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Molecular Analysis of the *period* Locus in Drosophila melanogaster and Identification of a Transcript Involved in Biological Rhythms

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Summary

We have isolated and analyzed DNA sequences encompassing the *period* (*per*) locus of Drosophila melanogaster. The location of this clock gene was delimited by the molecular mapping of chromosome aberrations at or very near the *per* locus. At least five RNAs are transcribed from this region. One of these transcripts, a 0.9 kb species, is strongly implicated in *per*'s control of biological rhythms. Two independently isolated arrhythmic mutations at the *per* locus dramatically reduce the level of this transcript. Furthermore, the level of the 0.9 kb transcript is strongly modulated during a light/dark cycle. We discuss evidence, from previously reported genetic and phenotypic analysis of *per*'s function, suggesting that this region may be complex and that several gene products from the *per* region, including this 0.9 kb transcript, may be involved in the different aspects of normal rhythmicity influenced by this clock gene.

Introduction

Biological rhythms in many types of metazoans and microbial organisms are widely known and investigated (Sweeney, 1983), but almost nothing is known about the molecular mechanisms that make up the endogenous cellular timers. Most studies of biological clocks focus on circadian rhythms and their analysis at behavioral, physiological, and anatomical levels. There are, in addition, some powerful genetic systems for analyzing these rhythms (reviewed by Feldman, 1982). In Drosophila, seven genetic loci have been mutated such that they perturb or abolish biological rhythms (Konopka and Benzer, 1971; Pittendrigh, 1974; Jackson, 1983).

The most salient clock gene in D. melanogaster is defined by several genetic variants at the X chromosomal *period* (*per*) locus (reviewed by Konopka, 1984). Mutations at this locus cause abnormally short or long rhythm periods or lead to arrhythmic phenotypes (Smith and Konopka, 1981, 1982; Konopka, 1984). Specifically, there is one short-period mutant (per^s), two long-period variants (per^{L1} and per^{L2}), two arrhythmic mutants (per^{o1} and per^{o2}), and a chromosome aberration (called *T(1;4)JC43*), one of whose X chromosomal breakpoints is near *per* and can cause a given fly to be long-period or arrhythmic. In addition to the circadian eclosion rhythms (the criteria on which the mutants were originally isolated by Konopka and Benzer, 1971) and locomotor activity rhythms, a short-term oscillation in the male's courtship singing behavior is also disrupted by these aberrant genotypes (Kyriacou and Hall, 1980). A defect somewhat analogous to this behavioral impairment is the erratic larval heartbeat which appears to be induced by per^{o1} (Livingstone, 1981; M. S. Livingstone and E. D. Aceves-Piñar, unpublished data).

Our analysis of the *per* gene was initiated by application of microexcision techniques to obtain clones from the 3B1-2 region of the X chromosome (Pirrotta et al., 1983). This was followed by mapping chromosomal breakpoints in or near the *per* locus. One of the most interesting of these variants is part of the *T(1;4)JC43* aberration, that is, an X chromosomal breakpoint at 3B1-2 which appears to cause lower than normal expression of *per* (Smith and Konopka, 1981). We mapped this breakpoint to the middle of our cloned DNA and concentrated on an analysis of transcripts from this region. We discuss our results with respect to the effects of *per* mutations on a variety of developmental and behavioral phenotypes. We suggest that a 0.9 kb RNA transcribed from the *per* region is necessary, but probably not sufficient, to control the array of cellular and rhythmic functions that seem to be under the influence of this clock gene.

Results

Molecular Mapping of Chromosomal Lesions in the Vicinity of *per*

The *per* gene has been mapped to the 3B1-2 region of the X chromosome (R. J. Konopka, Ph.D. thesis, California Institute of Technology, Pasadena, California, 1972; Young and Judd, 1978). The 3B1-2 interval is a subsegment of the *zeste-white* region which is well studied from both the genetic and molecular points of view (e.g., Judd et al., 1972; Pirrotta et al., 1983). Specifically, *per* has been mapped between sites defined by lethal mutations in the *l(1)zw3* and *l(1)zw6* complementation groups (e.g., Young and Judd, 1978). In addition, *per* can be assumed to lie distal to the left-hand breakpoint, in the 3B1-2 region, of $Df(1)w^{-64d}$ (Figure 1) and proximal to the right-hand breakpoint of *Df(1)K95,* since neither of these deletions uncovers *per* mutations (Young and Judd, 1978; Smith and Konopka, 1981).

The DNA corresponding to breakpoints that are near the *per* locus has been cloned in an analysis of DNA in the *zeste-white* region (Pirrotta et al., 1983). We began our study with two of the relevant recombinant phages, λ*a4715b* and λ*a4719b,* and a Charon 4 library in order to isolate DNA distal to sequences in λ*a4715b* (Figure 1). Genomic Southern blots of DNA from a variety of strains

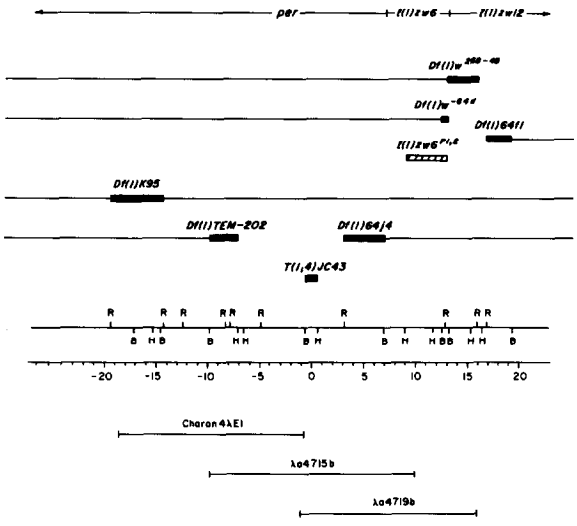


Figure 1. Mapping of Recombinant Phage and Chromosome Breakpoints near the *per* Locus

The Drosophila DNA contained in three overlapping recombinant phages is shown at the bottom, above which is a scale in kb. The two "λa47" phages are from Pirrotta et al. (1983); the remaining phage (Charon4 λ E1) was isolated by us in a short chromosomal walk (see text). "0" is at the approximate position of the *T(1;4)JC43* breakpoint in the X chromosomal region 3B1-2. Positive values proceed in the proximal direction and negative values in the distal direction (i.e., nearer to or farther from the X chromosome centromere, respectively). In the restriction map of this region, R's designate Eco RI sites, B = Bam HI, H = Hind III. Each breakpoint has been localized to a single restriction fragment, and all chromosome breaks were mapped with three enzymes (consistent results were obtained). Bold bars denote positions of the restriction fragments in which the breakpoints occur. The thin lines designate DNA indistinguishable from that derived from wild-type flies (in control Southern blots). The "empty" regions (adjacent to bold bars) designate the deleted DNA. In this regard, the thin lines proceeding in either direction from the lesion in *Df(1)K95* indicate that this is an inversion breakpoint (the deletion breakpoints on this chromosome are well to the left of this map; see text). Two of the deletion breakpoints (for $Df(1)w^{258\text{-}45}$ and *Df(1)64f1*) were previously reported by Pirrotta et al. (1983). The *T(1;4)JC43* translocation breakpoint was mapped to the 1 kb Bam HI/Hind III fragment at position 0, as indicated. The two hybrid dysgenesis-induced lesions near or at the *l(1)zw6* locus have been localized and are indicated here by the shaded bar. Since no "P" DNA is detectable in these strains (P. Reddy, unpublished data; see O'Hare and Rubin, 1983), these lethal mutations at the *l(1)zw6* locus could be deficiencies that were generated by a P element's insertion (Simmons and Lim, 1980) and a subsequent deletion event that removed all traces of the element as well as some adjacent chromosomal DNA.

