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A New Biological Rhythm Mutant of *Drosophila melanogaster* That Identifies a Gene With an Essential Embryonic Function

L. M. Newby and F. Rob Jackson

Neurobiology Group, Worcester Foundation For Experimental Biology, Shrewsbury, Massachusetts 01545

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ABSTRACT

To identify components of a circadian pacemaker output pathway, we have sought Drosophila mutations that alter the timing of eclosion but do not perturb circadian period or the expression of the activity rhythm. A mutant named *lark* has been identified, for which daily peaks of eclosion occur abnormally early while populations are synchronized to either light/dark or temperature cycles. The temporal phasing of locomotor activity in *lark* mutants, however, is entirely normal, as is the free-running period of the circadian pacemaker. The *lark* strain carries a single *P*-element insertion which, interestingly, has a dominant effect on the timing of eclosion, but is also associated with a recessive embryonic lethal phenotype. The analysis of excision-generated alleles suggests that the *lark* gene encodes an essential function. This function is apparently mediated by a transcription unit that is interrupted by the *P*-induced *lark* mutation. A combination of *in situ* hybridization analysis and reporter (β-gal) staining indicates that this transcription unit expresses mRNAs throughout the embryonic central nervous system and in a defined subset of cells in the nervous system of pharate adults. RNAs are first detected at about embryonic stage 11, just prior to the stage at which lethality occurs in *lark* homozygotes. Based primarily on the observed mutant phenotypes, a function is proposed for the LARK product(s) that is consistent with the pleiotropic nature of *lark* mutations.

ENDOGENOUS clocks (circadian pacemakers) that regulate daily rhythms in physiological, endocrine and behavioral processes exist within the central nervous systems of all animals (reviewed in EDMUNDS 1988). In recent years, both genetic and molecular approaches have been employed in attempts to understand the biochemical constituents of circadian pacemakers and their photic transduction (light entrainment) pathways (see reviews contained within YOUNG 1993). In particular, genetic studies in several different species including the fruit fly *Drosophila melanogaster* have identified products that may serve as integral components of the circadian pacemaker (BAYLIES *et al.* 1993; DUNLAP *et al.* 1993; HARDIN, HALL and ROSBASH 1993; JACKSON 1993; MENAKER and REFINETTI 1993).

Drosophila has proven to be a particularly attractive model system for molecular genetic investigations of neural pacemakers (reviewed in HALL 1990 and JACKSON 1993). In the fruit fly, the effects of circadian rhythm mutations can be assessed by monitoring two distinct behavioral periodicities. Rhythms are evident in the locomotor activity of single adults, or in the emergence of adults (*i.e.*, eclosion) from a population of mature pupae (KONOPKA and BENZER 1971; JACKSON 1983). This latter rhythm, which is a consequence of the precise regulation of a developmental event (eclosion) by an endogenous pacemaker, has been extremely well characterized from decades of studies (*e.g.*, see PITTENDRIGH 1981). This circadian pacemaker, which is known to be localized within the central nervous system of Drosophila (HANDLER and KONOPKA 1979; KONOPKA, WELLS and LEE 1983; EWER *et al.* 1992), can be synchronized (or entrained) to light/dark and temperature cycles, but regulates the expression of behavioral rhythms in the absence of periodic environmental stimuli (*i.e.*, in constant darkness).

These rhythm phenotypes have been used to select Drosophila mutations that alter the most fundamental properties of circadian pacemakers, including period, photic sensitivity and temperature compensation (reviewed in JACKSON 1993). All Drosophila mutations that alter circadian period do so for both the eclosion and the activity rhythm (KONOPKA and BENZER 1971; NEWBY *et al.* 1991; KONOPKA, SMITH and ORR 1991) consistent with the idea that these rhythms are regulated by a single pacemaker. Although evidence suggests that single light and temperature input pathways regulate the pacemaker itself, there are probably separate pacemaker output pathways for the neuroendocrine control of the activity and eclosion rhythms. This is suggested by the analysis of mutations that selectively perturb only one of the two rhythms (JACKSON 1983; NEWBY and JACKSON 1991; JACKSON 1993).

Primarily due to the work of Truman and colleagues on several different insects (reviewed in REYNOLDS and TRUMAN 1983 and TUBLITZ *et al.* 1986),

certain aspects of the neuroendocrine control of eclosion are understood. The best characterized component of the neuroendocrine pathway controlling eclosion is a peptide of about 4 kDa known as eclosion hormone (EH). In *Manduca*, the production of EH occurs within two pairs of CNS neurons (VM cells) whose axons project the entire length of the ventral nervous system and terminate in the proctodeal nerve. The release of EH and its subsequent action on targets within the CNS are responsible for the initiation of the program of motor activity which results in eclosion (HEWES and TRUMAN 1991). The circadian pacemaker regulates the action of EH and eclosion by restricting release of the hormone to a defined time of day (a few hours prior to eclosion; TUBLITZ *et al.* 1986). However, neither the mechanism by which the pacemaker regulates EH release nor the precise CNS targets of EH action are presently known. Moreover, EH is not the only factor influencing eclosion. The steroid 20-hydroxyecdysone (20-HE) participates by modulating the release of EH (TRUMAN *et al.* 1983). Thus, both EH and 20-HE participate in the orchestration of eclosion behavior.

In theory, genetic lesions affecting the regulation or action of EH or 20-HE could alter the timing of eclosion. In addition to the production or release of EH or the regulation by steroid hormone, these lesions might affect the relevant cellular targets of EH or 20-HE, or the potential intracellular signals that mediate the downstream effects of the hormones. The developmental appearance of two *Manduca* CNS proteins known as EGPs (*E*closion Hormone and Cyclic *G*MP-regulated *P*hosphoproteins), for example, is regulated by 20-HE (MORTON and TRUMAN 1988a). Moreover, the action of eclosion hormone in different insects is mediated by the second messenger cGMP (MORTON and TRUMAN 1985; SHIBANAKA *et al.* 1991), and the EGPs are phosphorylated in a cGMP-dependent manner in response to EH (MORTON and TRUMAN 1988b).

Genetic perturbations of the neuroendocrine pathway leading from the pacemaker to eclosion might cause at least two different selectable phenotypes: (1) *arrhythmic* behavior, in which there is no pacemaker control of eclosion, or (2) an alteration of *phase*, as a consequence of an effect on the daily timing of eclosion events. If such mutations are specific for the "eclosion output pathway," then they should alter the rhythmic pattern of eclosion but have no effect on the timing of locomotor activity. Such a selection should exclude mutations that perturb the pacemaker input (entrainment) pathway as well as those that alter the pacemaker itself (these types of lesions are expected to affect both activity and eclosion).

