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GENETICS OF BIOLOGICAL RHYTHMS IN *DROSOPHILA*

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CONTENTS

INTRODUCTION

The first examples of single gene mutations that altered the period of a circadian rhythm were reported by Konopka & Benzer in 1971 (16). In that report, three mutant alleles of one genetic locus in *Drosophila melanogaster*, named the *period (per)* locus, were described. The mutants identified were a short-period mutant, per^S, that shortened the period of the pupal eclosion and of adult locomotor activity rhythms from the normal 24 hours (hr) to 19 hr; a long-period mutant, per^L, that lengthened the period to 29 hr; and an arrhythmic mutant, per^{01}, that abolished the rhythms. Since then, clock mutants have been isolated in *Chlamydomonas* (5), *Drosophila pseudoobscura* (21), and *Neurospora* (8). Additional clock mutants have been isolated in *D. melanogaster*, both at the *period* locus (see below) and at other genetic loci (11, 15). The two best-studied loci are the *period* locus in *D. melanogaster* and the *frequency* locus in *Neurospora* (7). Owing to space limitations,

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this review focuses on studies that have involved the *per* locus, including genetic and neurobiological investigations that led to the recent molecular analyses of the *per* locus DNA and the *per* protein.

GENETICS OF THE *PER* LOCUS

Besides the original three *per* alleles described above, several additional alleles have been isolated. Such alleles include two additional arrhythmic alleles, per^{o2} and per^{o3} (9, 25), an additional long-period allele, per^{L2} (15), and an allele that variegates for a long-period phenotype, per^{Lv} (9, 14). All *per* alleles tested show a partially dominant phenotype. A per^{o} allele in a heterozygote lengthens the period by about half an hour, whereas a per^{L} allele in a heterozygote lengthens the period by about one hour (25). The per^{S} allele has the strongest effect; the period of the $per^{S}/+$ heterozygote is intermediate with respect to the period of per^{S}/per^{S} and $+/+$ homozygotes: 2.5 hr shorter than that of the wild type.

Two groups (24, 26) studied the phenotypes of chromosome aberrations with a breakpoint in the *per* region. These studies revealed that the *per* locus is a nonessential locus that can be deleted without producing lethality. Such deletions, produced by overlapping deficiencies, exhibit an arrhythmic phenotype for both the eclosion and locomotor activity rhythms. When heterozygous with per^{o}, deficiencies with a breakpoint at *per* have either an arrhythmic phenotype or a period close to that of the wild type. The arrhythmic phenotype is apparently due to a break within the *per* gene, whereas the deficiencies with a period close to that of wild type have an intact *per* gene. The single available translocation with a breakpoint in the *per* region, named JC43, has an anomalous phenotype. When heterozygous for per^{o} or for an arrhythmic deficiency, JC43 produces various activity profiles. Some are arrhythmic, some start with one or two transient long-period cycles and then become arrhythmic, and some have very long period rhythms that persist for longer than nine days with periods in the 32–36-hr range. The eclosion rhythms of JC43 heterozygotes are all arrhythmic, probably representing a mixture of the three phenotypes described above. In the mixture, the few rhythmic flies are not detectable because of the presence of the arrhythmic and long-arrhythmic flies and the variability of the period in persistent rhythms.

A dosage study was also carried out to determine how altering the number of *per* genes affects period (25). The *per* locus was found to be dosage compensated, like other X-linked genes in *Drosophila*. Males with one dose of per^{+} have periods similar to those of females with two doses. One extra dose in a female (a total of three doses) shortens the period by about half an hour, whereas one extra dose in a male, equivalent to two extra doses in a female, shortens the period by about an hour. Removing *per* doses has the

opposite effect; one dose (total) in a female lengthens the period relative to two doses in a female, or one dose in a male. These dosage effects are similar for both the eclosion and locomotor activity rhythms. From these studies it was concluded that per^{L} represents a decrease in the level of *per* gene expression, per^{S} represents an increase, and per^{o} represents a lack of *per* gene expression. Since the shortening effect of more than one per^{+} dose in a male appears to saturate at about −1.5 hr, it was also concluded that the per^{S} phenotype, with a period about 5 hr shorter than that of the wild type, is due to increased activity of the *per* gene product, rather than to overproduction of the wild-type gene product. Both of these conclusions are consistent with the results of the molecular analysis of the *per* gene discussed below.