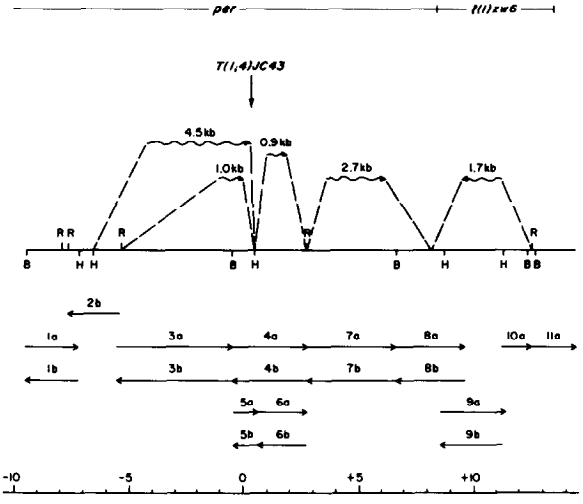


Figure 2. Summary of Transcript Mapping

The RNA species transcribed from various subsegments of the *per* region are indicated above their specific genomic sources. The restriction map and scale are as in Figure 1. Numbered fragments (between the restriction map and the scale) indicate pEMBL subclones. When indicated, a and b refer to the opposite directions of Drosophila DNA segments inserted into the pEMBL single-stranded phage used for RNA Northern blotting (see Experimental Procedures). In subsequent figures, subclones designated without a letter (e.g., subclone 4) refer to double-stranded probes. Subclones *8a* and *8b* are Bam HI/Eco RI subclones, constructed using an artificial Eco RI site from the phage. The directions of transcription (arrowheads on the wavy lines) are based on a positive Northern blot signal with pEMBL single-stranded phage probes containing an insert in one direction (a or b) and an absence of such a signal with the phage containing the same insert cloned in the opposite direction (see Dente et al., 1983). The indicated position of *T(1;4)JC43*'s 3B1-2 breakpoint defines "0" on the restriction map scale. The approximate location of a boundary between a 3B1-2 subsegment that is likely to contain *per* and an adjacent subsegment putatively containing the *l(1)zw6* gene is shown at the top (see Figure 1).

were probed with subclones (Figure 2) in order to localize breakpoints on the molecular map in Figure 1.

On the basis of our breakpoint analysis of strains carrying X chromosomal lesions associated with per^+ phenotypes, we conclude that the entirety of the *per* locus is bounded on the left side by either the right-hand deletion breakpoint of *Df(1)K95* (or conceivably the more proximally located inversion breakpoint; see Discussion), and on the right side by the molecularly mapped 3B1-2 breakpoint of the $Df(1)w^{-64d}$ chromosome. Results of the breakpoint mapping of *per* mutant strains imply that the *per* region may be limited to an approximately 15 kb region near the center of the restriction map (Figure 1). This is, of course, a narrower interval than that defined by mapping breakpoint sites in DNA from chromosomally aberrant, but per^+, strains.

RNAs Transcribed from the Vicinity of *per*

Subclones from the Drosophila DNA carried in the recombinant phages shown in Figure 1 were used to probe Northern blots of D. melanogaster RNA. Because *per*'s function is associated with several adult phenotypes (e.g., Konopka, 1984), our initial experiments used RNA from this stage of the life cycle. Flies hemizygous for the 3B1-2 breakpoint in *T(1;4)JC43* exhibit weak, long-period rhythms, or are arrhythmic (Smith and Konopka, 1981). Therefore, we searched for RNAs homologous to DNA that surrounds the position of this breakpoint. A summary of this transcript mapping is shown in Figure 2. Five transcripts, of sizes 4.5 kb, 2.7 kb, 1.7 kb, 1.0 kb, and 0.9 kb, were detected. The latter two are virtually indistinguishable under normal electrophoresis conditions, so that only four bands are visible in this blot (Figure 3A). However, subclone *5b* hybridizes to the 1.0 kb transcript (and the 4.5 kb transcript), whereas subclone *6b* hybridizes exclusively to the 0.9 kb transcript (Figure 3A). Another RNA of approximately 1.5 kb is also visible in Figure 3 (see figure legend). An additional complexity is that there is a third,

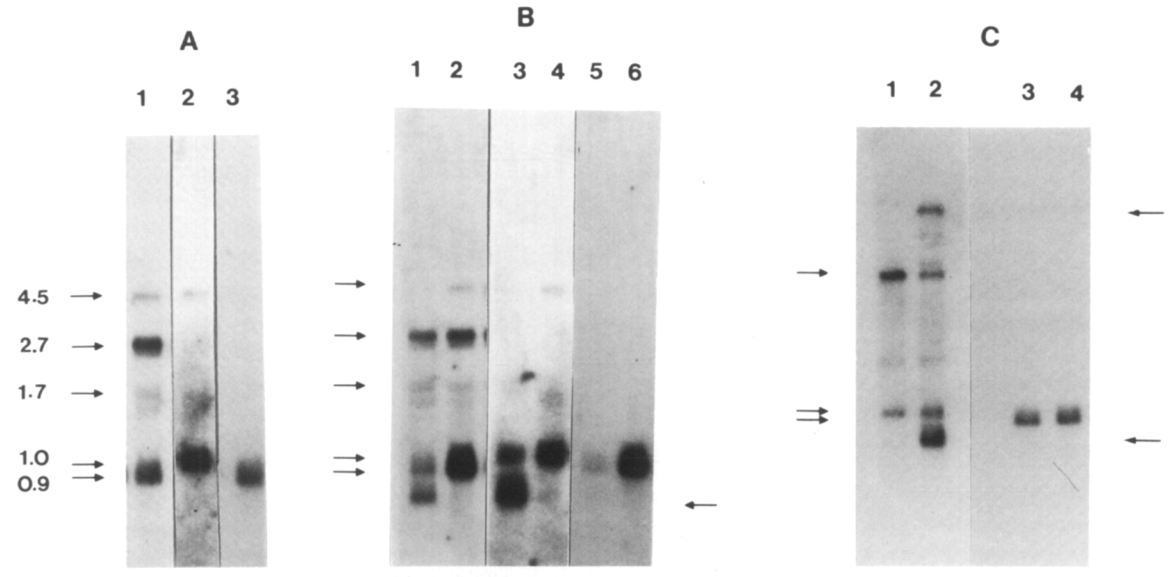


Figure 3. Northern Blot Analysis of Transcripts from the *per* Region

(A) Approximately 2–4 μg of pA$^+$ RNA from adult flies (the pA$^+$ RNA from approximately 50–100 flies) was electrophoresed on formaldehyde agarose gels and hybridized with the various subclones shown in Figure 2.