We have employed a transposon (*P* element) mutagenesis strategy to generate mutations affecting the

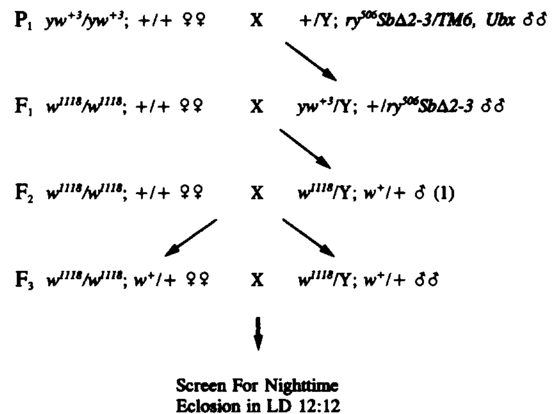


FIGURE 1.—Crossing scheme for the generation of *P* element-bearing autosomes. In this scheme, a single *X*-linked *P-lacW* (w^{+3}) element was mobilized using the transposase activity of the Δ2-3 chromosome. The chromosome balancer *In(3LR)TM6, Ubx* (TM6,Ubx) was carried by the P_1 males. In the F_1 generation, crosses of two dysgenic males to several w^{1118} females were set up in individual vials. Single w^+ males bearing new autosomal insertions were selected from the F^2 progeny of each vial, and used to establish *P* element-bearing autosomal lines.

Drosophila circadian system. Importantly, the manner in which our screen was conducted does not exclude mutations of essential genes whose products are also required for the timing of rhythmic events (see MATERIALS AND METHODS). This report describes the analysis of a new Drosophila mutant that might identify one element of the output pathway mediating the pacemaker control of eclosion.

MATERIALS AND METHODS

Stocks and culture conditions: Drosophila strains were reared in LD 12:12 at 24° and approximately 60% humidity on a standard cornmeal/sugar medium (NEWBY *et al.* 1991). The marker mutations and chromosome rearrangements used in this study have been described (LINDSLEY and ZIMM 1992).

***P*-element mutagenesis:** At least two arrhythmic variants of Drosophila as well as several mutations which alter the daily timing of eclosion have been mapped to the autosomal genome (JACKSON 1983; M. YOUNG, unpublished data; P. HARDIN, unpublished data). For this reason, we sought autosomal insertions that result in eclosion timing phenotypes. As illustrated in Figure 1, a single *X*-linked copy of *P-lacW* (BIER *et al.* 1989) was mobilized to search for autosomal insertion mutations. *P-lacW* is a *P* element-*lacZ* reporter construct containing a *white*$^+$ (w^+) minigene, a bacterial origin of replication, and the β-lactamase gene encoding ampicillin resistance. For our mutagenesis, we used *P*[ry^+ Δ*2-3*](99B) (or Δ2-3), a *P* element that produces high levels of transposase in the germline and soma but cannot excise from its third chromosome location (ROBERTSON *et al.* 1988). Two dysgenic F_1 males were mated to several w^{1118} females in individual vials to generate lines for screening. A single F_2 *white*$^+$ (w^+) male was selected from each F_1 cross to prevent multiple recoveries of the same insertion event.

Since most Drosophila circadian rhythm mutations have semidominant effects on eclosion rhythms, we did not attempt to produce lines for screening that were homozygous

for individual autosomal inserts (although unless the insertion was lethal, the lines usually contained homozygotes). We emphasize, therefore, that this genetic screen does not exclude insertions into essential genes that might have semidominant effects on rhythmicity. This is important, because the products of "rhythm" genes might actually execute essential functions during development or in the adult.

Selection of mutants and analysis of eclosion rhythms: The pacemaker control of eclosion is precise; in LD 12:12, individuals in a wild-type population eclose within an approximate 6-hr interval at the beginning of the photoperiod (Jackson 1983). This has greatly facilitated efforts to select eclosion-rhythm mutants. In the present studies, Drosophila strains carrying new *P-lacW* insertions were reared in vials at 18° while entrained to LD 12:12. To select for rhythm mutants, the eclosion behavior of mature pupal populations was monitored in an abbreviated manner over a 2-day period of time. On day 1, adults were cleared from the vials 2 hr prior to the lights-off signal. Forty-fifty minutes prior to the lights-on signal the next day, the vials were observed in dim red light (5–10 W with a Kodak GBX-2 filter) to which the circadian pacemaker is insensitive (Zimmerman and Ives 1971). In such conditions, there was little or no eclosion of wild-type adults during the late subjective day or subjective night. The same was true of the $y\ w^{+3}$ and Δ2-3 parental strains used in the present mutagenesis (data not shown). Therefore, vials in which a significant number of adults (at least 10–20 flies out of a daily total of ca. 50) emerged late in the day or during the subjective night were retained as candidate eclosion-rhythm mutants. The behavior of these candidate variants was then more completely characterized (in larger pupal populations) by counting eclosion events at 2-hr intervals over several days (Newby *et al.* 1991). Since X^2-periodogram analysis (see next section) is not sensitive enough to detect rhythms in eclosion (Dushay *et al.* 1990; Newby *et al.* 1991), the data were analyzed for rhythmicity using the Very Efficient Spectral Analysis (VESTAL) program (Runnel, Lee and Halberg 1974).

Locomotor activity rhythms: Our methods for monitoring locomotor activity in single flies have been described (Newby *et al.* 1991; Newby and Jackson 1991). In the present studies, we used the previously described Drosophila activity monitors (Newby and Jackson 1991) or monitors produced by Trikinetics (Waltham, Mass.). For a description of the Trikinetics monitors, see Ashburner (1989a). Prior to activity measurements, adults were entrained for 3–5 days in LD 12:12 at 22°, and then loaded into 5-mm² glass tubes containing a small amount of Drosophila medium. Each glass tube was placed in a monitor such that it bisected the infrared light beam which detects activity events. Activity data were collected for 6–10 days using an Apple IIe- or Macintosh-based collection system and then uploaded to a VAX/VMS 3900 mainframe computer for further analysis. The phases of activity peaks in LD 12:12 were determined using analytical phase-analysis software described in Hamblen-Coyle *et al.* (1992). The phase results shown in Table 1 are from an analysis of 9 days of data for each type of fly. Average circadian periods were estimated from data collected in continuous darkness and constant temperature using a X^2-periodogram program (Sulzman 1982) implemented on a VAX/VMS 3900 mainframe computer. These and other VAX/VMS rhythm analysis programs were gifts of David Wheeler and Jeffrey Hall (Brandeis University).

***P*-element excision studies:** Excisions of the *lark P-lacW* insertion were initially obtained by mobilizing the element according to the scheme depicted in Figure 2, using the transposase activity of the Δ2-3 chromosome. Males and

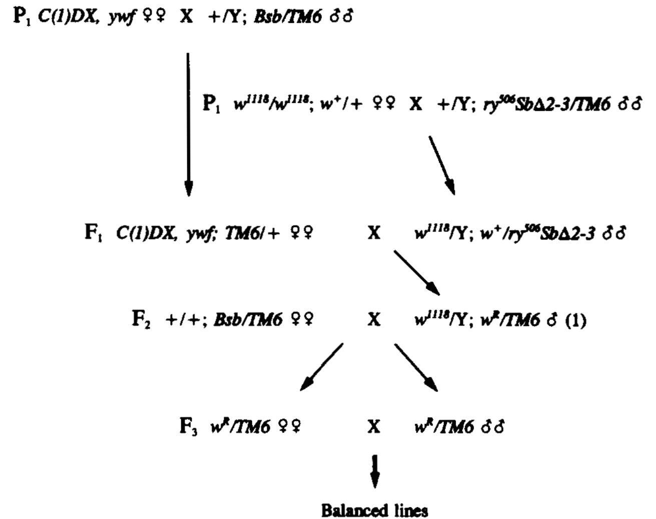


Figure 2.—*P* element excision scheme for the production of *lark* lethal revertants. Excisions of the *lark P-lacW* insertion were generated using the transposase activity of Δ2-3. Individual white-eyed revertants (w^R) were selected from the F_2 progeny of each vial cross, and mated to *Blunt short bristles* (*Bsb*)/TM6 females to establish balanced stocks.