NEUROBIOLOGY OF *PER* MUTANTS

In 1980 Kyriacou & Hall (20) reported that *per* mutations also affect the period of a short-term oscillation in the interpulse interval (ipi) of the male courtship song, which is produced by wing vibration. The ipi of the song is a temperature-dependent, species-specific characteristic; its mean value at 25°C is approximately 34 msec in wild-type *D. melanogaster*. Over the course of several minutes, the ipi undergoes a rhythmic fluctuation with an amplitude of 4–6 msec peak to trough, with a period of about 55 sec in the wild type. The ipi rhythm, like the circadian rhythm, is temperature compensated; its period remains nearly unchanged for temperatures from 16°C to 35°C. The effects of the *per* mutants on the ipi rhythm are similar to their effects on the circadian rhythms. However, *per* mutations cause a greater percentage change, from the wild type, in the ipi rhythm than in the circadian rhythm. The short-period allele, per^{S}, decreases the period of the ipi rhythm to 40 sec, whereas the long-period allele, per^{L1}, increases it to 76 sec. The arrhythmic allele, per^{o1}, produces arrhythmicity in the interpulse interval. Thus, for the first time, rhythms on two very different time scales, one with a circadian period and one with a period on the order of a minute, were shown to have some element of their mechanisms in common.

The circadian clock controlling the adult locomotor activity rhythm could, in principle, be located in the thoracic ganglia, where the motoneurons that directly control the leg movements are located. Another possibility is that it is located in the brain, where it controls activity of the motoneurons. Two lines of evidence, from transplant experiments and an analysis of mosaic flies, indicate that the circadian clock is located in the brain. Handler & Konopka (10) reported that short-period adult brains implanted into the abdomens of arrhythmic (per^{o}) hosts could, in some cases, confer a short-period rhythm on the activity of the host for at least four cycles. These results implied that although the driving oscillator was absent from arrhythmic flies, the circuits

(presumably in the thoracic ganglia) capable of responding to the output of the driver were still present. Likewise, the evidence suggested a humoral output from the brain, possibly neurosecretory, since the rhythms were observed before any direct neural connections could have been established. It was suggested that the humoral factor had an inhibitory effect on the activity rhythm, since arrhythmic flies have intermittent activity that is uniformly distributed throughout the 24-hr day.

Konopka & Wells (18) reported an abnormality in the morphology of a neurosecretory cell group associated with the arrhythmic per^{o} mutation as well as with two arrhythmic mutants of *D. pseudoobscura*. This cell group normally consists of four clustered cells in either side of the brain, roughly halfway between the top and bottom edge, in the posterior area of the brain. Cells in this cluster are occasionally located abnormally near the top edge of the brain (about 17% of cells in wild-type *D. melanogaster*). The per^{o} mutation significantly increases the percentage of abnormally located cells to about 40%. In two aperiodic strains of *D. pseudoobscura*, one from each of the two known complementation groups, the percentages of abnormally located cells are likewise significantly increased over those in the wild type. In *D. pseudoobscura*, however, there is a sexual dimorphism in the percentage of abnormally located cells: females have over twice the percentage of abnormal cells as males (16% vs 7% in wild type). This dimorphism is maintained in the aperiodic strains. These results suggest that neurosecretory cells may indeed be part of the *Drosophila* circadian system, and that the *per* gene product may influence the development of these cells.