Lane 1, hybridization with a mixture of subclones *4*, *7*, and *8*. Lane 2, hybridization with subclone *5b*. Lane 3, hybridization with subclone *6b*. The same lane was hybridized sequentially, resulting in the autoradiogram shown in lane 1, then lane 2, and, finally, lane 3. The hybridization in lane 2 was deliberately overexposed to show the 4.5 kb transcript.

The arrows indicate positions of the 4.5, 2.7, 1.7, 1.0, and 0.9 kb transcripts. The band for the 2.7 kb species may be a doublet, but its character is always identical, e.g., using different probes or in the RNA from different strains. A transcript migrating between the 1.7 and 1.0 species can also be seen. This transcript has weak homology to the relevant labeled probes (*4*, *5b*, *7*), i.e., it is not detected on increasing the stringency conditions of the hybridization washes (data not shown). The anomalous transcript is also seen in RNA from adults of the genotype *Df(1)64j4/Df(1)TEM-202*, which is deficient for DNA homologous to the subclones used in these Northern blotting experiments, suggesting that it derives from elsewhere in the genome.

(B) Approximately 4 μg of pA$^+$ RNA from *T(1;4)JC43/Df* and from wild-type adults was electrophoresed on two adjacent tracks. The filter was hybridized sequentially to generate the autoradiograms shown in lanes 1 and 2, then lanes 3 and 4, then lanes 5 and 6 (odd numbers, RNA from the mutant; even numbers, from wild type). Lanes 1 and 2 were hybridized with subclones *4*, *7*, and *8;* lanes 3 and 4 with subclone *5b;* lanes 5 and 6 with *6b* (see Figure 2). Note the fact that the band intensity of the 2.7 kb RNA is approximately equal in lanes 1 and 2. Arrows are as in (A).

(C) Lanes 2 and 4, each containing 30 μg pA$^+$ RNA from the mutant, were overloaded compared to lanes 1 and 3, which each contained 3 μg of wild-type pA$^+$ RNA. Lanes 1 and 2 were hybridized with subclones *3b* and *5b;* lanes 3 and 4 with subclone *6b* (Figure 2).

Arrows on the left indicate the 4.5, 1.0, and 0.9 kb transcripts from the wild type (always ordered from top to bottom), and arrows on the right indicate the two *T(1;4)JC43*-specific transcripts which are approximately 10 kb and 0.5 kb in length.

higher molecular weight transcript (approximately 7 kb in length), which is not well characterized but which has extensive overlap with the 4.5 and 1.0 kb transcripts (Figure 3, and data not shown).

The 0.9, 2.7, and 1.7 kb RNA species appear to arise from consecutive transcription units, from the genomic region proximal to the translocation's 3B1-2 breakpoint. That is, each of the transcripts is homologous to a separate genomic segment. In contrast, the 4.5 kb transcript and the 1.0 kb transcript hybridize to some of the same probes (Figure 2). The size of the probes, and in particular the small size of the cloned DNA in subclone *5* (the most proximal clone which hybridizes to both the 1.0 and 4.5 kb transcripts), suggests that the genomic sequences corresponding to these transcripts are overlapping or interspersed (see Vincent et al., 1984).

In order to verify that these RNAs originate from the *per* region and not from related loci elsewhere in the genome, we analyzed RNA from the doubly deficient *Df(1)64j4/Df(1)TEM-202* females. Southern blots of DNA from flies of this genotype (not shown) revealed that the DNA between the two *per* region breakpoints associated with these deletions (between approximately −10 and +5 on the molecular map in Figure 1) is absent and therefore not essential, as previously suggested by cytogenetic data (e.g., Smith and Konopka, 1981). Four of the transcripts, the 0.9, 1.0, 4.5, and 2.7 kb species, are undetectable in such flies (data not shown); this supports the contention that these transcripts originate as shown in Figure 2.

Since the 3B1-2 breakpoint in *T(1;4)JC43* leads to phenotypes whose components seem to stem from subnormal activity of the *per* locus (Smith and Konopka, 1981, 1982), we examined in some detail RNA from *T(1;4)JC43/Df(1)64f1* females (Figures 3B, 3C). The deletion (*Df*) eliminates DNA from this region, so all relevant transcripts derive from the *T(1;4)JC43* chromosomes. It is readily apparent that all three transcripts homologous to DNA flanking the breakpoint (i.e., the 4.5, 1.0, and 0.9 kb RNAs) are significantly decreased in abundance as compared to wild type. In contrast the 2.7 kb and the 1.7 kb RNA species, transcribed from more proximal subsegments, are unaffected. The 7 kb RNA (referred to above, and not

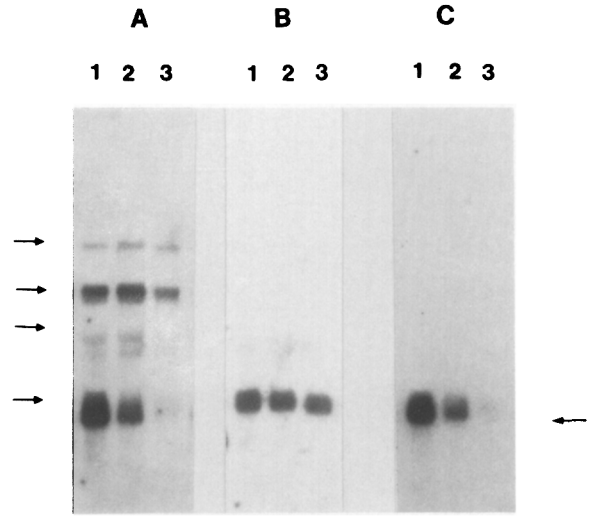


Figure 4. Analysis of Transcripts from *per*^{o} Strains

pA^{+} RNA was extracted from *per*^{o} flies and analyzed next to approximately equal quantities of pA^{+} RNA from wild-type adults. Four micrograms of pA^{+} RNA was applied to each lane. Lane 1, wild type; lane 2, *per*^{o2}; lane 3, *per*^{o1}. The same Northern blot was sequentially hybridized to three groups of probes:

(A) Hybridization to subclones *4*, *7*, and *8*.

(B) Hybridization to subclone *5b*.

(C) Hybridization to subclone *6b* (see Figure 2).

The arrow on the right indicates the 0.9 kb RNA species that is affected by the *per*^{o} mutations. Arrows on the left indicate the 4.5, 2.7, 1.7, and 1.0 kb transcripts whose levels are not visibly lower than normal in the mutants. In (A), lanes 2 and 3 reveal only that the 0.9 kb transcript is decreased in abundance by *per*^{o2} or *per*^{o1}; the 1.0 kb band is very faint in this autoradiograph; the absence of effects of these mutations on its abundance is revealed by use of the smaller, more specific, single-stranded probe in (B).