females in these crosses carried a *white* (*w*) mutation so that loss of the *P-lacW* w^+ element could be observed. F_1 males bearing both the *P-lacW* w^+ insertion and Δ2-3 were mated to females carrying attached-*X* [*C(1)DX, ywf*] chromosomes and the third chromosome balancer TM6 (marked with *Ubx*). Single white-eyed F_2 male progeny (one per vial) were collected as presumptive *P*-element revertants and mated to females carrying the dominant marker *Blunt short bristles* (*Bsb*) and the TM6 balancer. Balanced stocks were generated from the progeny of these crosses. In later excision studies, putative F_2 revertant males were mated directly to *lark/In(3L)TM3* females to identify chromosomes that were lethal in trans to the *P*-induced *lark* mutation.

DNA and RNA analysis: To isolate sequences adjacent to the *P-lacW* insertion of *lark* mutants, a novel 12-kb, *P*-element-containing, *Bam*HI fragment was cloned from *P-lacW/+* genomic DNA (see Figure 5). This fragment was predicted to include the entire *P-lacW* element (minus some polylinker sequence) as well as approximately 1.3-kb of flanking genomic DNA. It was cloned by fractionating *Bam*HI-digested *P-lacW/+* genomic DNA from the *lark* strain through low-melting agarose, extracting a size class of DNA that included the 12-kb fragment and ligating it to *Bam*HI-cut, phosphatased bacteriophage λ GEM-11 arms. DNA was packaged *in vitro* and the phage were infected into *Escherichia coli* LE392. The recombinant phages in this size-selected "minilibrary" were identified by plaque hybridization using radiolabeled *P*-element and *P-lacW* probes. Altogether, 37 phage clones containing inserts that hybridize to both *P* element and *P-lacW* sequences were isolated. One of these known as λB2 has been characterized in detail. The insert includes a 1.3-kb *Hind*III-*Bam*HI fragment that is mostly flanking Drosophila genomic DNA. A partial sequence analysis of this fragment, however, has demonstrated the presence of DNA derived from *P-lacW*, including the 31-bp terminal repeat diagnostic of *P*-element transposons.

The 1.3-kb *Hind*III-*Bam*HI fragment of λB2 was radiolabeled with ^{32}P, and used as a probe to isolate larger, wild-type genomic inserts from a bacteriophage λCharon 4A/Drosophila library (Maniatis *et al.* 1978). Two overlapping genomic inserts, designated λ2C and λ3-1 were obtained

and mapped with restriction endonucleases. These clones encompass a 25-kb genomic interval that spans the site of *P-lacW* insertion (see Figure 7).

For the analysis of genomic DNAs, Southern blotting was performed according to standard procedures (SAMBROOK, FRITSCH and MANIATIS 1989) using GeneScreen-Plus (NEN) membrane. The blots were hybridized (3–5 × 10^6 cpm/ml buffer) and washed using conditions of high stringency (hybridization in 5X SSC and 50% formamide at 37–42°, and washing in 0.1X SSC at 60–65°). For Northern analysis, Drosophila RNA was extracted by a standard method (CHOMCZYNSKI and SACCHI 1987) and enriched for poly(A)+ transcripts using oligo(dT)-cellulose chromatography (SAMBROOK, FRITSCH and MANIATIS 1989). RNA blots were prepared and hybridized to DNA or cRNA probes as previously described (JACKSON, NEWBY and KULKARNI 1990).

β-galactosidase staining and immunohistochemistry: β-galactosidase (β-gal) staining of fixed, permeabilized embryos was carried out according to protocol 76 of ASHBURNER (1989b) using the substrate X-gal at a final concentration of 0.2%. Hand dissected late-stage pupae (pharate adults) were frozen in OCT compound and cut into 10-μm sections on a Reichert-Jung cryostat to examine the spatial distribution of the P transposase-β-gal fusion protein. A purified mouse anti-*E. coli* β-gal serum (Promega) was employed at a dilution of 1:500 for these studies. Localization of the primary antibody was revealed using a sheep anti-mouse Ig (Amersham) conjugated to horse radish peroxidase (HRP) at a dilution of 1:300. HRP staining reactions included 0.7 mg/ml diaminobenzidine and 1.6 mg/ml urea hydrogen peroxide in 60 mM Tris-HCl.

Tissue and chromosome *in situ* hybridizations: Chromosome *in situ* hybridizations with biotin-14-dATP-labeled DNA probes were carried out exactly as previously described (ENGELS *et al.* 1986) using DNA labeling and detection kits from BRL.

Tissue *in situ* hybridization to whole embryos was performed using a standard protocol (TAUTZ and PFEIFLE 1989) but with longer and a greater number of post-hybridization washes (C. S. HONG and B. GANETZKY, unpublished data). Embryos were dechorionated in 50% bleach and rinsed thoroughly with tap water. They were then fixed while shaking for 20–30 min in a mixture of heptane and 10% formaldehyde, PBS and 50 mM EGTA. Embryos were removed from the aqueous phase and devitellinized by adding a large volume of methanol and shaking vigorously for several min. They were further rinsed with methanol and 100% ethanol before being stored at −20°. Prior to hybridization, the embryos were rinsed once in 50% ethanol/50% PBT and three times in PBT. After these treatments, they were fixed again in 5% formaldehyde/PBS for 20 min, and then for 20 min more in 5% formaldehyde/0.6% Triton X-100/PBS. After three additional 5-min washes in PBT, they were digested with 50 μg/ml proteinase K (BMB) for 3–5 min at room temperature. The proteinase K treatment was terminated by rinsing twice with 2 mg/ml glycine in PBT, followed by three rinses in PBT. They were subsequently fixed again with 5% formaldehyde and 0.2% gluteraldehyde in PBS for 15 min, and then rinsed five times (5 min each) in PBT.

The hybridizations were begun by washing the embryos in a 50:50 mix of PBT and hybridization solution consisting of 50% formamide, 5X SSC, 100 μg/ml salmon sperm DNA, 200 μg/ml tRNA and 0.1% Tween 20. The embryos were then prehybridized at 47° for 1 hr in the same hybridization solution. They were hybridized in a microfuge tube on a rocking shaker for 24–36 hr at the same temperature with about 0.1 μg/ml heat denatured, digoxygenin-labeled probe. DNA probes were prepared using a BMB labeling kit according to the suggested protocol. The labeling reactions, however, were continued for 1 hr at 15° and then for an additional 3 hr at 20°. Labeled DNA fragments were precipitated with 100% ethanol, washed in 70% ethanol, resuspended in hybridization solution, and denatured by boiling 5 min before use.