To determine the focus of the per^{S} mutation, mosaic flies were produced such that the male tissue was hemizygous for per^{S} (19-hr period) and the female tissue was heterozygous for per^{S} ($per^{S}/+$, 21.5-hr period) (19). A resulting fate-map indicated a focus for the per^{S} mutation that was consistent with a location in the brain, clearly distinct from thoracic markers. Some bilateral mosaics had double (male + female) rhythms, apparently the result of a male oscillator on one side of the brain and a female oscillator on the other. The relatively weak coupling of oscillators on the two sides of the brain allowed expression of both male and female rhythms in the locomotor activity pattern. Thus, both the transplant experiments and the mosaic analysis indicated that the locomotor activity clock was located in the brain. Preliminary results from mosaics in which both the locomotor activity rhythm and the courtship song ipi rhythm were monitored suggest that the oscillator controlling the ipi rhythm is not in the brain but in the thoracic ganglia (J. C. Hall, personal communication).

The circadian cycle is normally composed of a subjective day and a subjective night. Since the *per* mutations altered the period of the cycle, it was of interest to determine whether their effects occurred over the entire cycle or

were confined to a portion of it. When the light responses of the per^S mutant eclosion rhythm were compared with those of the wild type, two effects were found (13). First, the mutant clock could be reset by light pulses to a greater extent than the wild-type clock (about 10 hr as opposed to 3 hr for wild type). In addition, although the duration of the light-sensitive part of the cycle (the subjective night) was similar for per^S and the wild type, the duration of the light-insensitive part of the cycle (the subjective day) was 5 hr shorter in the mutant than in the wild type. Thus, the difference in period between per^S and wild type was entirely caused by a shortening of the subjective day in per^S (17). Likewise, when the durations of the active and inactive portions of the cycle were measured for per^S and wild type, the difference in period between the mutant and wild type could again be accounted for by a shortening of the active part of the cycle corresponding to the subjective day (17). Thus, separate molecular processes correspond to the subjective day and the subjective night; the per^S allele acts by shortening the subjective day. Whether the increased responsiveness of the clock to light is a direct effect of the per^S mutation remains to be determined.

A model for the action of the *per* gene was constructed on the basis of these results. The driving oscillation is interpreted in terms of a membrane gradient that is established during the subjective day and dissipated during the subjective night (17). The *per* gene product is active during the subjective day and functions, like a pump, to establish the gradient. When a high threshold is reached, the pump shuts off and light-sensitive channels open to dissipate the gradient. A light pulse during the subjective night closes the channels and starts the pump; the value of the gradient when the channels close is the same as the value when the pump starts, and thus a reset in the cycle is produced. For per^S, the extent of resetting predicted by this model is in good agreement with the experimental findings. For wild type, the observed resetting is less than that predicted by the model; this discrepancy may result from a masking effect that prevents the maximum reset from being attained. Interestingly this model predicts that the shortening of the subjective day is due to a more rapid attainment of the higher threshold, as a result of increased *per* product activity during the subjective day. This prediction is in complete agreement with the genetic evidence discussed above that indicates that per^S does in fact have increased *per* product activity.

MOLECULAR STUDIES

Cloning and Sequencing of Wild-Type and Mutant per *DNA*

With the advances in recombinant DNA technology using *Drosophila* DNA, the search for the *per* gene product intensified. In 1984 two groups (2, 23) reported the cloning of DNA from the *per* region and the functional rescue of

the arrhythmic phenotype using cloned per^+ DNA (1, 28). An important landmark for identifying the *per* gene was the JC43 translocation discussed above. The breakpoint of this translocation occurs very near to the DNA that codes for a 4.5-kb RNA transcript and a 1-kb transcript to the right of the 4.5-kb transcript in the wild type. In flies heterozygous for JC43 and for a deficiency for the *per* region, the levels of these two RNA transcripts are reduced, and two novel RNA transcripts are found, one smaller than 1 kb and one of about 11 kb. The 1-kb wild-type transcript has two unusual properties. First, the level of this RNA varies by an order of magnitude during a light–dark cycle; the level is much higher during the light than during the dark. This difference is not, however, a direct effect of the light; the rhythm in level of this transcript persists in constant darkness after entrainment in a light–dark cycle. Thus, the 1-kb transcript is somehow involved in the circadian organization of the fly. The second unusual property of this transcript is that its level is reduced in per^{o1} and per^{o2} flies to about 5% and 20% (respectively) of normal, whereas the level of the 4.5-kb transcript is unaffected. This finding is especially interesting in view of the fact that the site of the per^{o1} mutation maps to the 4.5-kb RNA, causing premature termination of the translated protein (see below). The protein produced from the 4.5-kb RNA, or part of it, may control the expression of the 1-kb RNA transcript.