Table 1. Spatial and Temporal Expression of Transcripts from the *per* Region

Developmental Stage or Body Region	Transcripts (kb)				
	0.9	1.0	1.7[a]	2.7	4.5
Embryos	−	−	++	++	−
Larvae, late third instar	−	−	++	++	−
Pupae, early	−	−	++	++	−
Pupae, mid/late	+	+	++	++	+
Adults, 2 hr post-eclosion	++	+	++	++	++
Adults, 1 week post-eclosion	++++	+	++	++	+++
Adult heads	++++	+	−	++	+++
Adult thoraces + abdomens	++++	+	++	++	+++

The detectability of the five transcripts, whose genomic sources are shown in Figure 4, was assessed by Northern blot analysis of pA^{+} RNA from several stages of development and from anterior vs. posterior body regions of adults (see Experimental Procedures). A semi-quantitative summary of the results is presented here wherein, at one extreme, "−" indicates no detectable hybridization of labeled single-stranded probes to the RNA in question; and the maximal detectability in these experiments (most intense bands) is indicated by "++++". This relatively high level is at least 10 times lower than the level of RNA from adults that hybridizes to double-stranded probes from the *Adh* locus in this species (our data, not shown; see Goldberg et al., 1983).

[a] Two P-element-induced mutations at the *l(1)zw6* locus map to a genomic subsegment (Figure 1) coincident with subclone *10a*, which hybridizes to the 1.7 kb RNA (Figure 4). This transcript is present from the earliest developmental stages onward (this table); *l(1)zw6* mutations are lethal to first instar larvae (T. C. Kaufman, Ph.D. thesis, University of Texas, Austin, Texas, 1970), i.e., at a stage shortly after the time when the RNA in question makes its initial appearance in normal development. The nondetectability of the 1.7 kb transcript in the fly's head is a further indication that this RNA species might not be involved in *per*'s expression (see Discussion).

yet well characterized) is not reduced in level in *T(1;4)JC43/Df* females (data not shown).

Two novel transcripts are apparent in RNA isolated from flies hemizygous for this translocation breakpoint (Figure 3C). Blotting with various subclones indicated that both such RNAs are derived from DNA distal to the central Hind III site (at approximately position 0 in Figure 1), and both are transcribed in the same direction as the three transcripts whose genomic sources flank the *T(1;4)JC43* chromosome break. The smaller of these novel transcripts (0.5 kb in length) derives principally, if not entirely, from the central 1.0 kb Bam HI/Hind III fragment (subclone *5* in Figure 2). The novel 10 kb transcript derives from the same general region as the wild-type 4.5 kb transcript, although its genomic source has not been mapped with precision.

The fact that the wild-type transcripts are apparently unchanged in size, under the influence of *T(1;4)JC43*'s 3B1-2 breakpoint, suggests (but does not prove) that none of the genomic sources of these three transcripts is interrupted by this breakpoint. Were a transcript to be produced by an interval spanning the *T(1;4)JC43* break, it is likely that it would not be present in RNA from *T(1;4)JC43/Df* females. This interpretation is consistent with Northern blotting data which demonstrate that none of these three transcripts is homologous to DNA on both sides of the central Hind III site (at position 0). (Other mapping data indicate that the 3B1-2 breakpoint in *T(1;4)JC43* is close to this same restriction site (P. Reddy, unpublished results; see Figure 2.) The results support the relatively conservative interpretation that the residual gene activity correlated with one or more of these transcripts is responsible for the weak rhythms manifested by *T(1;4)JC43/Df* females.

To examine the effects of other *per* variants on these transcripts, we analyzed RNA from flies expressing arrhythmic mutations at the locus (Figure 4). In *per*^{o1} and *per*^{o2} adults, the 0.9 kb transcript is dramatically reduced in level to approximately 5% or 20% (respectively) of the wild-type level. No other transcript from this region is visibly affected (Figure 4). This series of tests has been performed twice and similar results were obtained each time (even to the extent that the *per*^{o1} allele causes the greatest decrease in abundance of the 0.9 kb RNA). The reduced abundance of the 0.9 kb transcript in the two *per*^{o} mutants is similar to the low level in flies hemizygous for the 3B1-2 breakpoint in *T(1;4)JC43*, as determined by densitometric measurements on the autoradiograms shown in Figure 3.

Other aspects of the expression of the 0.9 kb RNA are appropriate for considering the involvement of this function in phenotypes of the adult (Table 1). This transcript is easily

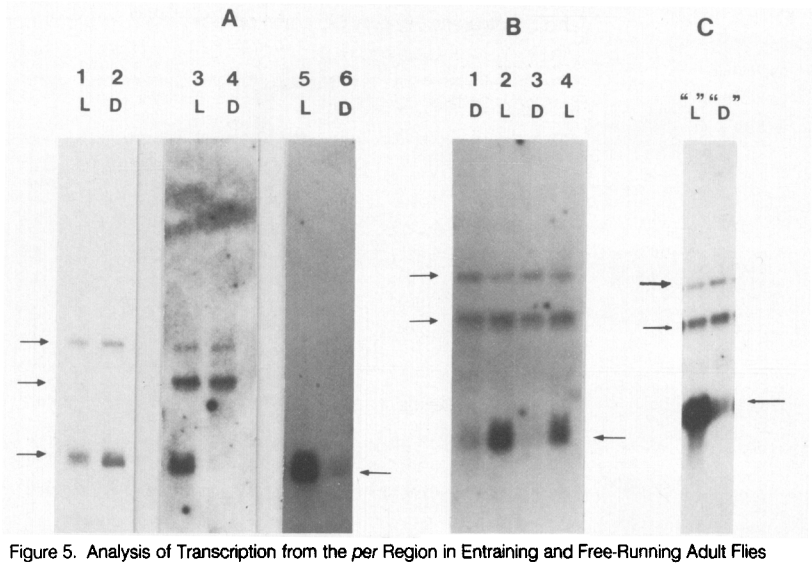


Figure 5. Analysis of Transcription from the *per* Region in Entraining and Free-Running Adult Flies

(A) Adult wild-type flies (approximately one week post-eclosion) were entrained through three cycles of a 12 hr/12 hr light/dark regime; the beginning of a cycle was always at 8:00 A.M. Flies were collected near the middle of the next light period (2:00 P.M.) and the middle of the next dark period (2:00 A.M.), then were quickly frozen at −70°C. RNA was prepared from both sets of flies and analyzed on Northern blots as described in Experimental Procedures.

Lanes labeled L, RNA from light-period collections; D, from dark-period collections.

The pair of lanes was hybridized sequentially to the three sets of probes, first to those shown in lanes 3 and 4; then as shown in lanes 1 and 2; then in lanes 5 and 6. Lanes 1 and 2, hybridization to probe *5b;* lanes 3 and 4, hybridization to probes *4* and *7;* lanes 5 and 6, hybridization to probe *6b* (see Figure 2). As in Figure 4, the 1.0 kb RNA is revealed clearly by use of the small, specific, single-stranded probe in lanes 1 and 2. Arrows on the left indicate the 4.5, 2.7, and 1.0 kb transcripts. The arrow on the right indicates the 0.9 kb transcript.