After hybridization, embryos were washed at 47° for 30 min in hybridization solution, and then again for 30 min in 50% hybridization solution/50% PBT. Following this treatment, they were washed extensively (8 washes for 1 hr each) in 1 ml of PBT, and incubated while rocking at room temperature for 1 hr in 1 ml of a 1:2000 dilution of phosphatase conjugated anti-digoxygenin antibody (BMB), which had previously been preabsorbed against a large volume of fixed embryos. After the antibody incubation, the embryos were briefly washed three times with PBT, three times more (for 1 hr each) and finally rocked gently overnight in PBT. Enzymatic detection of phosphatase was carried out using the BMB detection kit exactly according to the manufacturer's protocol in the presence of 1 mM levamisol (Sigma). The embryos were then rinsed several times in PBT, once in 40% glycerol in PBT for 30 min and finally twice in 80% glycerol/0.1 M NaCl. They were viewed using a Zeiss Axioskop and DIC optics.

RESULTS

Phenotypes of the *lark* mutant: We mobilized an *X*-linked insertion of the *P*-element vector *P-lacW* (BIER *et al.* 1989) in order to identify eclosion-rhythm mutants. Eclosion was characterized in strains derived from 1063 independent autosomal insertions of *P-lacW* (see MATERIALS AND METHODS for a description of genetic crosses and the selection of eclosion-rhythm mutants). Of about a half dozen initial candidates, one strain exhibited a significantly altered eclosion profile while entrained to a light/dark (LD 12:12) cycle. In this strain, the daily peaks of eclosion for flies bearing an autosomal *P-lacW* insertion [white$^+$ (w^+) in eye color] occurred abnormally early in the cycle (essentially during the subjective night) relative to white-eyed (w) siblings that did not carry the *P*-element insertion. One representative profile for the insertion-bearing population is shown in Figure 3A. This "early-eclosion" phenotype is very reproducible and was observed during every cycle of three independent experiments (for a total of ten days altogether). The strain carrying this *P-lacW* insert has been designated *lark*, because of the early-eclosion phenotype.

lark populations also exhibit an early-eclosion phenotype while entrained to a temperature cycle. Figure 3B shows a comparison of the daily eclosion profiles for *P-lacW*-bearing (w^+) and w sibling populations that were maintained in constant light (LL) and a temperature cycle consisting of 12 hr at 25° and 12 hr at 18° (temperature entrainment of the Drosophila eclosion rhythm is described in ZIMMERMAN, PITTENDRIGH and PAVLIDIS 1968). In these conditions, the eclosion of the w^+ population is early relative to w siblings, similar to the results obtained in the previous

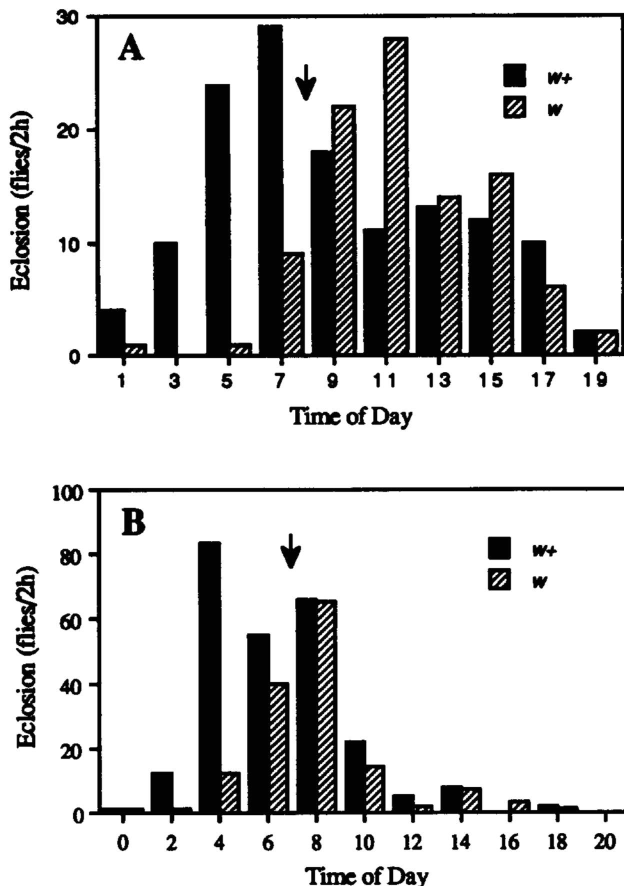


FIGURE 3.—Early eclosion phenotype of the *lark*/+ strain. The two populations represent siblings, only one of which (w^+) is heterozygous for a *P-lacW* insert on the third chromosome. The *w* siblings, which have a normal eclosion profile, do not carry the *P-lacW* insertion. Panels A and B illustrate eclosion behavior during entrainment to an LD 12:12 light/dark cycle and a 12:12 temperature cycle (18°:24°), respectively.

LD entrainment studies. Although Figure 3B shows only one (day 2) of the two days of data collected in this experiment, the eclosion of w^+ progeny was similarly early on day 1. On both days of this experiment, however, there was an apparent temperature-induced increase in eclosion events at about 8 a.m. (the time of the step-up in temperature during the cycle). This results in an additional minor peak for the w^+ population at this time of day (Figure 3B). Nonetheless, as similar early eclosion profiles are seen in both LD and temperature cycles, it seems unlikely that the mutant phenotype is due to an alteration of the pacemaker light input pathway.

Locomotor activity rhythms were characterized for the *lark* strain to determine if the phasing of activity was also abnormal in *P* element-bearing individuals. In three independent experiments, the timing of activity was similar in w^+ (*lark*/+) and *w* individuals; on average, the peaks for both the morning and the evening activity bouts occurred within approximately 1 bin (30 min) of one another. The results for one experiment are shown in Table 1. This experiment

TABLE 1

Phases of peak activity for *lark*/+, +/+ siblings, and wild-type flies

Genotype	Evening phase (Bin no. ± SEM)[a]	N[b]	Morning phase (Bin no. ± SEM)	N
+/+ (wild type)	23.9 ± 0.1	34	2.1 ± 0.2	24
lark/+	23.7 ± 0.1	22	2.5 ± 0.1	22
+/+ (white)	25.3 ± 0.1	25	2.1 ± 0.1	25

[a] Peak phase is expressed as the bin number (30-min internal) ± standard error in which activity levels are highest. Bin 1 corresponds to lights-on.

[b] *N* = the number of flies in which distinct morning or evening bouts of activity could be discerned. In many strains, including the wild type, the morning bout of activity is not as coherent as the evening bout.

also included wild-type Canton-S individuals for which peaks of activity were indistinguishable from those of *lark*/+ flies (Table 1). Thus, the *lark P-lacW* insertion has little or no effect on the timing of activity bouts.