The per^S, per^L, and per^{o1} mutations all map to the gene that codes for the 4.5-kb transcript. This DNA contains eight exons. The per^L mutation maps to exon three and represents a thymine-to-adenine change that results in substitution of aspartic acid for valine (3). The per^{o1} mutation maps to exon four and represents a cytosine-to-thymine change that results in a translational stop codon instead of the normal glutamine (3, 27). Thus, in per^{o1}, the resulting protein has approximately 400 amino acids, compared with about 1100 in wild type. The per^S mutation maps to exon five, the same exon that contains a glycine–threonine repeated region (see below). In per^S, a guanine-to-adenine change results in a serine-to-asparagine change in the mutant protein. Although it is not yet clear how the amino acid substitutions result in the observed period changes produced by per^S and per^L, it is intriguing that these mutations map to different exons. There may be separate domains in the *per* protein for short-period and long-period phenotypes. Sequencing of the per^{L2} and per^{Lv} mutations may shed light on this aspect of *per* protein function.

Reversal of the Arrhythmic Phenotype by Transformation

Wild-type DNA clones from the *per* region have been used in transformation experiments to restore rhythmicity to genetically arrhythmic (per^{o1}, per^{o2}, per^{o3}, per^{Lv}, and overlapping-deficiency) flies (1, 9, 28). Some of these clones contain most, but probably not all, of the DNA that codes for the

4.5-kb RNA; yet, they are capable of rescuing both the locomotor activity circadian rhythm and the courtship song ipi rhythm.

The 4.5-kb RNA has a repeated sequence in exon four that codes for a glycine–threonine repeat (12, 22). Repeats of glycine and serine have been found in proteoglycan molecules (4). Small regions of repeated glycine and serine residues also appear in the putative *per* protein. A search for a proteoglycan produced by the *per* region yielded an antigen that was recognized by antisera to a fusion protein containing part of the *per* protein. The biochemical properties of the antigen were altered by treatment with heparitinase, but not chondroitinase ABC (22); these results indicated that the *per* region does indeed produce a proteoglycan. How is the repeated sequence of glycine and threonine involved in rhythmic behavior? To answer this question, the DNA coding for this amino acid repeat was deleted from a DNA fragment that could reverse arrhythmicity with the repeat intact (J. C. Hall, personal communication). Surprisingly, the deletion had little or no effect on the ability of the DNA fragment to restore the circadian locomotor activity rhythm in arrhythmic per^{o1} flies. However, the period of the courtship song ipi rhythm was reduced by the deletion to 40 sec from the 60-sec period, as measured using a 13.2-kb transforming fragment with the repeat intact, and to 46 sec from 71 sec using the 8.0-kb transforming fragment with the repeat intact. Thus, the glycine–threonine repeat is not necessary for proper expression of circadian rhythmicity, but may be involved in the process by which different species acquire different courtship song parameters. The results from the deletion of the glycine–threonine repeat and the fact that some transforming DNA fragments can restore circadian rhythmicity without the complete sequence coding for the 4.5-kb transcript argue that only part of the 4.5-kb transcript is involved in the control of circadian rhythmicity.

