± s.d.	cycles/ run	period	cycles/ run
Df(1)w^{-64d}	24.8 ± 0.4	11	24.1 ± 0.4	7	24.4 ± 0.5	6	24.1 ± 1.0	4
Df(1)62d18	arrhythmic	19	25.2 ± 0.3	11	arrhythmic arrhythmic	5 3[b]	24.8 ± 0.3	4
Df(1)TEM-202	arrhythmic	15	--		arrhythmic	7	--	
X^{D}*4*^{P}*JC43;* *X*^{P}*2*^{D}*RC45*	23.0 long-arr arrhythmic	1 1 8	--		--		--	

a 18°C
b 22°C

Table 5. Eclosion rhythm phenotypes: complementation behavior of 3B1-2 chromosome aberrations. (18°C)

	per^{0}		*T(1;4)JC43*		*per*$^{+}$ *(FM7)*	
	period ± s.d.	cycles/ run	period	cycles/ run	period	cycles/ run
per^{+} *(C-S)*	24.6 ± 0.8	7	24.9 ± 0.5	6	24.7 ± 0.7 24.1 ± 1.0	6 7[b]
Df(1)K95	24.7 ± 0.6 25.3 ± 1.0	7 6	24.6 ± 0.4	6	24.5 ± 0.9	4
Df(1)w^{-} *64d*	24.2 ± 0.5 24.2 ± 0.5	7 7	24.0 ± 0.7	6	23.8 ± 0.7	6
l(1)zw3^{h2}	25.8 ± 1.2	3[a]	25.3 ± 0.8	3[a]	25.3 ± 0.6	3[a]
l(1)zw6^{h15}	25.5 ± 1.5	3[a]	25.7 ± 0.6	3[a]	24.3 ± 1.9	3[a]
per^{0}	arrhythmic arrhythmic arrhythmic	6 5 4	arrhythmic arrhythmic	6 3[a]	24.9 ± 1.7 24.5 ± 1.4	4 2[a]
Df(1)w^{rJ1}	arrhythmic	6	arrhythmic	3[a]	24.8 ± 3.9	2
Df(1)64f1	arrhythmic	6	--		--	
Df(1)64j4	arrhythmic arrhythmic	6 4	arrhythmic arrhythmic	4 4[a]	25.0 ± 1.0 24.7 ± 0.8	3 3[a]
Df(1)62d18	arrhythmic arrhythmic	6 5	arrhythmic	4[a]	24.7 + 1.3	3[a]
Df(1)TEM-202	arrhythmic	7	--		--	
T(1;4)JC43	arrhythmic arrhythmic	6 3[a]	(lethal)		24.0 ± 0.7	6

a 22°C
b *(C-S/C-S)*

Table 6. Activity periods and alphas (in hr) of wild-type males with or without an additional per^+ dose

Genotype	# per^+ doses	period[a] ± s.d.	alpha ± s.d.	n
per^+ (C- S) / Y	1	24.0 ± 0.4	14.8 ± 0.9	12
per^+ (C- S) / w^+Y	2	23.2 ± 0.4**	11.7 ± 2.2**	21
per^+ ($w\ sn^3\ m$) / Y	1	23.7 ± 0.3	13.2 ± 1.8	8
per^+ ($w\ sn^3\ m$) / w^+Y	2	22.9 ± 0.4**	13.7 ± 2.1NS	10
per^+ ($w\ sn^3\ m$) / Y; $Dp(1;3)w^{67k27}$	2	22.6 ± 0.2**	13.0 ± 0.3NS	6

a Determined from activity offsets
** $p < .001$
NS Not significant

Table 7. Activity and eclosion rhythm periods

	Activity Rhythm		Eclosion Rhythm	
Genotype	period ± s.d.	n	period ± s.d.	cycles/ run
Df(1)64f1 / w^+ *Y*	24.1 ± 0.4	16	24.2 ± 0.6	6
Df(1)K95 / w^+ *Y*	23.7 ± 0.6	14	23.7 ± 0.8	5

Chapter 3

Effects of Dosage Alterations at the per Locus on the Period of the Circadian Clock of Drosophila *

* Smith RF, Konopka RJ (1982) Mol Gen Genet 185:30-36

3.1 Summary

The normal 24-h period of the circadian rhythms of locomotor activity and eclosion of *Drosophila melanogaster* is altered by changes in *per* gene dosage. Females with only one dose of per^+ or per^s (the 19-h short-period mutant allele) or per^l (the 29-h long-period mutant allele) have periods which are about 1-2 h longer than the corresponding females with 2 doses. Females with 3 doses of per^+ and males with 2 doses of per^+ or per^s have periods which are ½ to 1 h shorter than the corresponding individuals without the extra dose. Males with three per^+ doses have periods which are about 1.5 h shorter than wild-type males; additional per^+ doses do not shorten period further. The observation that decreased *per* dosage lengthens period while increased dosage shortens period suggests that the long- and short-period mutations alter period by respectively decreasing and increasing *per* gene or gene product activity. The per^+ dosage results and the complementation behavior of per^s indicate that the hypermorphic phenotype of per^s results from increased activity of the per^s gene product rather than an overproduction of per^+ product. This is the first report of such a mutant action in *Drosophila*.

3.2 Introduction

In *Drosophila melanogaster*, several EMS-induced mutations that alter the normal 24-h period of the circadian rhythms of locomotor activity and eclosion have been mapped to a single locus in the 3B1-2 region of the distal X-chromosome. Two mutant alleles, per^{o} and per^{o2}, completely abolish rhythmicity, per^{l} and per^{l2} both lengthen period to about 29 h, while per^{s} shortens period to about 19 h (Konopka and Benzer 1971; Konopka 1972, 1979 and unpublished results). In addition, several chromosome aberrations with a breakpoint in the 3B1-2 region have mutant *per* phenotypes (Young and Judd 1978, Smith and Konopka 1981). Deficiencies of the entire *per* region and four deficiencies and one duplication with 3B1-2 breakpoints have arrhythmic clock phenotypes. The only available translocation with a 3B1-2 breakpoint, *T(1;4)JC43*, produces activity-rhythm records that are totally arrhythmic, very long-period (31-39 h), or transiently long-period before becoming arrhythmic (Smith and Konopka 1981). The *per* mutations are particularly interesting in that they can cause period changes in both directions away from the wild-type period as well as the elimination of periodicity. Arrhythmic alleles appear to be null mutants since they behaviorally and genetically act as a deficiency of the *per* locus. In this paper we present the results of a dosage and complementation analysis of wild-type and mutant *per* alleles which indicate that long- and short-period mutations alter period by respectively decreasing and increasing *per* activity. In addition our results suggest that the hypermorphic phenotype of per^{s} results from increased activity of the per^{s} gene product rather than an overproduction of per^{+} product.

3.3 Materials and Methods

The *per* mutants used in this study are described in Konopka and Benzer (1971) except for per^{o2} which has not been previously described. The per^{o2} mutation was isolated from a screen for X-linked locomotor-activity mutants. Mutagenesis was conducted as described previously (Konopka and Benzer 1971) and stocks were examined for ones with abnormal activity phenotypes. An arrhythmic strain was isolated and complementation and mapping tests identified this mutant as a second arrhythmic *per* allele.

The per^{+} per^{s} and per^{s} per^{s} tandem duplications were derived from *Dp(1;1)w,108* as described in the footnote to Table 4. The tandem duplications *Dp(1;1)w,108* and *Dp(1;1)w,129* and the tandem quintuplications *Qn(1;1)w,126* and *Qn(1;1)w,144* (all derivatives of *Dp(1;1)w*) were generously provided by Dr. E.B. Lewis. All other duplication and deficiency strains were generously provided by Dr. Burke Judd. A description of the chromosome aberrations and other mutants used in this study can be found in Lindsley and Grell (1968) and Young and Judd (1978).

Locomotor-activity rhythms of individual *Drosophila melanogaster* adults and the eclosion rhythms of populations of individuals were monitored and periods calculated as described previously (Smith and Konopka 1981). Locomotor-activity rhythms were monitored at constant temperature (± 0.5° C) in the range 23-25° C. Eclosion rhythms were monitored at 18° C in order to maximize run duration (see Smith and Konopka 1981). In this study the locomotor-activity periods of some genotypes were obtained from records showing a minimum of 5, rather than 6, cycles of rhythmicity.