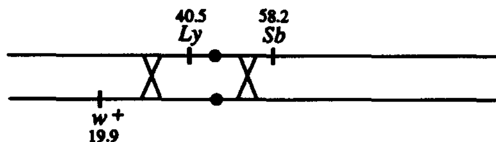
Since the aberrant phasing of eclosion events could, in theory, result from a change in pacemaker properties (*e.g.*, see HAMBLEN-COYLE *et al.* 1992), we characterized free-running circadian periods for the eclosion rhythm in *P* element-bearing (w^+) and *w* sibling populations of the *lark* strain. Eclosion events were monitored at 2-hr intervals in continuous darkness and a constant temperature of 18° for 5 days (see NEWBY *et al.* 1991). The average free-running circadian period (τ), as determined by a VESTAL analysis (see MATERIALS AND METHODS), was 23.5 hr for both populations. Similarly, individual *P-lacW*-bearing *lark*/+ flies also express locomotor activity with a normal circadian periodicity (τ=24.1 ± 0.06 h, n = 15). Thus, the only obvious behavioral defect of the *lark* strain is an abnormal daily phasing of eclosion.

We noticed that all of the individuals bearing *P-lacW* in the *lark* strain had similar eye color. This is significant, because usually it is possible to distinguish between heterozygotes and homozygotes carrying *P-lacW* based on the dosage of the w^+ gene that is carried as a marker by this *P*-element construct. Therefore, we suspected that the insertion might be associated with recessive lethality, but have a dominant effect on eclosion behavior. Subsequent genetic crosses showed that a lethal was associated with the *P-lacW* (w^+-bearing) chromosome. When presumed heterozygous w^+ flies from the *lark* strain were crossed *inter se*, the resulting ratio of w^+:*w* adults was approximately 2:1, consistent with the inheritance of a recessive, autosomal lethal factor (in two independent crosses, ratios of 417:213 and 618:298 were observed). Thus, in all respects, the *lark* mutation behaves as a recessive lethal allele that has dominant or semidominant effects on the timing of eclosion when it is carried in viable *lark*/+ heterozygotes.

Mapping the *lark* insertion: The *P-lacW* element of the *lark* strain was mapped to chromosome *3* by

A Cross 1: *w/w; w+/Ly Sb* X *w/Y; +/+*

	Parental Types		Recombinant Progeny					
	w+	Ly Sb	w+ Ly Sb	±	w+ Sb	Ly	w+ Ly	Sb
Night	721	302	171	93	119	55	11	3
Day	276	620	75	170	25	69	1	29



B Cross 2: *w/w; w+/R D* X *w/Y; +/+*

	Parental Types		Recombinant Progeny					
	w+	R D	w+R	D	w+D	R	w+R D	±
Night	918	138	141	103	244	48	40	10
Day	83	468	34	117	51	154	10	95

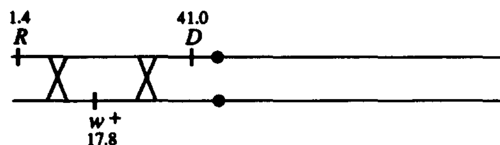


FIGURE 4.—Recombination mapping of the w^+ element and behavioral phenotype of the *lark* mutant. In both crosses (A and B), progeny were collected at approximately 1 hr before lights-on and again at 2 hr prior to lights-off each day of the experiment; the progeny numbers shown in panels A and B are pooled data from 9 days of eclosion. The visible phenotypes of recombinant progeny were scored in order to determine the map position of the *P-lacW* w^+ element. The temporal distribution of eclosion in the various progeny classes demonstrated that the early-eclosion phenotype of the *lark* mutant segregated with the w^+ element.

following the segregation of the w^+ phenotype and the dominant markers *Cy* (second) and *Ubx* (third) in a standard linkage analysis. Such crosses demonstrated coincident chromosomal inheritance patterns for the *P-lacW* w^+ element and the factors responsible for lethality and abnormal phase. In "balanced" *lark/In(3LR)TM6* (TM6) stocks (established by two generations of outcrossing), the *lark* w^+ third chromosome is lethal and associated with altered behavior (not shown).

Recombination mapping of the *P-lacW* w^+ factor was accomplished using the third chromosome dominant markers *Roughened* (*R*; 3-1.4), *Lyra* (*Ly*; 3-40.5), *Dichaete* (*D*; 3-41.0), and *Stubble* (3-58.2). The mapping was performed in the presence of a *w* mutation (w^{1118}) so as to be able to observe the segregation of the w^+ element associated with *P-lacW*. The results from these two crosses are shown in Figure 4. An initial cross with a doubly marked chromosome bearing *Ly* and *Sb* mapped the factor to the left of *Ly* at position 19.9. A subsequent cross with an *R D* third chromosome placed it at map position 17.8. These data demonstrate that the w^+ gene carried by *P-lacW* maps between positions 17.8 and 19.9 on the left arm of chromosome *3*. The estimate of 17.8 might be a more accurate value, since it was derived from a cross in which the markers flanked the *P-lacW* insert. This map position is in reasonable agreement with the cytogenetic location for the *lark* gene that was determined from chromosome *in situ* hybridization (see below).

Importantly, the same recombination data show that the early-eclosion phenotype cosegregates with the w^+ marker of the *P-lacW* insert. When recombinant progeny were classified according to whether they eclosed during the dark (2 hr before lights off until 1 hr before lights on) or during the light (1 hr before lights on until 2 hr before lights off), there was a marked difference in the eclosion profiles of the w^+ and *w* parental types and reciprocal recombinants

(Figure 4). Recombinants from either cross that carried *P-lacW* (and the w^+ gene) preferentially eclosed early and during the dark portion of the cycle. This behavior is indistinguishable from that of *P-lacW*-bearing flies of the *lark* strain.

Individual recombinants from the *R D* mapping experiment were also crossed to *lark*, w^+/TM6 flies to be certain that the factor causing lethality segregated with the *P-lacW* w^+ element. *R* w^+ or w^+ *D* recombinant chromosomes, in all cases (15 of each), produced lethality in trans to a *lark* w^+ homolog. In contrast, *D* or *R* recombinant chromosomes (15 of each) that lacked the *P-lacW* w^+ insert produced viable progeny when combined with a *lark* w^+ homolog. These results together with the *P*-element excision studies described below indicate that a single insertion of *P-lacW* causes both the behavioral and lethal phenotypes of the *lark* mutant.

Reversion of the *lark* phenotypes by *P*-element excision: We also conducted a *P*-element excision analysis to confirm that an insertion of *P-lacW* caused the behavioral and lethal phenotypes of the *lark* strain. Excisions of the *lark* *P-lacW* element were selected as described in MATERIALS AND METHODS to determine if loss of the element was associated with a reversion to wild type. From a total of 16 characterized excision events, 15 simultaneously restored normal viability and eclosion behavior. In contrast, a single lethal revertant also had abnormal eclosion as judged by the number of adults emerging in the night *vs.* the day portions of the cycle. The behavior of strains carrying other excision-induced lethal alleles also indicates that a single insertion event causes both of the *lark* mutant phenotypes (see below).