(B) In a separate set of experiments, one-week-old flies were maintained under the same kind of 12 hr/12 hr light/dark regime (as in A) for three cycles. The entrainment was continued; and four consecutive collections of the flies were made, twice per day, over a 48 hr period. Lane 1 (D), collection at 11:30 P.M. day 1; lane 2 (L), 2:00 P.M. day 2; lane 3 (D), 2:00 A.M. day 3; lane 4 (L), 11:30 A.M. day 3. After collection and freezing, RNA was prepared, electrophoresed, and probed with subclones *2, 6,* and *7.*

Arrows on the left indicate the 4.5 kb and 2.7 kb transcripts, whose levels are relatively unchanged. The arrow on the right designates the 0.9 kb RNA whose levels, in contrast, fluctuate.

(C) One-week-old flies were maintained under a 12 hr/12 hr light/dark regime for three cycles (as in A and B). The flies were then maintained in complete darkness for three further cycles, after which two consecutive collections were made of these free-running flies at 2:00 A.M. (subjective night, "D") and 2:00 P.M. (subjective day, "L") over a 12 hr period. RNA was prepared, electrophoresed and probed with subclones *4* and *7.* Arrows are as in (B).

detected in very young as well as older adults. It is also detectable, albeit with difficulty, in late pupae (data not shown), from which we conclude that the abundance of this transcript increases dramatically at approximately the pupal/adult boundary. Table 1 notes that the 0.9 kb transcript is present in the heads of adults as well as in the posterior body regions; these data, too, would be consistent with the involvement of this function in more than one rhythmic behavioral phenotype (see Discussion).

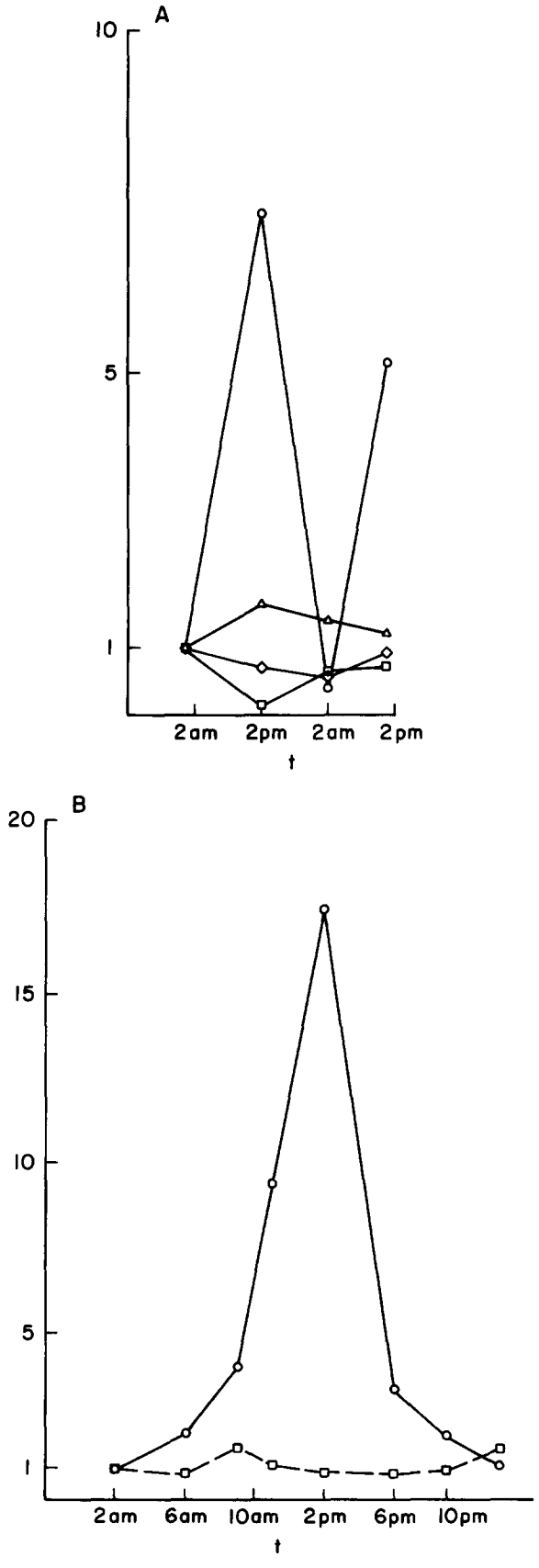
There is additional evidence that draws attention to the importance of the 0.9 kb transcript as part of the *per* region's clock controlling function. All of the experiments shown above, and our normal protocol for RNA extraction, involve flies collected near the middle of a given day (see Experimental Procedures). These RNAs were compared to RNA isolated from flies collected near the middle of the night. Remarkably, the 0.9 kb transcript is reduced dramatically in abundance in the RNA from nighttime fly collections vs. those performed during daylight (Figures 5A and 5B and Figure 6).

Several aspects of this finding merit additional comment. First, the cycling of the abundance of the 0.9 kb RNA continues during "free-funning," i.e., in the absence of an endogenously applied light/dark cycle (Figure 5C). This observation indicates that the abundance fluctuations are not responding solely to external light/dark cues, but rather are coupled to the endogenous clock. Second, the other transcripts from this region, when compared to each other or to the level of mRNA transcribed from the *Adh* locus (see Goldberg et al., 1983), manifest no similar fluctuations (Figures 5 and 6). Third, analysis of a more detailed time course reveals that the cycling is circadian, i.e., there is one cycle per day (Figure 6B). These observations, taken together with the effect of the per^{o} mutations on the abundance of this RNA, strongly implicate this 0.9 kb transcript in rhythmic functions.

Discussion

Genomic Segments near the *per* Locus

The molecular mapping of lesions surrounding, and in some cases probably within, the *period* gene suggests that much and perhaps all of this locus is within a specific X chromosomal subsegment that spans approximately 25 kb of DNA in the 3B1-2 region (Figure 1). The most important genetic variants that we used to draw this conclusion are those whose lesions do not appear to disrupt *per*'s expression. On *per*'s left (distal) there is the right-



hand breakpoint of *Df(1)K95*, and on *per*'s right (proximal), there is the left breakpoint of *Df(1)w*$^{-64d}$ as well as the site of the small deletions (induced by hybrid dysgenesis) near or within the *l(1)zw6* locus.

There are uncertainties with respect to the nature of DNA distal to the *Df(1)K95* lesion shown in Figure 1. This aberrant chromosome is associated with a small, 30 kb inversion that lies distal to the breakpoint shown in Figure 1 (i.e., to the left of the indicated *Df(1)K95* lesion, M. Goldberg, personal communication). Thus DNA associated with *per*'s function could lie in the inverted segment located to the right of the deletion per se. We consider this interpretation unlikely, however, based on the positions of the other breakpoints and on the transcript analysis presented above.