3.4 Results

3.4.1 Effects of Genetic Background on Period

Wild-type doses of the *per* locus can be added to and subtracted from genotypes using X-chromosome duplications and deficiencies of the 3B1-2 region. Several different deficiencies, duplications, and wild-type chromosomes were used in the dosage studies in order to determine and control for the effects of genetic background on period. The activity-rhythm periods of these chromosomes are shown in Table 1. The periods of two per^+ strains are not significantly different (Table 1, a vs. b). When two deficiencies of the *per* locus, $Df(1)w^{rJ1}$ and *Df(1)64f1*, and the two arrhythmic alleles, per^o and per^{o2}, are combined with the w^+Y duplication of the *per* locus, the resulting periods are also not significantly different from the *C-S* wild-type strain (Table 1, a vs c-f). The period phenotypes of three duplications used in this study were assayed when combined with per^o, which acts as a recessive null mutation of the *per* locus (Konopka 1972, Smith and Konopka 1981). One of these duplications, $Dp(1;3)w^{67k27}$, did produce a significantly shorter period than wild-type (Table 1h). Part of this difference appears to be the result of a shortening of period produced by the genetic background of the per^o strain since all three combinations of per^o and a duplication displayed periods slightly shorter than the *C-S* wild-type strain (Table 1f-h) and shorter than both deficiency/w^+Y combinations (Table 1c,d). Thus there appears to be 1) a 0.3 h period difference between the genetic backgrounds of $Dp(1;3)w^{67k27}$ and the other two duplications, and 2) a 0.4-0.5 h difference between the genetic backgrounds of per^o and the deficiencies (Table 1, f vs. c,d).

3.4.2 A Reduction in per$^+$ *Dosage Lengthens Period*

The period of one per^+ dose in a female was determined in four different genetic backgrounds by combining the two deficiencies, $Df(1)w^{rJ1}$ and *Df(1)64f1*, with two different per^+ chromosomes, a Canton-S X-chromosome and $w\ sn^3\ m$. In each case females with only one dose of per^+ had periods which were about 1 h longer than females with two doses (Table 2a,b,f,g). A per^+ dose was also deleted using the per^o allele, which behaves as a null mutant in all of the genetic studies we have conducted (see Smith and Konopka, 1981). This mutant also behaved as a deficiency in our dosage studies. One dose of per^+ when present in per^+/per^o females produced a period lengthening of 0.4 and 1.0 h in two different genetic backgrounds (Table 2c,i). The shortening effect of the genetic background of per^o (described above) is best controlled in the genotypes shown in Table 2j, where individuals of both genotypes were siblings generated from the same cross (see footnote to Table 2). In this comparison, females with one dose of per^+ in a per^o background had a period which was 1.3 h longer than females with 2 per^+ doses in a per^o background. The second arrhythmic allele, per^{o2}, also significantly lengthened period when heterozygous with per^+ (Table 2d). When differences in genetic background are taken into account the dosage effects of both of the null *per* alleles are as great as those of the *per* deficiencies, thus the dosage effects of the deficiencies appear to be the result of the *per* locus rather than a separate closely-linked locus.

3.4.3 The per *Locus is Dosage Compensated in Males*

Males with the normal one dose of per^+ have periods similar to females with two doses of per^+ (Table 2e,h) rather than females with one dose of per^+. Thus the *per* locus is dosage compensated in males. Dosage compensation is a general property of X-linked gene activity in *Drosophila* males (Stewart and Merriam 1980).

3.4.4 Extra per$^+$ *Doses Shorten Period*

When present as an extra *per*$^+$ dose in males, the duplications *Dp(1;2)w*^{70h31}, *Dp(1;3)w*^{67k27}, and *w*$^+$*Y* shorten period by about 1 h (Table 3a-d). When present as an extra (third) *per*$^+$ dose in female siblings, however, *Dp(1;3)w*^{67k27} only shortens period by 0.5 h (Table 2k vs. 3b). This difference between sexes can be explained by dosage compensation; 2 *per*$^+$ doses in a male when dosage compensated would be equivalent to 4 doses in a female. Two tandem duplications of the *per* locus, derivatives of *Dp(1;1)w*, were also studied (Table 3e,f). *Dp(1;1)w* is cytologically duplicated for the *per* locus (see footnote to Table 3) and we have shown genetically that it is duplicated for *per* since we have been able to exchange both of the original *per* alleles with mutant *per*^{s} alleles (see below). *Dp(1;1)w,129* produces a 1-h shortening of period similar to the other examples of 2 *per*$^+$ doses in a male. *Dp(1;1)w,108* shortens period by almost 2 h; the extra 1-h shortening observed for this duplication is most likely the result of genetic background since all of the 5 other examples of 2 *per*$^+$ doses in a male shorten period by about 1 h.

The period phenotypes of males with 3 doses of *per*$^+$ are shown in Table 3g,h. In the first example, individuals with 3 *per*$^+$ doses exhibited periods which were 0.8 h shorter than siblings with 2 doses. In the second example, an extra *per*$^+$ dose in addition to a tandem duplication did not produce a significant difference in period compared to the tandem duplication alone. The periods of two tandem *per*$^+$ quintuplications, also derivatives of *Dp(1;1)w*, were also examined (Table 3i,j). Both quintuplications exhibited periods which were only about 0.5 h shorter than wild-type and thus have periods which are longer rather than shorter than either of the tandem duplications.

3.4.5 Dosage and Complementation Analysis of the per^s *and* per^l *Mutant Alleles*

Females with one dose of per^s have periods which are approximately 1.5 h longer than females with two doses (Table 4a,b). One dose of per^s in the combination per^s/per^o, however, produces a period which is only about 0.5 h longer than 2 doses of per^s (Table 4c). This difference is apparently the result of the shortening effect of the genetic background of per^o described above and not the result of residual activity of the per^o allele since the combination per^s/per^o has a period which is shorter than the two per^s/deficiency combinations (Table 4, c vs. a,b) while the per^s/per^+ combination has a longer period (Table 4, e vs. a,b).

One per^s dose in a male produces periods similar to 2 doses of per^s in a female (Table 4d,f) rather than one dose in a female, thus the per^s mutant phenotype is dosage compensated in males. A tandem duplication was constructed by crossing a per^s allele into both of the 3A-C segments of the *Dp(1;1)w,108* tandem duplication (see footnote to Table 4). Males with this tandem per^s duplication have periods which are 1 h shorter than males with one per^s dose (Table 4j).

The complementation behavior of per^s when combined with per^+ is also shown in Table 4. A per^+ allele when combined with per^s in a female lengthens period by 2.8 h relative to homozygous per^s females (Table 4e). In contrast, a per^+ allele when combined with per^s in a male (as a duplication) lengthens period by only 1-1.5 h relative to per^s males (Table 4g-i). This difference in complementation behavior between sexes can also be explained by dosage compensation: one dose of both per^s and per^+ in a male would be equivalent to two doses of each of these alleles in a female and we have shown above that increased dosage of per^s and per^+ shortens period.

The dosage and complementation behavior of per^l is shown in Table 5. Females with one dose of per^l have periods which are approximately 1 h longer than females

with two doses (Table 5a,d). The direction and magnitude of change in period produced by a deficiency is thus similar for per^l, per^s and per^+. The per^o allele has the same dosage phenotype as a deficiency when combined with per^l (Table 5b). As with per^+ and per^s, the per^l allele is dosage compensated in males (Table 5, e vs. a,d). Thus neither the per^s nor the per^l mutation affects this regulatory process. A per^+ allele when combined with per^l in a female shortens period by 4 h relative to homozygous per^l females (Table 5c).

3.4.6 Activity and Eclosion Rhythms Have Similar per *Dosage Phenotypes*

The effects of altered *per* dosage on eclosion-rhythm periods are nearly identical to those observed for the activity rhythm. When heterozygous with per^+, deficiencies produce a lengthening of period of about 1 h (Table 6a,c) while per^o lengthens period by 0.5 h (Table 6b,d). Duplications, which shorten the period of males by about 1 h (Table 6f-h), have a slightly smaller effect in female siblings (Table 6e). The quintuplication *Qn(1;1)w,126* has an eclosion-rhythm period similar to 2 doses of per^+ (Table 6i), thus showing a slightly shorter period than that observed for the activity rhythm.

Dosage and complementation analysis of the per^s mutant allele was also performed for the eclosion rhythm (Table 7). The eclosion and activity-rhythm periods of per^s genotypes differ slightly as a result of the lower temperature at which the eclosion rhythms were monitored. This is because the short-period phenotype of per^s is lengthened at temperatures less than 25°C while the period of wild-type remains constant (Konopka et al 1982). Since the direction of change in period as a result of altered dosage and of complementation with per^+ is similar for both rhythms, there does not appear to be a significant difference between rhythms in the dosage and complementation behavior of per^s other than that produced by the

difference in temperature.