New lethal *lark* alleles and eclosion behavior: To identify new mutations that were likely to be null alleles, we generated additional lethal revertants of the *lark* *P-lacW* insertion. Using the previously described genetic scheme, 279 excision events were obtained; six of these revertants were lethal when homozygous or in combination with the original *lark* *P-lacW* insertion. Strains heterozygous for any one of these six revertant chromosomes (R/TM3) exhibit an early-eclosion phenotype as judged by comparing eclosion during the two portions of an LD 12:12 cycle (Table 2). In the same experiment, however, flies of the control w^{1118} strain were indistinguishable from the wild type, emerging almost entirely within the day portion of the cycle. These results indicate that the behavioral alteration of *lark* heterozygotes is due to a decrease in the amount of normal gene product (50% of normal), whereas lethality probably represents the null phenotype.

The *lark* *P-lacW* insertion is lethal during embryogenesis: Embryos were collected from the cross of w^{1118}/Y; *P-lacW* w^+/+ males to w^{1118}/w^{1118}; *P-lacW* w^+/+ females to determine when during development lethality occurred in the homozygous *P-lacW*-bearing individuals. From a total of 538 embryos, 407 (75.7%) hatched into first instar larvae whereas 131 (24.3%) were arrested during embryogenesis. The frequency of developmentally arrested embryos is close to the value expected (25%) for a recessive lethal factor. We conclude, therefore, that most or all of the lethality associated with the *lark* *P-lacW* insertion occurs during embryogenesis. More detailed observations of unhatched embryos revealed that lethality occurred at stage 12 of embryogenesis (approximately 7–9 hr into development). In greater than 90% of the arrested embryos, internal morphology was almost unrecognizable and the developing nervous system was severely disorganized as visualized by anti-horse radish peroxidase (anti-HRP) immunostaining (not shown).

TABLE 2

Eclosion of *lark* lethal revertants

Strain	Eclosion events[a]	
	Night	Day
w^{1118}	3	34
R1	27	16
R2	54	17
R3[b]	15	14
R4	22	4
R5	34	15
R6[b]	20	19

[a] Night and day were defined as 7 p.m.–7 a.m. and 7 a.m.–7 p.m., respectively, in an LD12:12 cycle in which lights-on occurred at 8 a.m.

[b] For R3 and R6, eclosion events were counted at 6 a.m. and 7 p.m.; thus, the night portion of the cycle was 7 p.m.–6 a.m. and day was 6 a.m.–7 p.m.

Isolation of genomic DNA spanning the *lark* *P-lacW* insertion: A single *X*-linked copy of *P-lacW* was mobilized in our *P*-element mutagenesis. Therefore, the expectation was that only one insert (or at most a few) would be present on the *lark* mutant third chromosome. A Southern blot analysis was entirely consistent with this expectation; *i.e.*, a labeled *P*-element plasmid (pπ25.1) detected a single novel fragment per restriction enzyme digest in genomic DNA of the *P-lacW*-bearing w^+ flies. The hybridization of this probe to two different digests of w^+ or w genomic DNA is shown if Figure 5A. Novel bands present in w^+ DNA are indicated by arrows; the other bands detected in both w and w^+ DNA are a result of sequences in the pπ25.1 plasmid that are known to be derived from cytogenetic region 17C (SPRADLING and RUBIN 1982). Further analysis demonstrated that a *P-lacW* probe also detected novel fragments in genomic DNA from *lark* w^+ progeny and hybridized to a single cytogenetic interval (region 65EF) of chromosome *3L* (data not shown). These data thus strongly suggest that a single

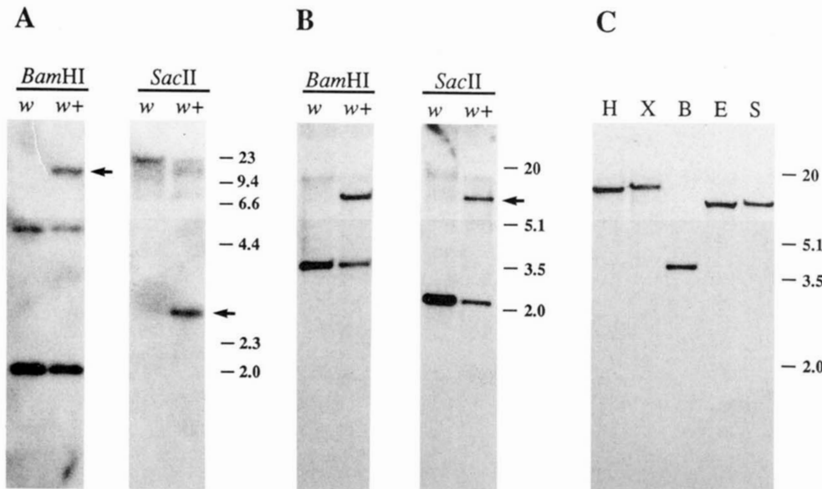


FIGURE 5.—Southern blot analysis of genomic DNAs from *w* (+/+) and w^+ (*lark*/+) sibling populations. Genomic DNAs were digested with the indicated enzymes and hybridized to radiolabeled pπ25.1 sequences (A) or the 1.3-kb *Hind*III-*Bam*HI fragment from the λB2 phage clone (B and C). Panels A and B illustrate hybridization to DNAs from *w* and w^+ progeny of the *lark* strain, whereas panel C shows hybridization to wild-type C-S DNA. Note the presence of single novel bands in each w^+ (*lark*/+) lane. In addition to *P* element sequences, the pπ25.1 plasmid clone contains a Drosophila genomic DNA fragment from chromosome region 17C (SPRADLING and RUBIN 1982), which as expected hybridizes to common bands in the *w* and w^+ lanes.

copy of *P-lacW* is present in the genome of the insert-bearing mutants.

Because the *P-lacW* construct carries a bacterial origin of replication and a β-lactamase gene encoding ampicillin resistance (BIER *et al.* 1989), it is theoretically possible to clone genomic DNA sequences flanking the site of insertion by direct plasmid rescue. Using this strategy and standard plasmid rescue techniques (PIRROTTA 1986), we obtained several clones from *lark* w^+ DNA that contained genomic DNA sequences. However, none of these genomic DNA clones spanned the *P-lacW* insertion site as judged by Southern blot analysis of mutant DNA even though some of them contained both *P* element and white-gene sequences. We have not determined why plasmid rescue failed for the *lark* strain.

As we were unable to utilize direct plasmid rescue techniques to isolate this *P-lacW* insert, we screened a size-selected library constructed from *lark* w^+ genomic DNA fragments with *P*-element and *P-lacW* probes to obtain appropriate clones (see MATERIALS AND METHODS for details of library construction and screening). A 12-kb novel *Bam*HI fragment (see Figure 5A) containing most of *P-lacW* and more than 1 kb of flanking Drosophila genomic DNA was obtained as a phage insert from this screen. This larger clone known as λB2 contains A 1.3-kb *Hind*III-*Bam*HI fragment which, based on the known structure of *P-lacW*, was predicted to include the very end of a *P*-element (including the 31-bp terminal repeat that characterizes this class of transposons) as well as flanking genomic sequences. This 1.3-kb fragment was subcloned and partially sequenced to confirm the presence of the *P*-element repeat. Moreover, a Southern blot analysis utilizing DNAs from both w^+ (*P-lacW*-containing) and *w* flies of the *lark* strain demonstrated that sequences had been cloned from a region adjacent to the site of *P-lacW* insertion. As shown in Figure 5B, labeled 1.3-kb sequences hybridized to novel fragments in w^+ DNA, as expected for a clone that originates from a region adjacent to the insertion (see arrows). The novel *Bam*HI band identifies the same 12-kb fragment as that detected by pπ25.1 plasmid sequences (see Figure 5A). In contrast, the novel *Sac*II bands detected by the pπ25.1 and the 1.3-kb probes are expected to differ in size, because they contain flanking sequences from opposite sides of the *P-lacW* insertion.