Alternate processing at the *per* locus has been demonstrated by analysis of cDNA clones of the *per* gene transcripts (6). These studies show that, besides the major 4.5-kb transcript described above (type A), two other transcripts (types B and C) are produced from the *per* region. They are also about 4.5 kb each, but are less abundant than type A. In the type-B transcript cDNA, a splicing site results in the splitting of exon eight of the type-A transcript into two exons separated by an 89-bp intron. This splitting does not, however, change the coding sequence of the putative *per* protein. In the type-C transcript cDNA, however, three of the introns present in the type-A transcript are not spliced out, which results in a very large exon and a different amino acid sequence at the end of the putative *per* protein. Nevertheless, both type-A and type-C transcripts can rescue circadian rhythmicity in per^{o} flies, when both genomic DNA and cDNA are used to produce transformants in separate lines.

Although genetically arrhythmic flies can be made rhythmic by transform-

ing DNA from the wild-type *per* region, most, if not all, of the transformants are characterized by incomplete penetrance and a longer than normal period. The one possible exception is a 13.2-kb genomic fragment (6); even in this case, a few arrhythmic flies are observed, and the period is about half an hour longer than that of per^+ flies. The increase in period may be accounted for by the observation, discussed in relation to the dosage studies above, that long periods are associated with decreased *per* gene or gene-product activity. Thus, *per* sequences present in an abnormal genetic environment, or missing some regulatory sequences, may function at less than wild-type levels. In fact, an inverse correlation between *per* RNA titer and the period of transformed rhythms has been demonstrated (3). Transformed lines with low *per* RNA titers have longer than normal periods, whereas the lines with the highest RNA titers have periods closer to normal, although still slightly longer than normal. The reason for the lack of complete penetrance is more difficult to discern; perhaps an event early in development determines whether the *per* gene will be expressed at proper levels in the proper cells. Besides being present in the adult nervous system (2, 23), the 4.5-kb *per* transcript is also expressed during the second half of the embryonic stage.

The Per ^{Lv} Mutation

Although the 4.5-kb *per* transcript is of central importance in *per* gene function, sequences to the left and right of the DNA coding for this transcript also seem to be involved in the control of the expression of rhythmicity. In the case of the per^{Lv} mutation, for example, sequences to the left and right of the *per* region enhance the expression of rhythmicity (14). When present in the hemizygous state, or when heterozygous with a deficiency for the entire *per* region, per^{Lv} produces no strong rhythms. Most such flies are arrhythmic; a few are weakly rhythmic. When per^{Lv} is heterozygous with arrhythmic deficiencies that have breakpoints within the *per* region, rhythmicity is enhanced whether sequences occur to the left or to the right of *per*, and a significant fraction of such flies have strong rhythms. The nature of the interactions between such deficiencies and per^{Lv} remains to be elucidated.

FUTURE PROSPECTS

Although much information concerning the nature and expression of the *per* gene has been obtained, the mechanism of the circadian clock and the mechanism by which the sequence changes in the *per* gene result in period changes remain unknown. The model of Konopka & Orr (17), discussed above, has not yet been disproved. Future experiments will be directed toward answering the following questions (and others). Since each of the three *per* alleles that have been sequenced map to a different exon, are there domains in

the *per* gene or protein that are associated with particular phenotypes, such as short period and long period? What is the molecular basis of the per^{Lv} mutation? What is the role of the 1-kb, oscillating RNA in the circadian organization of the fly? What is the relationship of other *Drosophila* clock loci to the *per* locus, and are there sequence homologies or molecular domains in these loci? How does differential *per* gene expression result in both a long-term (circadian) and a short-term (courtship song ipi) rhythm? The most intriguing part of the *Drosophila* rhythm story is still to come.

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NOTE ADDED IN PROOF The experiments involving the deletion of the DNA coding for the amino acid repeat in the *period* gene, discussed on page 233, have since been published in *Nature*. (Yu, Q., Colot, H. V., Kyriacou, C. P., Hall, J. C., Rosbash, M. 1987. Behaviour modification by *in vitro* mutagenesis of a variable region within the *period* gene of *Drosophila*. *Nature* 326:765–69.)

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