3.5 Discussion

We have observed that decreased dosage of per^+, per^s, or per^l lengthens period while increased *per* dosage shortens period. It is not surprising that period is sensitive to changes in *per* dosage since in *Drosophila* gene activity (as measured by enzyme activity) is dosage dependent (see O'Brien and MacIntyre 1978). For those gene-enzyme systems which have been mapped, an extra dose is observed to increase enzyme activity by about one-half (or more for dosage compensated X-linked genes in males; see Stewart and Merriam 1980) while the deletion of a dose is observed to reduce activity by about one-half. Since an extra per^+ dose shortens period while the deletion of a dose lengthens period, the short- and long-period mutations apparently alter period by respectively increasing and decreasing *per* gene or gene-product activity.

The dose-response phenotypes of the per^s and per^l alleles also demonstrate that these mutations respectively increase and decrease the level of *per* expression. The dependence of gene activity on gene dosage in *Drosophila* allows mutations that decrease, increase, or eliminate gene expression to be distinguished by the following dose-response phenotypes (cf. Muller 1932; Lifschytz and Green 1979): (i) mutations that when combined with a deficiency express a mutant phenotype that is more extreme than the phenotype of the mutation when homozygous are classified as hypomorphic (having reduced activity); (ii) mutations that when combined with a deficiency express a phenotype that is less extreme than the phenotype of the mutation when homozygous are classified as hypermorphic (having increased activity); (iii) mutations that when combined with a deficiency of the locus express a mutant phenotype that is as extreme as the phenotype of the mutation when homozygous are assumed to be amorphic (i.e. null). Based on these criteria, per^l, per^s, and per^0 can be classified as a hypomorph, hypermorph, and amorph, respec-

tively: the per^l phenotype is enhanced when combined with a deficiency, the per^s phenotype is partially suppressed by a deficiency, while per^o behaves as a deficiency of the locus. The translocation *T(1;4)JC43*, which produces mutant *per* activity phenotypes that are both very long-period and/or arrhythmic, displays a dose-response phenotype which is consistent with an extreme hypomorphic mutant action: the degree of arrhythmicity (both penetrance and expressivity) is enhanced when *T(1;4)JC43* is combined with a deficiency (Smith and Konopka 1981). While the classification scheme described above is based on the phenotypes produced by a reduction in dosage, our observation that increased dosage of per^+ or per^s shortens period provides additional support for the proposal that short-period mutant action results from increased *per* activity.

Hypermorphic mutant alleles are extremely rare for loci of any genetic system. The *frq* locus of *Neurospora*, which has mutant alleles that both increase and decrease circadian period (Feldman et al. 1979) may be another (perhaps homologous) example of a locus having both hyper- and hypomorphic mutations. The only *Drosophila* locus having both hypermorphic and hypomorphic "point" mutations besides *per* is Beadex (1:59.4). At the Beadex locus, hypermorphic alleles (*Bx*) have a dominant mutant phenotype that affects wing scalloping while hypomorphic or amorphic alleles (*hdp*) have a recessive mutant phenotype that affects wing positioning (Lifschytz and Green 1979). As with per^s, the hypermorphic phenotype of *Bx* is suppressed by a deficiency and is mimicked by extra wild-type doses.

While both per^s and *Bx* behave as hypermorphs, the type of mutant effect by which the hypermorphic phenotype is produced appears to be different for these two mutations. Gene activity could be decreased or increased by either a change in the activity of each molecule (e.g. by an alteration in the structural gene) or by a change in the rate at which wild-type molecules are produced (e.g. by an alteration in a regulatory element). If per^s were an overproducer of wild-type product, then

(1) per^s males with a per^+ duplication should produce more product and thus have a shorter period than per^s/Y males, and (2) in a similar manner per^s/per^+ females should have a shorter period than per^s/deficiency females. However, such results are not observed (Table 4). Instead it appears that the shortening effect of per^s is averaged rather than summed when combined with per^+, as though half of the total *per* product has increased activity and half has wild-type activity. In contrast, wild-type duplications enhance the mutant phenotype of *Bx* (Lifschytz and Green 1979). Furthermore, multiple per^+ doses cannot mimic the full extent of the short-period phenotype. The shortening effect on period of more than one per^+ dose in a male appears to saturate at a level of about -1.5 h (Table 3). In the case of the *Bx* locus, however, each increase in wild-type dose produces an even more extreme mutant phenotype (Lifschytz and Green 1979). These observations suggest that the hypermorphic phenotype of per^s results from an increase in the functional activity of each molecule of product while the hypermorphic phenotype of *Bx* results from an overproduction of wild-type product (see Lifschytz and Green 1979 for additional genetic evidence that suggests that the hypermorphic phenotype of *Bx* results from an overproduction of wild-type product).

Unfortunately, it is not possible to distinguish between the possibilities of decreased product activity vs. decreased amount of wild-type product for hypomorphic mutants using the type of analysis described above for hypermorphic mutants. This is because, when combined with a hypomorphic mutation, deficiencies should enhance and wild-type alleles should suppress the phenotype of mutations having either type of hypomorphic mutant effect. This is indeed observed for both per^l and *T(1;4)JC43* (see Smith and Konopka 1981).

Mutational and dosage alterations at the *per* locus also affect a very short-term rhythm in the *Drosophila* courtship song. The courtship song of *Drosophila* males (and females phenotypically transformed into males by the mutation *tra*) has a

rhythm in the duration of inter-pulse intervals with a period of about 54 s (Kyriacou and Hall 1980). Kyriacou and Hall also found that each of the *per* mutants affects the period of this song rhythm in a fashion parallel to that observed for circadian rhythmicity: per^S shortens the period of the song oscillation to 42 s, per^l lengthens period to 82 s, and per^0 abolishes rhythmicity. In contrast with circadian periodicity, however, both per^0 and deficiencies of the *per* locus *shorten* song period when combined with per^+, per^S, or per^l (to about 40, 34, and 52 s, respectively). If the level of *per* activity had an opposite effect on the period of these rhythms, then the *per* mutants should also have an opposite effect on period. However, the mutants produce the same direction of change in period for both rhythms; only the dose response is reversed. We have no explanation for the difference in the direction of the dosage response between these two rhythmic systems.

Another difference in the effects of the *per* locus on these two rhythms is that the period of the song rhythm is much more dosage sensitive than circadian periodicity. The change in song period from 54 to 40 s described above for 1 vs. 2 per^+ doses in a (transformed) female is a 26% reduction in period, while for circadian rhythmicity this dosage difference produces less than a 5% lengthening of period. Although the effects of duplications were not reported for the song rhythm, we have shown that an extra per^+ dose in males or females produces less than a 5% shortening of circadian period. The relative insensitivity of circadian periodicity to changes in *per* dosage suggests that there might exist for the circadian system, but not the song rhythmic system, a mechanism which regulates the amount of *per* product and thus partially compensates for the effects of *both* increased and decreased *per* dosage in *both* sexes. Although this type of genetic regulation has not been previously observed for any other *Drosophila* locus, the *per* locus may be unique: we have shown that the proper level of *per* activity is critical for the maintenance of a

precise 24-h period and such a mechanism may act under wild-type dosage conditions to precisely regulate *per* product levels.

This model of *per* regulation also provides an explanation for the unusual dosage and complementation behavior of per^S (see Addendum = § 3.8, p. 108). If *per* product levels were strictly dosage dependent then per^S/deficiency individuals would be expected to produce less *per* product and have a longer period than per^S/per^+ heterozygotes. However per^S/deficiency individuals are observed to have shorter periods than per^S/per^+ heterozygotes (Table 4). If, according to our model, the amount of *per* product produced by one *per* dose in a female is partially compensated, per^S/deficiency individuals could produce nearly as much per^S product as per^S/per^S homozygotes. This could then account for the observation that per^S/deficiency individuals have periods nearly as short as per^S homozygotes and shorter than per^S/per^+ heterozygotes. Partial compensation of increased *per* dosage could in a similar fashion account for the observation that per^+ duplications lengthen rather than shorten period when present in per^S males.