The same 1.3-kb probe detects single bands in each of five different restriction enzyme digests of wild-type DNA (Figure 5C), indicating that this region is uniquely represented in the Drosophila genome. Moreover, the sizes of the detected bands are consistent with the molecular map of the wild-type genomic interval (see Figure 6A). Chromosome *in situ* hybridizations have demonstrated that the 1.3-kb fragment contains sequences from cytogenetic region 65EF on chromosome *3L*, a position coincident with the previously determined location of the *P-lacW* insert in the *lark* strain.

DNA sequences from the λB2 clone were employed as a probe to screen a phage library containing larger wild-type Drosophila genomic DNA inserts. In this way, several overlapping clones spanning the site of *P-lacW* insertion were obtained. They were characterized by digestion with restriction endonucleases to derive the map shown in Figure 6A. Limited DNA sequencing of the *lark* insertion allele indicates that the *P-lacW* element resides precisely 59 bp to the left of the *Cla*I site depicted in this figure.

Transcription of sequences from the site of *P-lacW* insertion: Each of three different labeled DNA fragments from the region including the site of *P-lacW* insertion (depicted by rectangles in Figure 6A) hybridizes to a pair of poly(A)$^+$ mRNAs of approximately 1.3 and 1.8 kb that are transcribed in the same direction. As shown in Figure 6B, a labeled *Kpn*I-*Cla*I fragment detects both transcripts in RNA from em-

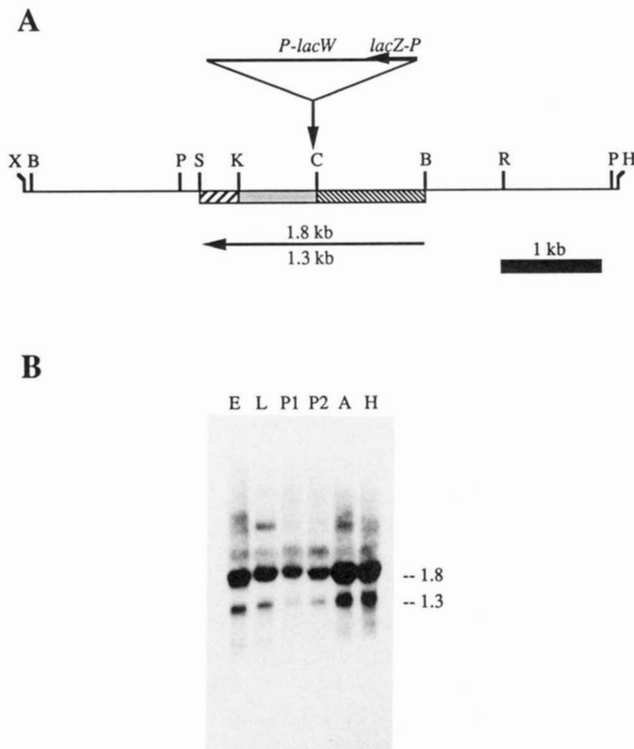


FIGURE 6.—Molecular map and transcription of the region encompassing the *lark P-lacW* insert. (A) Map of the region showing the position (vertical arrow) and orientation of the *P-lacW* insertion. The insert resides 59 bp to the left of the indicated *Cla*I site. The rectangles below the map show the positions of fragments used in tissue *in situ* and RNA blot analyses. The horizontal arrow below the restriction map of the locus indicates the direction of transcription for the two putative *lark* mRNAs. B = *Bam*HI; C = *Cla*I; H = *Hind*III; K = *Kpn*I; *P* = *Pst*I; R = *Eco*RI; Sal = *Sal*I; X = *Xho*I. (B) Northern blot analysis of staged poly(A)$^+$ RNAs using the *Kpn*I-*Cla*I fragment shown in panel A. This fragment and the others indicated in the top panel hybridize to mRNAs of 1.3 and 1.8 kb. The fainter bands seen in this photograph are not reproducibly detected with these probes.

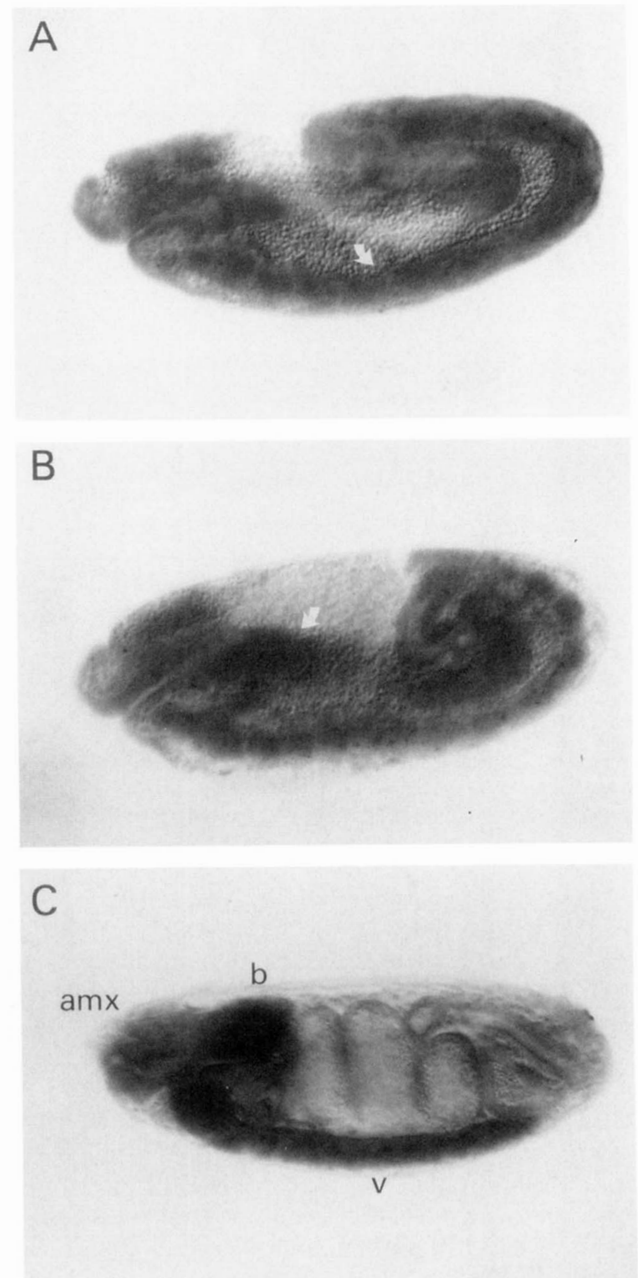


FIGURE 7.—Expression of putative *lark* transcripts. A population of 0–24 hr wild-type (C-S) embryos was hybridized to a digoxygenin-labeled *Kpn*I/*Cla*I fragment (see Figure 6). Anterior is to the left and dorsal is up. The three panels illustrate the hybridization patterns of stage 11 (A), stage 12 (B) and stage 16 (C) embryos. The arrows in panels A and B point to the ectoderm and part of the developing brain, respectively. amx, antenno-maxillary complex; b, brain; v, ventral nervous system.

bryos, third instar larvae, pupae, whole adults and adult head tissues. Because probes from both sides of the site of insertion detect these mRNAs, the *P-lacW* element must reside within this transcription unit. Furthermore, we did not detect abnormally sized transcripts in w^+ RNA (not shown). This result suggests that the elimination of one or both of the normal mRNAs is responsible for the *lark* mutant phenotypes.