Effect of *T(1;4)JC43* on RNAs Transcribed from the *per* Region

The 3B1-2 breakpoint of *T(1;4)JC43* would, from prior phenotypic evidence, be assumed not to abolish a portion of the *per* locus that is essential for its function. This is because the activity of *per* seems only to be subnormal, not absent, in a fraction of the flies heterozygous for this breakpoint and a *per*$^{-}$ deletion (Smith and Konopka, 1981). Consistent with this phenotype is the effect of *T(1;4)JC43* on the RNA species transcribed from the DNA in and around the *per* locus. The three transcripts (4.5 kb, 1.0 kb, and 0.9 kb) homologous to segments adjacent to the 3B1-2 lesion in *T(1;4)JC43* are all dramatically reduced in abundance by this breakpoint. We suggest that the reduced levels of one or more of the three transcripts are responsible for the weak rhythms associated with the *per* region lesion in *T(1;4)JC43*.

Figure 6. Quantification of RNA Abundance Changes

(A) Several different exposures of the Northern blot autoradiograms shown in Figure 5B were analyzed by densitometry. Also the blot was hybridized to an alcohol dehydrogenase (*Adh*) probe (see Goldberg et al., 1983) and the resultant autoradiogram similarly analyzed. The values obtained for the three *per* region transcripts (4.5, 2.7, and 0.9 kb) were divided by the value obtained for *Adh* transcript in each lane and normalized to the ratio computed for lane 1 (first 2:00 A.M. point).

Δ; *Adh* values, normalized to lane 1.

□; 4.5/*Adh*, normalized to the ratio obtained in lane 1.

◇; 2.7/*Adh*, normalized to the ratio obtained in lane 1.

○; 0.9/*Adh*, normalized to the ratio obtained in lane 1.

The blot was also hybridized to a probe specific for the 1.0 kb RNA. It varies by no more than a factor of 2, i.e., the results obtained for the 1.0 kb RNA are similar to those for the 4.5 kb and the 2.7 kb transcripts.

(B) One-week-old flies were entrained in a 12 hr/12 hr light/dark regime for three cycles. Flies were then harvested at eight different times (see abscissa) during the next cycle. RNA was extracted, subjected to Northern blot analysis, and probed with subclones 2, 6, and 7. The autoradiograms were analyzed by densitometry as in (A). The intensities of the 4.5 kb, 2.7 kb, and 0.9 kb transcripts were determined. For each lane, the values for the 4.5 and the 0.9 kb RNAs were divided by the value for the 2.7 kb transcript and the ratios normalized to that computed with respect to lane 1 (which was set equal to 1.0).

○; 0.9/2.7

□; 4.5/2.7.

Effects of Arrhythmic Mutations on an RNA Transcribed from the *per* Region

It is significant that one of the three transcripts, the 0.9 kb RNA derived from a region just to the right of the translocation breakpoint (Figure 2), is at much lower than normal levels in flies expressing either per^o mutation (Figure 4). Since per^{o1} and per^{o2} were obtained in two different mutant screens, separated by approximately ten years, it is unlikely that the specific RNA phenotype induced by these mutations is fortuitous (e.g., caused by "background genotype" differences from the wild type). None of the other identified transcripts, and in particular neither of the remaining two transcripts affected in *T(1;4)JC43/Df* adults, is at appreciably subnormal levels in either per^{o1} or per^{o2} flies.

Several interpretations could explain the effect of per^o mutations on the 0.9 kb RNA species. Perhaps both mutations are in a specific genomic interval which is directly responsible for the transcription of this 0.9 kb transcript. Thus the mutations might disrupt a promoter region and lead to lowered transcriptional activity of the genomic segment that codes for this RNA. Alternatively, these could be nonsense mutations which have an effect on mRNA stability (see Zitomer et al., 1979). In this regard, the notion that the per^o variants are null mutations (e.g., Smith and Konopka, 1981, 1982) is germane. A very different view would be that the per^o mutations are in a segment of DNA near to but entirely separate from that which encodes the 0.9 kb RNA. This speculation implies that the level of the 0.9 kb transcript might be regulated by another gene product from the *per* region.

An examination of the molecular effects of another variant of this locus, per^s, will be interesting. Smith and Konopka (1982) hypothesized that the short-period per^s allele is a hyperactive mutated gene, in part because extra doses of per^+ lead to shorter than normal rhythm periods (see also Konopka and Orr, 1980). Against the background of our data on *per* region transcripts in normal flies and those expressing hypoactive *per* variants, it may be possible to demonstrate increased levels of one or more of these RNAs in per^s. In this general context, it is notable that an extra dose of per^+ does not lead to increased levels of tyrosine decarboxylase, an enzyme that is present at lower than normal levels in per^{o1} adults (Livingstone and Tempel, 1983). Since dosage changes of protein-coding loci in this species generally lead to alterations in the level of the relevant products (O'Brien and MacIntyre, 1978), the data of Livingstone and Tempel (1983) suggest that tyrosine decarboxylase levels (and octopamine synthesis) are affected indirectly by *per* mutations. The availability of DNA cloned from the *per* region, and of transcripts homologous to this material, should allow a definitive examination of this issue.

Temporal and Spatial Regulation of Transcripts from the *per* Region

One of the most interesting features of temporal changes in expression of RNAs transcribed from the vicinity of *per* is that the 0.9 kb RNA (Figure 2) changes in levels at different stages of a light/dark cycle (Figures 5 and 6). This regulation could be indirect, that is, influenced by the eventual product of a separate transcript that is encoded within a nearby DNA segment. Yet it is also possible that the striking fluctuations in the levels of this RNA species occur without the involvement of other *per* gene products. In a sense, this is part of the general question of whether the 0.9 kb transcript drives the circadian cycle or whether the transcription of this RNA species is a consequence of the cycle. An examination of the details of this regulation, including experiments involving phase-shifting light pulses (see Konopka and Orr, 1980), will be particularly informative. Similarly, it will be interesting to examine the effects of *per* mutants on the cycling of the 0.9 kb RNA.

We have detected the 0.9 kb transcript only in adults and late pupae (Table 1). In this regard, we note that it seems sufficient for a *per* allele associated with rhythmicity to be active only during the adult stage, as shown by transplant experiments (Handler and Konopka, 1979) and the effects of the temperature-sensitive per^{L2} allele (D. P.-Y. Orr, Ph. D. thesis, California Institute of Technology, Pasadena, California, 1982). Thus some elements of *per*'s expression seem to control the ongoing adult behaviors that are affected by *per* mutations (Konopka, 1984; Kyriacou and Hall, 1980). That is, *per* is not required solely during development to set up the nervous system for running these rhythms.

Yet, other effects of mutations at this locus seem to be manifest during larval development and possibly as early as the embryonic stage. Konopka and Wells found that a circadian rhythm of cell division, in lines established from Drosophila embryos, is perturbed by the per^s mutation. Livingstone (1981) reported not only that the per^{o1} allele affects heartbeating in third instar larvae, but also that the pericardial cells of this mutant organ are aberrant in morphology. Other cell types seem to develop or function abnormally under the influence of this same mutation. Neurosecretory cells in the posterior brain of per^{o1} adults are, more often than in per^+ flies, scattered in ectopic locations (Konopka and Wells, 1980). There is a suggestion that cells of this type are "born" in embryos (White and Kankel, 1978), implying that per^{o1} might affect these cells as early as this stage. Cells of the larval salivary gland exhibit rhythmic fluctuations in membrane potential in per^+, and these cells in per^{o1} larvae were shown to be mildly disturbed in this rhythm (Weitzel and Rensing, 1981). Thus RNAs detected only in pupae and adults (e.g., the 0.9 and 4.5 kb transcripts, Table 1) seem as if they cannot totally account for all of the biological activities of the *per* region. The adjacent 2.7 kb transcript (Figure 2), expressed throughout the fly's life cycle (Table 1) and ostensibly unaffected by *per* variants, is a viable candidate for participating in the control of rhythmic phenotypes expressed during relatively early developmental stages.