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3.7 Tables

Table 1. Activity rhythm periods of males with one per^+ dose. (Period in h)

	Genotype[a]	per^+ doses	Period ± SD	n	Δ Period[c]
a	per^+_a / Y	1	24.0 ± 0.4	12	--
b	per^+_b / Y	1	23.7 ± 0.3	7	-0.3 NS
c	$Df(1)w^{rJ1}/w^+Y$	1	24.2 ± 0.4	15	+0.2 NS
d	$Df(1)64f1/w^+Y$	1	24.1 ± 0.4	16	+0.1 NS
e	per^{o2}/w^+Y	1	23.9 ± 0.3	8	-0.1 NS
f	per^o/w^+Y	1	23.7 ± 0.3	12	-0.3 NS
g	per^o / Y; $Dp(1;2)w^{70h31}$	1	23.7 ± 0.4	6	-0.3 NS
h[b]	per^o / Y; $Dp(1;3)w^{67k27}$	1	23.4 ± 0.2	11	-0.6 ***

[a] Genotypes are abbreviated as follows:
per^+_a = Canton-S (*C-S*)
per^+_b = $w\ sn^3\ m$
per^o = $per^o\ w\ spl$
per^{o2} = per^{o2} (unmarked)
(w^+Y (2D1-2; 3D3-4), $Dp(1;2)w^{70h31}$ (3A6-8; 3C2-3), and $Dp(1;3)w^{67k27}$ (3A4-5; 3E8-F2) are per^+ duplications ; $Df(1)w^{rJ1}$ (3A1-2; 3C2-3) and $Df(1)64f1$ (3A10-B1; 3B3-4) are deficiencies of the *per* locus)

[b] Siblings of the genotypes shown in Table 2j

[c] Compared to per^+_a / Y (a)

*** $p \leq .001$

NS Not significant

Table 2. Activity rhythm periods of females with 2 per^+ doses (on left) vs. females with 1 or 3 doses or males with 1 dose (on right). Period differences between matched genotypes are shown in the last column.

	Genotype[a]	per^+ doses	Period ± SD	n	Genotype	per^+ doses	Period ± SD	n	Δ Period[d]
a	per^+_a / per^+_a	2	24.2 ± 0.2	13	$per^+_a / Df(1)w^{rJ1}$	1	25.1 ± 0.1	5	+0.9
b					$per^+_a / Df(1)64f1$	1	25.4 ± 0.3	10	+1.2
c					per^+_a / per^o	1	24.6 ± 0.2	9	+0.4
d					per^+_a / per^{o2}	1	24.8 ± 0.2	7	+0.6
e					per^+_a / Y (males)	1	24.0 ± 0.4	12	-0.2 NS
f	per^+_b / per^+_b	2	24.1 ± 0.4	5	$per^+_b / Df(1)w^{rJ1}$	1	25.1 ± 0.4	9	+1.0
g					$per^+_b / Df(1)64f1$	1	25.1 ± 0.2	5	+1.0
h					per^+_b / Y (males)	1	23.7 ± 0.3	7	-0.4 NS
i	per^+_c / per^+_b	2	23.8 ± 0.3	7	per^+_c / per^o	1	24.8 ± 0.4	7	+1.0
j[b]	per^+_c / per^o; $Dp(1;3)w^{67k27}$	2	23.5 ± 0.4	7	per^+_c / per^o	1	24.8 ± 0.4	7	+1.3
k[c]	per^+_c / per^+_b	2	23.8 ± 0.3	7	per^+_c / per^+_b; $Dp(1;3)w^{67k27}$	3	23.3 ± 0.2	7	-0.5

[a] per^+_c = $y\ In(1)w^{m4L}rst^{3R}$

See Table 1 for other genotype abbreviations

[b] F_1 siblings of the cross per^o / per^o X $per^+_c / Y; Dp(1;3)w^{67k27}$

[c] F_1 siblings of the cross per^+_b / per^+_b X $per^+_c / Y; Dp(1;3)w^{67k27}$

[d] $p \leq .01$ except where noted

NS Not significant

Table 3. Activity rhythm periods of males with 1-5 doses of per^+.

	Genotype[a]	per^+ doses	Period ± SD	n	Genotype	per^+ doses	Period ± SD	n	Δ Period[e]
a[b]	per^+_b / Y	1	23.6 ± 0.2	5	per^+_b / Y; $Dp(1;2)w^{70h31}$	2	22.5 ± 0.3	6	-1.1
b[c]	per^+_b / Y	1	23.7 ± 0.2	8	per^+_b / Y; $Dp(1;3)w^{67k27}$	2	22.7 ± 0.2	9	-1.0
c	per^+_b / Y	1	23.7 ± 0.3	7	per^+_b / w^+Y	2	23.0 ± 0.4	15	-0.7
d					per^+_a / w^+Y	2	23.2 ± 0.4	21	-0.8
e	per^+_a / Y	1	24.0 ± 0.4	12	$Dp(1;1)w,129$ / Y	2	23.1 ± 0.2	7	-0.9
f					$Dp(1;1)w,108$ / Y	2	22.2 ± 0.4	7	-1.8
g[d]	per^+_c / w^+Y	2	23.2 ± 0.6	14	per^+_c / w^+Y; $Dp(1;3)w^{67k27}$	3	22.4 ± 0.2	11	-0.8
h	$Dp(1;1)w,108$ / Y	2	22.2 ± 0.4	7	$Dp(1;1)w,108$ / w^+Y	3	22.5 ± 0.4	11	+0.3 NS
i	per^+_a / Y	1	24.0 ± 0.4	12	$Qn(1;1)w,126$ / Y	5	23.4 ± 0.2	7	-0.6
j					$Qn(1;1)w,144$ / Y	5	23.6 ± 0.3	5	-0.4 *

[a] $Dp(1;1)w,108$ = $Dp(1;1)w$, y ac $(w^{ch}\ rst^+)\ (w^+\ rst^2)$ = X-108 of E.B. Lewis stock collection
$Dp(1;1)w,129$ = $Dp(1;1)w$, y^2 sc $(w^i\ rst^+)\ (w^i\ rst^+)$ = X-129
$Qn(1;1)w,126$ = $Dp(1;1)w$, y^2 sc $(w\ rst^+)_4\ (w^+\ rst^+)$ fa = X-126
$Qn(1;1)w,144$ = $Dp(1;1)w$, y $(w^i\ rst^+)_5$ ec = X-144
(all of the above are derivatives of $Dp(1;1)w$ which is a tandem duplication of the region 3A-C ; see Lindsley and Grell, 1968)
See Tables 1 and 2 for other genotype abbreviations

[b] F_1 siblings of the cross per^+_b / per^+_b X per^+_c / Y ; $Dp(1;2)w^{70h31}$

[c] F_1 siblings of the cross per^+_b / per^+_b X per^+_c / Y ; $Dp(1;3)w^{67k27}$

[d] F_1 siblings of the cross C(1)DX, y f / w^+Y X per^+_c / Y ; $Dp(1;3)w^{67k27}$

[e] $p \leq .01$ except where noted

* $p \leq .05$

NS Not significant

Table 4. Activity rhtyhm periods: dosage and complementation behavior of per^s.

	Genotype[a]	Period ± SD	n	Genotype	Period ± SD	n	Δ Period[c]
a				per^s / $Df(1)w^{rJ1}$	20.2 ± 0.2	8	+1.5
b				per^s / $Df(1)64f1$	20.3 ± 0.2	6	+1.6
c	per^s / per^s	18.7 ± 0.3	7	per^s / per^0	19.2 ± 0.4	4	+0.5 *
d				per^s / Y	19.2 ± 0.4	6	+0.5
e				per^s / per^+_a	21.5 ± 0.2	10	+2.8
f	per^s_b / per^s_b	19.4 ± 0.1	5	per^s_b / Y	19.1 ± 0.3	6	-0.3 NS
g[b]	per^s / Y	18.6 ± 0.3	7	per^s / Y; $Dp(1;3)w^{67k27}$	20.1 ± 0.2	8	+1.5
h				per^s / w^+Y	20.4 ± 0.5	4	+1.2
i	per^s / Y	19.2 ± 0.4	6	$Dp(1;1)w$, per^+ per^s / Y	20.2 ± 0.2	9	+1.0
j				$Dp(1;1)w$, per^s per^s / Y	18.2 ± 0.2	20	-1.0

[a] per^s = per^s w spl

per^s_b = y per^s sn^3 m

$Dp(1;1)w$, per^+ per^s = $Dp(1;1)w$, y ac (per^+ w^{ch} rst^+) (per^s w rst^+) spl
(recombinant of $Dp(1;1)w,108$ / per^s w spl)

$Dp(1;1)w$, per^s per^s = $Dp(1;1)w$, (per^s w^+ rst^+) (per^s w rst^+) spl
(recombinant of $Dp(1;1)w$, per^+ per^s / per^s)

See Table 1 for other genotype abbreviations.

[b] F_1 siblings from the cross per^s / per^s X per^+_c / Y ; $Dp(1;3)w^{67k27}$

[c] $p \leq .01$ except where noted

* $p \leq .05$

NS Not significant

Table 5. Activity rhythm periods: dosage and complementation behavior of per^l.