While this manuscript was in preparation, a paper describing similar work appeared (Bargiello and Young, 1984). Whereas many of our data are similar, a few salient differences are noteworthy. The other investigators identi-

fied a male-specific 1.2 kb transcript that comes from a region distal to the genomic source of the RNAs we have identified (see Figure 2). This transcript might be related to male-specific components of Drosophila rhythms (see Kyriacou and Hall, 1980). If so, we predict that this RNA species would be located in the thoracic nervous system, since mosaic focusing of *per*^{s}'s effect on the courtship song rhythm implies a direct effect of this mutation on the thoracic ganglia (R. J. Konopka, J. C. Hall, and C. P. Kyriacou, unpublished data), whereas the focus of this same mutation's effects on adult circadian rhythms is in the fly's head (Konopka et al., 1983). An alternative possibility for a molecular correlate of these results is that a single transcript (e.g., the 0.9 kb RNA) might be responsible for all the adult activities, which would be consistent with the fact that this RNA species is expressed in the head plus other body segments (Table 1).

Whereas our results point to the 0.9 kb transcript as a component of *per*'s influence on rhythmic phenotypes in the adult, we recognize that there are phenogenetic data which indicate that the portion of the X chromosome adjacent and distal to the 3B1-2 breakpoint in *T(1;4)JC43* makes a weak but detectable contribution to behavioral rhythms (Smith and Konopka, 1981). This part of the *per* region does not include material homologous to the 0.9 kb RNA but does code for the 4.5 and 1.0 kb transcripts (Bargiello and Young, 1984; Figure 2). It is interesting to note that all three of these transcripts have similar if not identical developmental profiles (Table 1). Perhaps two or all three of these transcripts are required to organize robust rhythmic phenotypes of adults.

Nevertheless, we emphasize that our data lead to the strong suggestion that one of these RNAs, the 0.9 kb species, is involved in certain rhythmic/behavioral phenotypes. We anticipate that experiments which exploit DNA-mediated transformation will identify both the biological contribution of this transcript and its specific relationship to DNA which is altered in *per* mutants.

Experimental Procedures

Drosophila Stocks and Their Genetic Manipulation

D. melanogaster strains were raised on a cornmeal/agar/molasses/yeast medium at 25°C, 60% relative humidity, in a 12 hr/12 hr light/dark cycle. Our source of X chromosomes carrying the normal *per*$^{+}$ allele was a Canton-S wild-type stock (the genetic background in which all *per* mutations have been induced). The following chromosome aberrations were used in conjunction with *per*$^{+}$ or other genetic variations mapping in or near this gene: *Df(1)64j4*, *Df(1)TEM-202*, *Df(1)64f1*, *Df(1)w*^{rJ1}, *Df(1)w*$^{-64d}$, and *Df(1)K95*. These X chromosomal deletions have been reported (Young and Judd, 1978; Smith and Konopka, 1981) either to delete or possibly interrupt *per* (the first four listed), or to contain a functionally normal version of this locus (the last two, which nevertheless do have breakpoints very near to *per*). Two of the deleted X chromosomes are free of other genetic variants; but *Df(1)64j4* is flanked by the *yellow* and *white-apricot* markers; *Df(1)TEM-202* is linked to *yellow*^{2}, *cut*^{6}, and *forked*; *Df(1)w*^{rJ1} carries *yellow*^{2}, *split*, *echinus*, and *singed*^{3}; and *Df(1)K95* is linked to *yellow*^{2}. These lethal deficiencies were maintained with *Df*-bearing males that had their particular deletion covered by a Y chromosome carrying a distal segment of the X chromosome (i.e., *w*$^{+}$Y); the females in each stock were *C(1)DX,y f/w*$^{+}$Y (see Lindsley and Grell, 1968, for a description of these and other standard genetic variants).

The aberrant genotype *T(1;4)JC43* involves an X-4th chromosome translocation plus an X chromosomal inversion; one of the breakpoints on the X, at 3B1-2, is very near to *per* and disrupts its normal expression (Young and Judd, 1981; Smith and Konopka, 1981). The other breakpoint on *T(1;4)JC43*'s X chromosome is several bands removed from *per* (Smith and Konopka, 1981). This translocation/inversion is a homozygous lethal variant (because of the non-*per* breakpoint on the X chromosome and/or the 4th chromosomal lesion); thus *T(1;4)JC43* was maintained by balancing it, in females, with the marked, multiply inverted chromosome *In(1)FM7*. Flies have good viability if the only breakpoint in *T(1;4)JC43* that is uncovered is the one at 3B1-2. For this, we used *Df*s which are completely deleted of the *per* locus and do not have breakpoints extremely near to it, i.e., *Df(1)64f1* or *Df(1)w*^{rJ1} (see Smith and Konopka, 1981). We also generated females for this purpose which were heterozygous for a lethal *Df* and a particular Canton-S derived X. This *per*$^{+}$-bearing chromosome had been obtained from one male who was crossed to attached X (*C(1)DX/Y*) females. Thus a series of flies could be generated with each separate type carrying different chromosome aberrations in heterozygous condition with the same "normal" X. We produced *Df(1)64j4/Df(1)TEM-202* females, each of whose X chromosome is deleted of *per* (or at least carries an ostensibly inactive *per* locus; see Young and Judd, 1978; Smith and Konopka, 1981). To generate these doubly deficient flies, deletion-bearing males of the appropriate genotype were crossed to females that had been produced by previous crosses of males from the other *Df* stock to *FM7* females.

Other kinds of females, which we constructed to be heterozygous for X chromosome lethal factors, carried *l(1)zw6*^{P1} or *l(1)zw6*^{P2} mutations. These are allelic, hybrid dysgenesis-induced variants that were likely to have been caused initially by P-element transposition (Simmons and Lim, 1980). The two lethals are at a locus which is just to the right of *per* on the standard genetic map (Young and Judd, 1978; see Lindsley and Grell, 1968). We confirmed that these two mutations are *l(1)zw6* alleles, by complementation tests with other mutations in this gene, *l(1)zw6*^{e5} and *l(1)zw6*^{m57}. In a similar manner, we verified that we were working with the deletions (see above) that were missing the normal alleles of the appropriate, respective "*zeste-white*" (*zw*) gene loci, but which covered *zw*-lethal mutations outside the reported *Df* boundaries (see Judd et al., 1972; Young and Judd, 1978). The lethal mutations we used in these complementation tests were *l(1)zw2*^{b11}, *l(1)zw3*^{b12}, *l(1)zw6*^{e5}, *l(1)zw12*^{k3}, and *l(1)zw7*^{63g20}. These loci are listed in their proper left–right order and surrounded *per*, in the sense that this clock gene—which appears not to be allelic to any of the lethal mutations—is located between *l(1)zw3* and *l(1)zw6* (Young and Judd, 1978; Smith and Konopka, 1981).