	Genotype[a]	Period ± SD	n	Genotype	Period ± SD	n	Δ Period[b]
a				per^l_a / $Df(1)w^{rJ1}$	30.3 ± 0.6	7	+1.2
b	per^l_a / per^l_a	29.1 ± 0.5	7	per^l_a / per^o	30.1 ± 0.3	12	+1.0
c				per^l_a / per^+_a	25.1 ± 0.2	10	-4.0
d	per^l_b / per^l_b	29.1 ± 0.7	9	per^l_b / $Df(1)G4f1$	30.4 ± 0.4	4	+1.3
e				per^l_b / Y	28.6 ± 0.3	11	-0.5[NS]

[a] per^l_a = per^l (unmarked)
per^l_b = $y\ per^l\ sn^3\ m$
per^o = per^o (unmarked)
See Table 1 for other genotype abbreviations

[b] $p \leq .01$ except where noted

[NS] Not significant

Table 6. Eclosion rhythm periods of females with 1-3 per^+ doses (above) and males with 1-5 per^+ doses (below).

	Genotype[a]	per^+ doses	Period ± SD	cycles/ run	Genotype	per^+ doses	Period ± SD	cycles/ run	Δ Period[f]
a[b]	per^+_a/per^+_a	2	24.1 ± 1.0	7	$per^+_a/Df(1)w^{rJ1}$	1	24.9 ± 1.1	10	+0.8
b					per^+_a/per^o	1	24.6 ± 0.8	7	+0.5
c[c]	per^+_b/per^+_b	2	24.4 ± 0.5	7	$per^+_b/Df(1)w^{rJ1}$	1	25.4 ± 0.7	6	+1.0
d[d]	per^+_c/per^o; $Dp(1;3)w^{67k27}$	2	23.9 ± 0.7	5	per^+_c/per^o	1	24.4 ± 0.8	5	+0.5
e[e]	per^+_b/per^+_c	2	24.3 ± 0.7	6	per^+_b/per^+_c; $Dp(1;3)w^{67k27}$	3	23.6 ± 0.5	7	-0.7
f[e]	per^+_b/Y	1	24.1 ± 0.6	7	per^+_b/Y; $Dp(1;3)w^{67k27}$	2	23.1 ± 0.8	7	-1.0
g[c]	per^+_b/Y	1	24.3 ± 0.7	7	per^+_b/w^+Y	2	23.2 ± 1.0	7	-1.1
h[b]	per^+_a/Y	1	24.1 ± 0.9	7	per^+_a/w^+Y	2	23.1 ± 0.8	7	-0.9
i					$Qn(1;1)w,126/Y$	5	23.1 ± 0.7	7	-0.9

[a] per^o = per^o *w spl*

per^o_b = *y* per^o

See Tables 1, 2 and 3 for other genotype abbreviations

[b] $per^+_a/Df(1)w^{rJ1}$ and per^+_a/w^+Y are F_1 siblings of the cross per^+_a/per^+_a X $Df(1)w^{rJ1}/w^+Y$

[c] $per^+_b/Df(1)w^{rJ1}$ and per^+_b/w^+Y are F_1 siblings of the cross per^+_b/per^+_b X $Df(1)w^{rJ1}/w^+Y$

[d] F_1 siblings of the cross per^o/per^o X per^+_c/Y; $Dp(1;3)w^{67k27}$

[e] F_1 siblings of the cross per^+_b/per^+_b X per^+_c/Y; $Dp(1;3)w^{67k27}$

[f] p values are not given since periods were obtained from single eclosion runs.

Table 7. Eclosion rhythm periods: dosage and complementation behavior of per^s.

	Genotype[a]	Period ± SD	cycles/ run	Genotype	Period ± SD	cycles/ run	Δ Period[d]
a[b]				per^s / *Df(1)64f1*	21.8 ± 1.0	7	+1.9
b				per^s_b / *Df(1)64f1*	22.0 ± 0.7	7	+2.1
c	per^s / per^s	19.9 ± 1.5	8	per^s_b / $Df(1)w^{rJ1}$	22.1 ± 1.0	8	+2.2
d				per^s / Y	19.9 ± 1.4	8	0.
e				per^s / per^+_a	22.1 ± 0.9	7	+2.1
f[c]	per^s / per^+_c	21.9 ± 0.6	7	per^s / per^+_c; $Dp(1;3)w^{67k27}$	22.0 ± 0.6	7	+0.1
g[c]	per^s / Y	20.3 ± 0.8	8	per^s / Y; $Dp(1;3)w^{67k27}$	20.8 ± 1.3	7	+0.5
h[b]	per^s / Y	19.9 ± 1.4	8	per^s / w^+Y	20.7 ± 1.3	7	+0.8

[a] per^s = per^s *w spl*

per^s_b = *y* per^s sn^3 *m*

See Table 1 for other genotype abbreviations

[b] per^s/ *Df(1)64f1* and per^s / w^+Y are F_1 siblings of the cross per^s / per^s X *Df(1)64f1* / w^+Y

[c] F_1 siblings of the cross per^s / per^s X per^+_c / Y ; $Dp(1;3)w^{67k27}$

[d] p values are not given since periods were obtained from single eclosion runs.

3.8 Addendum — Discussion of the unusual dosage and complementation behavior of the *per*^{s} allele

A summary of the complementation and dosage behavior of the *per* alleles is shown in Table 3.8. When the *per* alleles are arranged in order of effecting an increase in period length (with the arrhythmic phenotype ≡ ∞ period), they seriate in a consistent order when tested opposite *per*$^{+}$, *per*^{l}, or *per*^{o} (≡ Df) but in a different order when seriated opposite *per*^{s}. While there are differences in genetic background in these comparisons, these differences cannot account for unusual ordering of the *per*^{s} combinations. As described above in §3.5, our model of *per* dosage regulation provides one possible explanation of the unusual dosage and complementation behavior of *per*^{s}. Another interpretation is that the the *per*^{s} mutant affects more than one attribute of *per* gene activity/function. For example, one mutant site(s) could account for the hypomorphic and amorphic mutant action of *per*^{l} and *per*^{o} and another separate mutant site could account for the non-additive effect on period displayed by some *per*^{s} combinations. The presence of at least two sites within the *per* locus with qualitatively different actions implies some type of genetic complexity at the *per* locus.

Table 3.8. Complementation and dosage behavior of the *per* alleles.
Activity rhythm periods (in h)
Females above (a-e); males below (f,g)

		1	2	3	4	5
		per^s period	per^+ period	per^l period	per^o period	$Df(1)w^{rJ1}$ period
a	per^s	18.7 ± 0.3	21.5 ± 0.2	22.9 ± 0.4[a]	19.2 ± 0.4	20.2 ± 0.2
b	per^+	21.5 ± 0.2	24.2 ± 0.2	25.1 ± 0.2	24.6 ± 0.2	25.1 ± 0.1
c	per^l	22.9 ± 0.4[a]	25.1 ± 0.2	29.1 ± 0.5	30.1 ± 0.3	30.3 ± 0.6
d	per^o	19.2 ± 0.4	24.6 ± 0.2	30.1 ± 0.3	arrhy.	arrhy.
e	$Df(1)w^{rJ1}$	20.2 ± 0.2	25.1 ± 0.1	30.3 ± 0.6	arrhy.	arrhy.
f	$w^+ Y$	20.4 ± 0.5	23.2 ± 0.4	--	23.7 ± 0.3	24.2 ± 0.4
g	Y	19.2 ± 0.4	24.0 ± 0.4	28.6 ± 0.3	arrhy.	arrhy.

a from Konopka and Benzer 1971

Chapter 4

Andante

- A New Circadian Clock Mutant of Drosophila melanogaster *

* Dominic P-Y Orr and Ronald J Konopka have contributed to portions of the studies described in this chapter.

4.1 Summary

By screening mutagenized sex-linked and autosomal stocks for ones in which the normal period or phase of the circadian rhythm of eclosion (adult emergence) has been altered, a new X-linked clock mutant has been isolated which lengthens the normal 24-h period of both the the eclosion and adult locomotor-activity rhythms to about 25.5 h. This mutant, which we have named Andante (*And*), is not an allele of the other previously identified clock mutant locus (*per*, at 3B1-2); recombination and deficiency mapping has placed the Andante locus at a separate site between polytene chromosome bands 10E2 and 10F1 (tentatively at 10E3, just proximal to the *m-dy* complex at 10E2-3). Andante, like all of the *per* mutant alleles, has a semi-dominant effect on period. The eclosion rhythm of Andante, like wild-type, has a low-amplitude (Type 1) phase-resetting response to light pulses, but compared to wild-type the Andante phase-resetting curve (PRC) is lengthened by 1-2 h per cycle.

4.2 Introduction

In the first attempt to isolate clock mutants in any organism, approximately 2000 mutagenized X-chromosomes of *Drosophila melanogaster* were screened for mutations altering the phase of entrainment of the eclosion rhythm in a LD 12:12 cycle (Konopka and Benzer 1971). Under LD 12:12 entrainment wild-type flies eclose during the light phase while very few flies eclose during the dark phase. By examining mutagenized strains for those with an abnormally high rate of eclosion during the night, three mutants were isolated that drastically altered circadian periodicity of both the eclosion and the locomotor-activity rhythm when examined under constant ("free-running") conditions. One mutant was arrhythmic, another had a period of about 19 h, while the third had a period of about 29 h. All three mutants were mapped to a single locus (called *per*) on the distal X-chromosome and were designated per^0, per^s, and per^l, respectively.

In an attempt to isolate additional clock mutants, 3219 mutagenized chromosomes have been screened for altered clock phenotypes by one of three different protocols. As a result, one new X-linked clock mutant has been isolated which lengthens the period of both the eclosion and locomotor-activity rhythms by about 1.5 h.

4.3 Mutant Screening Procedures

Mutagenized sex-linked and autosomal chromosomes were screened for clock mutants by one of three methods. The first screen is similar to that used by Konopka and Benzer (1971): eclosion patterns were monitored in stock bottles maintained in a LD 12:12 (12h light, 12h dark each day) cycle at room temperature. Stock bottles were cleared of adults at the end of each light period then the bottles examined at the end of the dark phase to estimate the number of flies which eclosed during the dark phase. As described above, very few wild-type flies eclose during the dark phase. Attached-X females served as an internal control for the eclosion pattern of males bearing identical mutagenized X-chromosomes. Examining mutagenized strains for those with an abnormally high rate of eclosion during the dark phase would allow the detection of arrhythmic clock mutants, such as *per*^{0}, or mutants with an altered phase of eclosion with respect to the entraining cycle.

Although the advantage of screening under LD 12:12 is that is technically very easy to perform, period mutants that do not push the phase of eclosion into the night would not be detected by such a screen. For this reason we also performed a second screen under the conditions of constant darkness (DD) after previous LD 12:12 entrainment, which allows the free-running period of the clock to be expressed. Under these conditions mutant strains with an altered period would show an altered phase of eclosion with respect to wild-type after a few cycles of free-run.

For the third screen mutagenized stocks were raised in LD 12:12 at 22° C, transferred to DD at the end of a light phase, given a 12h, 29° C temperature pulse starting 12h after the last seen light-dark transition (i.e. during the phase corresponding to the light phase of the previous LD 12:12 cycle), then returned to

22° C to monitor eclosion. Wild-type strains return to the phase of entrainment within two cycles after the temperature pulse allowing the detection of conditional mutants for which period or steady-state phase is altered while at a restrictive (high) temperature. With such conditional mutants it might be possible to determine the time of gene action during development and during course of each cycle. Since this screen was conducted under constant darkness, non-temperature-sensitive period mutants could also be detected.

4.4 Results and Discussion

The number of mutagenized chromosomes screened under the 3 conditions were 1795 hemizygous X-chromosomes for the first screen, 769 hemizygous X-chromosomes for the second screen and 439 hemizygous X-chromosomes and 555 homozygous second and third-chromosomes for the third screen. The clock phenotypes of putative mutants obtained from the first two screens were monitored in locomotor-activity tests. However no confirmed mutants were recovered.

The eclosion rhythm phenotypes of putative conditional mutants obtained from the third screen were monitored in bang-boxes under conditions similar to the screening conditions. One confirmed mutant, which we have named Andante (*And*) did show an eclosion pattern significantly different than wild-type (Fig. 1). On the third day after a 12 h, 31° C temperature pulse, the median of the eclosion peak of male siblings bearing an identical mutagenized X-chromosome showed a distinct 4 h phase delay compared to the wild-type internal control, females bearing a *y f* attached-X chromosome. This phase delay observed in the mutant strain under these conditions appears to be secondary result of a primary mutant effect on period. Under conditions of constant darkness and temperature, *And* lengthens the free-running period of both the eclosion and locomotor-activity rhythms by 1.2-1.5 h. The eclosion pattern of this mutant strain under conditions of constant temperature and darkness is shown in Fig. 2. Mutant females have periods which are about 1.5 h longer than the wild-type females. The eclosion rhythm periods of *And* males and females are shown in Table 4 a-d. The period of the locomotor-activity rhythm of this strain is also about 1.5 h longer than wild-type under constant conditions (Fig. 3; Table 1 a,i). When combined with a wild-type allele in *trans*-heterozygotes, *And* has a semi-dominant effect, lengthening period by 0.6 h compared to wild-type (Table 1b).

The mutant strain also has a morphological mutant phenotype (short, dark wings) which was mapped by recombination analysis to the miniature-dusky region (1-36.1). Complementation tests showed that the morphological phenotype is the result of a dusky (*dy*) mutation. The clock phenotype, however, does not appear to result from the dusky mutation. In locomotor-activity tests, two different dusky alleles, *dy* and m^D (see Lindsley and Grell 1968; Dorn and Burdick 1962) have periods which are similar to wild-type (Table 3a,b vs. 1b). In addition, *And/dy* and *And/*m^D heterozygotes (Table 1l,m) have periods which are not significantly longer than the *And/And*$^+$ heterozygotes (Table 1b; *And/And*$^+$ heterozygotes have a period which is 0.6 h longer than wild-type). In the eclosion tests, *And* does not lengthen period when combined with *dy* or m^D (Table 4h-k). Unfortunately, tests of allelism, which depend upon the uncovering of a recessive mutant phenotype when two mutants are combined in a *trans* configuration, are hampered by the semi-dominant phenotype of *And*.

Although the mutant clock phenotype of *And* does not appear to result from the dusky mutation, our mapping studies demonstrate that *And* is located just proximal to the dusky locus on the X-chromosome. Recombination experiments were first conducted in order to obtain a gross localization of clock mutation on the X-chromosome. F_1 recombinant males recovered from *y cho cv v f /And* females were pair-mated to virgin attached-X females in order to produce stocks of identical recombinant chromosomes. The periods of various recombinant types are shown in Table 2. Table 2a-d shows that the clock mutant maps to the right (proximally) of *v* and to the left of *f*. In addition only the double recombinant types that include dusky exhibit the mutant clock phenotype (Tables 2e-h and 4e).

Cytogenetic mapping was then conducted using deficiencies and duplications that cover the *m-dy* region (see Fig. 4). The results are shown in Table 1. The deficiency and duplication chromosomes were combined with *And* and *And*$^+$, the

latter to control for the non-specific effects of genetic background on period length. Unfortunately the cytogenetic mapping, which involves the uncovering of a recessive mutant phenotype in mutant/deficiency or mutant/duplication heterozygotes, is hampered by the semi-dominant phenotype of *And*. In addition, the effects on genetic background are relatively large compared to the 0.6 h difference in period between *And* homozygotes and *And/And*$^{+}$ heterozygotes. The results of Table 1 do allow a tentative mapping, however. Since *Df(1)KA7*, *Df(1)KA6* and *Df(1)HA85* when combined with *And* have periods $\geq$ that of *And/And*, these deficiencies would appear to uncover *And*. *Df(1)KA6* and *Df(1)HA85* show similar behavior for the eclosion rhythm (Table 4f,g). When combined with *And*$^{+}$, however, only *Df(1)HA85* shows a dominant mutant phenotype similar to that produced by *And* (Table 1e). Since the cytological extent of *Df(1)HA85* is entirely included within that of *Df(1)KA7* (Fig. 4), this difference in period between these two deficiencies would appear to be the result of differences in genetic background. Since *Df(1)m*$^{259-4}$, *Df(1)RA47*, and *Df(1)N105* have periods $\leq$ that of *And/And*$^{+}$, these deficiencies would not appear to include the *And* locus. This then limits the *And* locus to that region between polytene chromosome bands 10E2-3 (the right breakpoint of *Df(1)m*$^{259-4}$) and 10F1 (the left breakpoint of *Df(1)RA47*). Given this localization, the duplication *Dp(1;3)v*$^{+74c}$ (Fig. 4) must cover the *And* locus. Since the Y-borne duplication, *v*$^{+3}$ *Y y*$^{+}$ has approximately the same period when combined with *And* as *Dp(1;3)v*$^{+74c}$, especially if the background effects on period observed for both of the *And*$^{+}$/duplication combinations are subtracted from the respective periods of the *And*/duplication combinations, both duplications would also appear to cover the *And* locus. These results would thus tentatively limit *And* to 10E3, just proximal to the *m-dy* complex at 10E2-3.

The results of a complementation tests between *And* and two mutant *per* alleles are shown in Table 1n,o. When *And* is combined with *per*^{o} or *per*^{s} in *trans-*

heterozygotes, period is lengthened by 1.1-1.3 h compared to the And^{+}/per^{0} or per^{s} heterozygotes. Since *And* lengthens period by only about 0.6 h when combined with a wild-type chromosome (Table 1b), the two *trans*-heterozygous combinations of *And* and the *per* mutants exhibit a longer period than would be expected from a simple additive effect on period.