We tested the previously unanalyzed *l(1)zw6*^{P1}*/per*^{o1} genotype for circadian rhythmicity. Rhythms of adult locomotor activity were measured essentially as described in Konopka and Benzer (1971) and Jackson (1983). We confirmed, first of all, that *per*^{o1} flies are arrhythmic (see Konopka and Benzer, 1971; Smith and Konopka, 1981), and then that the heterozygous females just described are rhythmic. Other pertinent genotypes, whose effects on locomotor phenotypes we confirmed, were *Df(1)64j4/per*^{o1}, *Df(1)TEM-202/per*^{o1}, *Df(1)w*^{rJ1}, and *Df(1)64j4/Df(1)TEM-202*. Each of these female types was arrhythmic (see Young and Judd, 1978; Smith and Konopka, 1981). Finally, we reexamined the case of *T(1;4)JC43/Df(1)w*^{rJ1}; such females were essentially arrhythmic or expressed rhythms with very long periods that blended into arrhythmicity (see Smith and Konopka, 1981).

Bacteria, Plasmids, and Phage Strains

E. coli strain DP50, *supF*, was obtained from W. Bender; strain JM101 was from J. Messing. The pEMBL vectors 8+ and 9+, and single-stranded phage IR1 (Dente et al., 1983), were a gift from R. Cortese. The molecular clones λ*a4715b* and λ*a4719b*, from the 3B1-2 region of the X chromosome (Figure 1), were originally isolated by Pirrotta et al. (1983). The overlapping phage Charon4 λ E1 was isolated from the Canton S–Charon 4 library of Maniatis et al. (1978) using the distal-most 1.5 kb Eco RI fragments of λ*a4715b* (Figure 1) in the molecular walking procedure of Maniatis et al. (1978). The three phages were propagated in DP50 *supF* cells, and their DNA was purified as described by Yamamoto et al. (1970).

DNA and RNA Extraction

Flies to be used as sources of DNA or RNA were quick-frozen in liquid N_2 and stored at −70°C until processed. Such flies had been aged for

approximately 7 to 14 days as adults and harvested at the middle of the lights-on period of a given 12 hr/12 hr light/dark cycle, unless otherwise indicated. The protocol of Levy and Manning (1981) was followed to obtain head- and body-specific RNAs. In order to examine modulations in the levels of transcripts from the *per* region during or after development, we used RNA from the following developmental stages: 1–16 hour embryos, late third instar larvae, and early or mid/late pupal stages as described in Vincent et al. (1984).

DNA was prepared as described by Pirrotta et al. (1983). RNA was extracted by the method of Barnett et al. (1980), with modifications. Briefly, flies were homogenized in a Sorvall homogenizer in 100 mM Tris-HCl (pH 7.0), 0.15 M sodium acetate, 2% (w/v) sodium dodecylsulfate (SDS), 100 μg/ml polyvinylsulfate, and 2% (v/v) diethyl pyrocarbonate. The homogenate was centrifuged at 10,000 rpm for 10 min in a Sorvall SS-34 rotor. The supernatant was then extracted three times with phenol:chloroform:isoamyl alcohol (50:50:1), and RNA was precipitated by the addition of three volumes of 100% ethanol and incubation at −20°C. Poly(A)$^+$ (pA$^+$) RNA was prepared by passing total RNA through an oligo(dT) cellulose column (see Aviv and Leder, 1972, and Barnett et al., 1980, for details; the oligo (dT) cellulose was from Collaborative Research). We have been unable to detect the *per* region transcripts in total RNA. Therefore, the abundance changes described in Results cannot formally be distinguished from changes in adenylation.

Plasmid Constructions

Restriction fragments, isolated from λ*a4715b* and λ*a4719b* and ranging in size from 1–4 kb, were ligated to linearized pEMBL 9+ and 8+ DNA. The ligated DNA was transformed into JM101 (*amp*^{s}) cells and assayed on selective plates (re: antibiotic resistance and beta-galactosidase expression). DNA obtained from ampicillin-resistant, white colonies was analyzed with respect to restriction fragments (see below) to identify clones containing the Drosophila inserts in the required orientations. Single-stranded DNA from pEMBL 9+ and 8+ clones was prepared by superinfection of transformed cells with intact f1 phage IR1, as described by Dente et al. (1983).

Preparation of Radiolabeled Probes for Hybridization

Restriction fragments were generated with various endonucleases (see Results) purchased from New England Biolabs, Bethesda Research Laboratories, and Boehringer Mannheim. The fragments were purified from low melting agarose (see Pirrotta et al., 1983). They were radiolabeled with 50 μCi α-^{32}P-dCTP (800 Ci/mmole, from New England Nuclear) by nick translation (Golden et al., 1980). The same technique was used for labeling phage or plasmid DNA. Strand-specific hybridization probes were prepared from single-stranded pEMBL clones by the procedure described for phage M13 (Hu and Messing, 1982). One picomole of the hexadecanucleotide probe primer (New England Biolabs), complementary to the sequence 50 bp 5′ from the Drosophila insert, was hybridized to 200 ng of the single-stranded DNA and extended with the Klenow fragment of E. coli DNA Polymerase I (from Boehringer Mannheim) for 30 min at 20°C in the presence of 50 μCi α-^{32}P-dCTP and the other three unlabeled nucleotides.

Blot Hybridizations

Ten micrograms of genomic DNA, isolated from approximately 10–15 adult flies (see above), was digested with a given (indicated) restriction endonuclease and fractionated on an 0.8% agarose gel. DNA blots on nitrocellulose were performed by the method of Southern (1975). Hybridization was carried out as described in Colot and Rosbash (1982), and filters were subsequently exposed to Kodak XAR-5 film at −70°C with an intensifying screen (DuPont Cronex). For Nothern blots, pA$^+$ RNA was fractionated on 0.8%–1.0% agarose gels containing 2 M formaldehyde, 0.2 M morpholinopropanesulfonic acid (MOPS, from Sigma) and 50 mM sodium acetate (pH 7.0) (Colot and Rosbash, 1982). Approximately 4 μg of pA$^+$ RNA (from approximately 50–100 adults) was fractionated per lane, unless otherwise stated. Prior to loading, the RNA samples were denatured in 2 M formaldehyde, 50% deionized formamide and 0.2 M MOPS for 5 min at 65°C. After electrophoresis, the gel was soaked in 10X SSC (1X SSC = 150 mM NaCl, 15 mM sodium citrate) for 30 min and blotted in 20X SSC onto nitrocellulose filters (Thomas, 1980). Hybridizations were carried out as described above, with the addition of 10% (w/v) Dextran sulfate. RNA sizes were measured by comparison to yeast rRNA and pBR322 standards. The relative amounts of transcripts were determined by densitometric measurements of autoradiographs, as described by Hereford et al. (1981).

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