In order to characterize the manner in which *And* affects the underlying pacemaker system, we have conducted a phase-resetting study of the eclosion rhythm of *And* individuals (Fig. 5). Light pulses of 40' duration were administered to pupae of an *And/y f XX/Y* stock at various times after a transition from DL 12:12 to DD. The eclosion pattern of wild-type siblings (*y f XX/Y* females) serve as an internal control for phase-resetting response of *And/Y* males. As described previously (Konopka 1979), the wild-type phase-resetting curve (PRC) for saturating 40' light pulses is of the low-amplitude type (Type 1 or "weak"; Winfree 1980) with one 24-h cycle consisting of a 12 h a light-sensitive phase with a delay and an advance portion followed by a 12 h phase which is relatively insensitive to light. Since the magnitude of the phase shifts produced by 40' and 80' light pulses are similar in the *And* PRC, pulses of 40' duration produce maximum phase-shifting, as is observed for wild-type. The Andante PRC is similar to that of wild-type; the maximum amplitude of the advances and delays, the time of the shift from the delay portion of the cycle to the advance portion (at about 6-7 h), and the end of the advance phase (at about 15 h) are similar for both strains. While the 1.5 h lengthening of period produced by the mutant is not evident during the first cycle (the beginning of the delay portion of the second cycle, at about 24-26 h, is not very distinct for either strain), the time of maximum delay during the second cycle does occur later in the mutant (at about 30 h vs. 27-29 h for wild-type) and the time of crossover from delays to advances in the second cycle is about 2 h later for the mutant (at about 32-33 h vs. 30-31 h).

The similarity in the eclosion PRCs of Andante and wild-type is in contrast to the resetting curve of the per^{s} clock mutant which shows both a significant shortening of only one portion of the cycle, the light-insensitive phase, and a significant increase in the amplitude of the resetting response (Konopka, 1979).

4.5 References

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4.6 Figures

Fig. 1 Eclosion rhythm record of an *And/y f XX/Y* stock given a 12 h, 18-31-18° C temperature pulse 12 h after a DL-DD transition. The stock was reared previously in a LD 12:12 cycle at 18° C and the phase of the 12 h, 31° c temperature pulse corresponds to the light phase of the previous LD 12:12 cycle.

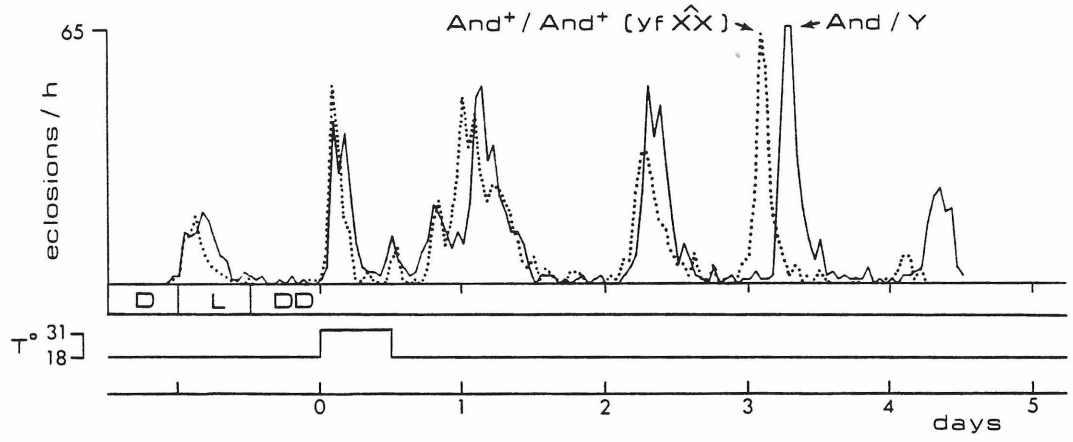

And+ / And+ (yf X̂X)
And / Y
65
eclosions / h
D
L
DD
T° 31
18
0
1
2
3
4
5
days

Fig. 2 Superimposed eclosion rhythm records of Canton-S (C-S) wild-type females and *And / And* females monitored at 18° C and in DD after previous entrainment in a LD 12:12 cycle at 18° C. Time 0 corresponds to the time of the DL-DD transition.

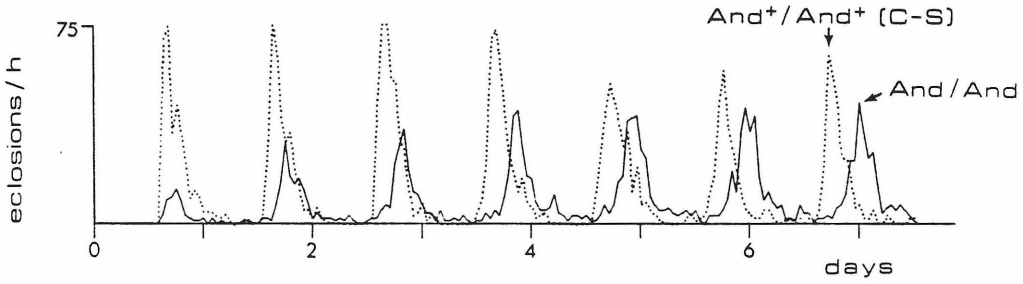

And+/And+ (C-S)
And/And
75
eclosions / h
0
2
4
6
days

Fig. 3 Superimposed locomotor-activity rhythm records of Canton-S (C-S) wild-type females and *And/And* females monitored at 24° C and in DD after previous entrainment in a LD 12:12 cycle at 24° C. Time 0 corresponds to the time of the DL-DD transition.

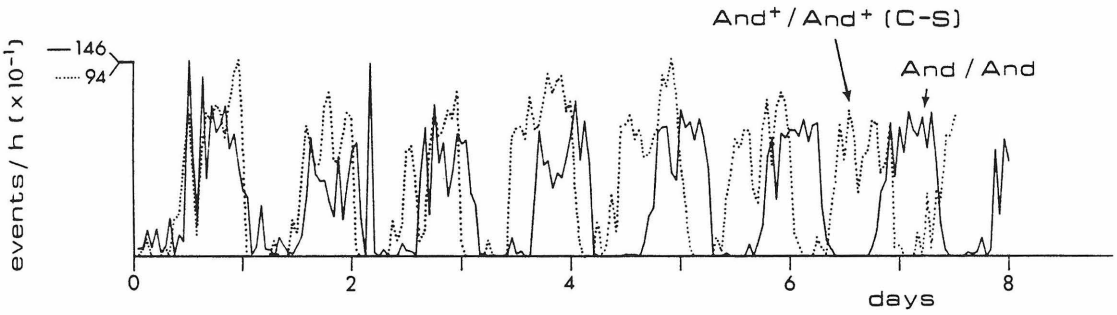

And+ / And+ (C-S)
And / And
146
94
events / h (x 10-1)
0
2
4
6
8
days

Fig. 4 Cytological extents of the X-chromosome deficiencies and duplications used in the cytogenetic mapping of the *And* locus.

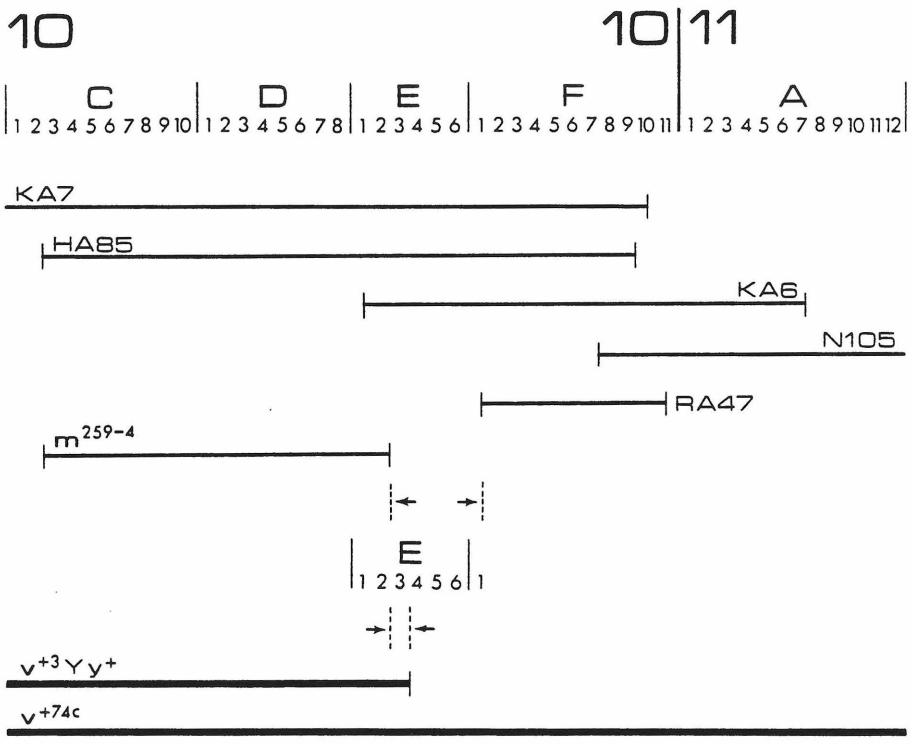
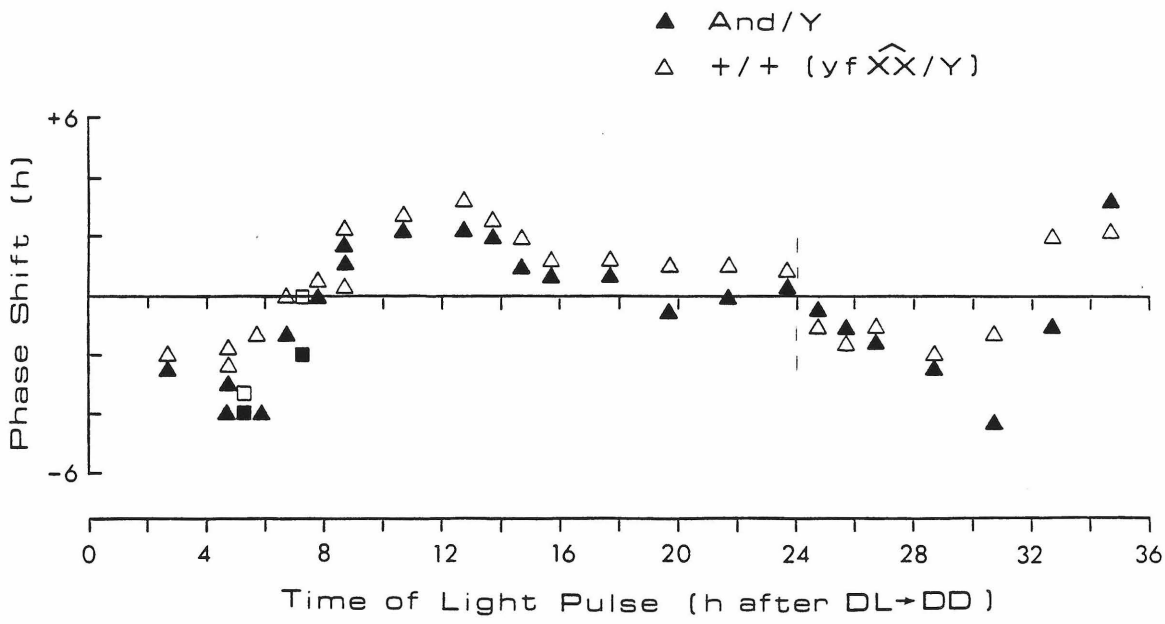

10
10|11
C
D
E
F
A
1 2 3 4 5 6 7 8 9 10
1 2 3 4 5 6 7 8
1 2 3 4 5 6
1 2 3 4 5 6 7 8 9 10 11
1 2 3 4 5 6 7 8 9 10 11 12
KA7
HA85
KA6
N105
RA47
m259-4
E
1 2 3 4 5 6 1
v+3 Yy+
v+74c

Fig. 5 The phase-resetting response of *And/Y* males (dark symbols) and the wild-type internal control, y f XX females (open symbols), to 40' (triangles) or 80' (squares) light pulses administered at various times after a transition from DL 12:12 to DD (18° C). The points are plotted at the pulse endpoints.


And/Y
+/+ (yf$\widehat{XX}$/Y)
Phase Shift (h)
+6
-6
0
4
8
12
16
20
24
28
32
36
Time of Light Pulse (h after DL→DD)

4.7 Tables

Table 1. Activity rhythm phenotypes: deficiency mapping results and complementation behavior of *And*. (Period in h)

		And		*And*$^+$		
		period ± s.d.	n	period ± s.d.	n	Δ period
a	*And*	25.7 ± 0.3	10	24.8 ± 0.2	9	+0.9
b	*And*$^+$	24.8 ± 0.2	9	24.2 ± 0.3	7	+0.6
c	*Df(1)KA7*	26.6 ± 0.3	6	24.4 ± 0.4	10	+1.2
d	*Df(1)KA6*	25.8 ± 0.2	7	24.0 ± 0.2	6	+1.8
e	*Df(1)HA85*	25.9 ± 0.2	6	25.2 ± 0.4	8	+0.6
f	*Df(1)m*$^{259-4}$	25.1 ± 0.2	6	24.5 ± 0.2	7	+0.6
g	*Df(1)RA47*	24.6 ± 0.2	7	24.3 ± 0.3	8	+0.3
h	*Df(1)N105*	24.3 ± 0.3	5	23.8 ± 0.8	9	+0.6
i	*Y* (males)	25.5 ± 0.4	4	24.0 ± 0.4	12	+1.5
j	*Y; Dp(1;3)v*$^{+74c}$	24.6 ± 0.2	7	23.7 ± 0.3	7	+0.9
k	*v*$^{+3}$ *Y y*$^+$	24.9 ± 0.4	8	24.6 ± 0.2	7	+0.3
l	*dy*	25.0 ± 0.3	7	24.5 ± 0.4	9	+0.5
m	*m*^{D}	24.8 ± 0.5	5	23.9 ± 0.3	6	+0.9
n	*per*^{0}	25.9 ± 0.7	8	24.6 ± 0.2	9	+1.3
o	*per*^{l}	26.2 ± 0.3	17	25.1 ± 0.2	10	+1.1

Table 2. Activity rhythm phenotypes: Recombination mapping of *And*.

	parental types							
	y	*cho*	*cv*	*v*	dy^+	*f*		
	+	+	+	+	*dy*	+		
	recombinant types						period ± s.d.	n
a	*y*	*cho*	*cv*	*v*	*dy*	+	25.6 ± 0.6	6
					x			
b	+	+	+	+	dy^+	*f*	23.9 ± 0.3	6
c	*y*	*cho*	*cv*	*v*	dy^+	+	23.8 ± 0.2	6
						x		
d	+	+	+	+	*dy*	*f*	25.4 ± 0.3	7
e	*y*	*cho*	*cv*	+	*dy*	*f*	25.4 ± 0.3	7
			x			x		
f	+	+	+	*v*	dy^+	+	24.0 ± 0.2	5
g	*y*	*cho*	*cv*	+	dy^+	*f*	24.4 ± 0.4	7
			x		x			
h	+	+	+	*v*	*dy*	+	25.2 ± 0.3	5

Table 3. Activity rhythm periods of *dy* and m^D homozygotes.

	Genotype	period ± s.d.	n
a	*dy*/*dy*	24.6 ± 0.4	9
b	m^D/m^D	23.7 ± 0.4	9

Table 4. Eclosion Rhythm Periods.

	Genotype[a]	Period ± s.d.	Genotype	Period ± s.d.	cycles/ run
a	*And/Y*	25.1 ± 0.6	*And$^+$/And$^+$ (y f XX)*	23.9 ± 1.1	4
b	*And/Y*	25.6 ± 0.5	*And$^+$/And$^+$ (y f XX)*	24.3 ± 0.6	4
c	*And/Y*	25.3 ± 0.8	*And/And*	25.2 ± 1.0	5
d	*And/Y*	25.3 ± 0.8	*And/And*	25.1 ± 0.2	5
e	*y cho cv v$^+$ dy (And) f/Y*	24.9 ± 0.6	*And$^+$/And$^+$ (y f XX)*	23.8 ± 0.9	6
f	*And/Df(1)KA6*	25.0 ± 0.4	*And/And$^+$*	24.3 ± 0.5	6
g	*And/Df(1)HA85*	25.0 ± 0.5	*And/And$^+$*	24.4 ± 0.4	5
h	*And/dy*	24.6 ± 0.5	*dy/Y*	24.1 ± 0.5	6
i	*And/m^D*	24.9 ± 0.5	*m^D/Y*	24.4 ± 0.2	6
j			*And/And$^+$*	24.8 ± 0.5	
k	*dy/Y*	24.6 ± ?.?	*dy/dy*	24.6 ± ?.?	5
l	*m^D/Y*	24.8 ± ?.?	*m^D/m^D*	24.8 ± ?.?	5

[a] *And$^+$/And$^+$* of **c, d, e** = *C(1)DX, y f (= y f XX)*
And$^+$ of **f, g** = *FM7*
And$^+$ of **j** = *FM3*