Spatial expression of transcripts: Whole embryos *in situ* hybridizations were performed to determine the spatial distribution of transcripts in embryos. For these experiments, DNA fragments from either side of the *P-lacW* insertion site were employed as hybridization probes. A *Kpn*I-*Cla*I probe (see Figure 6A), for example, first detects transcripts at about embryonic stage 11 (CAMPOS-ORTEGA and HARTENSTEIN 1985), just prior to the time at which *lark* homozygotes die during development. At this stage, signal is evident throughout the ectoderm/neuroectoderm as well as in cephalic regions in the vicinity of the developing brain (Figure 7A). In stage 12 embryos (Figure 7B), expression can be detected in the brain, the developing ventral nervous system and to a lesser extent in cells associated with the developing gut. At later stages of development (stage 16, Figure 7C), expression is apparently restricted to cells of the brain, ventral nervous system and regions in the developing

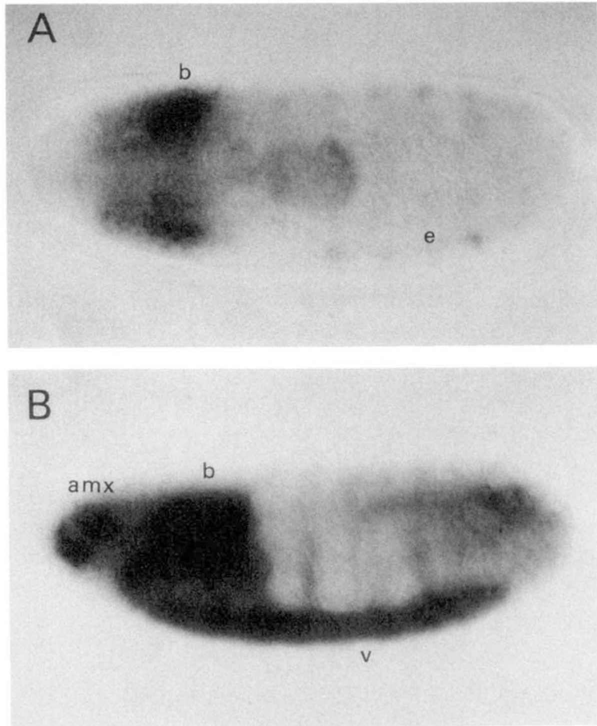


FIGURE 8.—β-galactosidase staining patterns of *lark*/+ embryos. Whole embryos were stained for β-gal activity using X-gal as substrate according to a standard procedure (ASHBURNER 1989b). (A) stage 10/11, (B) stage 16. e, ectoderm; other abbreviations are the same as in Figure 7.

head including those in the vicinity of an anterior sensory organ known as the antenno-maxillary complex. We did not detect signal within the peripheral nervous system.

The pattern of β-galactosidase activity in *lark* embryos: A major advantage of *P-lacW* and other enhancer-trap elements is the ability to characterize patterns of gene expression for identified loci. This is possible because the P transposase-lacZ fusion comes under the control of adjacent cis-acting regulatory elements. Given the orientation of the *P-lacW* insertion in the *lark* mutant (see Figure 6A), this gene fusion is situated near the 5′ end of the putative *lark* transcription unit, and perhaps close to cis regulatory sequences. Therefore, it is likely that the spatial distribution of β-galactosidase would reflect the normal pattern of *lark* gene expression. To determine whether β-gal staining might be a useful technique for studies of *lark* gene expression, we have characterized staining patterns in *lark*/+ embryos. In general, the pattern of β-gal staining is similar to the documented pattern of transcript distribution. Prominent staining, for example, is first seen in stage 10/11 embryos within the ectoderm/neuroectoderm as well as in cephalic regions (Figure 8A). In late stage embryos (Figure 8B), staining is almost entirely restricted to neural tissues including the brain, the ventral nervous system, and the antenno-maxillary complex (note that the nuclear localization of the β-gal stain is a consequence of a nuclear targeting signal present in the P element transposase-β-gal fusion of *P-lacW*). Because the spatial distributions of β-gal and *lark* transcripts are identical in late-stage embryos, we have employed the *lark* strain to characterize expression patterns at the pupal stage of development.

The distribution of β-galactosidase in *lark*/+ pharate adults: We were interested in examining the β-gal expression pattern in late-stage *lark*/+ pupae, since this is the developmental stage at which eclosion is regulated by the *Drosophila* circadian pacemaking system. Accordingly, we examined the distribution of the transposase-β-gal fusion protein in brains of pharate adults (*i.e.*, pupae in which adult development has been completed). The fusion protein was immunohistochemically detected (see MATERIALS AND METHODS) in frozen tissue sections of *lark*/TM6 (*i.e.*, *lark*/+) pharate adults that had been dissected from the pupal case. As shown in Figure 9A, immunoreactive product can be detected within several regions of the differentiated nervous system. For example, staining is observed in large cells located in medial (open white arrows) and lateral portions (solid white arrows) of the central nervous system (Figure 9, A and B). Staining is also seen in cells of the medulla (M) optic lobe (arrow heads, Figure 9) and in certain sections within cells of the optic lamina (L, Figure 9C). It is likely that some of the immunoreactive cells within the medulla and lamina are glia based on their size and positions. Finally, staining is evident within retinal (R) neurons associated with sensory bristles of the eye (white arrows, Figure 9D) but is not detectable in photoreceptor nuclei (black arrow, Figure 9D). In sections of w^{1118} pupae that were employed as controls, there was no detectable immunoreactivity.

DISCUSSION

A new *Drosophila melanogaster* eclosion-rhythm mutant has been isolated from a behavioral screen of approximately 1000 autosomal inserts of the *P-lacW* enhancer trap element. The *lark* mutation, which maps to the third chromosome, alters the phase of the eclosion rhythm but does not affect circadian period or the expression of the activity rhythm. Thus, it is a good candidate for a lesion specifically affecting the pacemaker output pathway mediating the timing of eclosion events. With respect to the behavioral phenotype, the *lark* mutant is similar to another Drosophila eclosion-rhythm variant known as *psi-3* (JACKSON 1983). The *psi-3* variant, which was chemically (ethyl methanesulfonate) induced, causes a similar early eclosion phenotype and maps to the third chromosome although it has not been precisely localized to a defined genetic interval. Unfortunately, the dominance of both *psi-3* and *lark* precludes a rigorous genetic

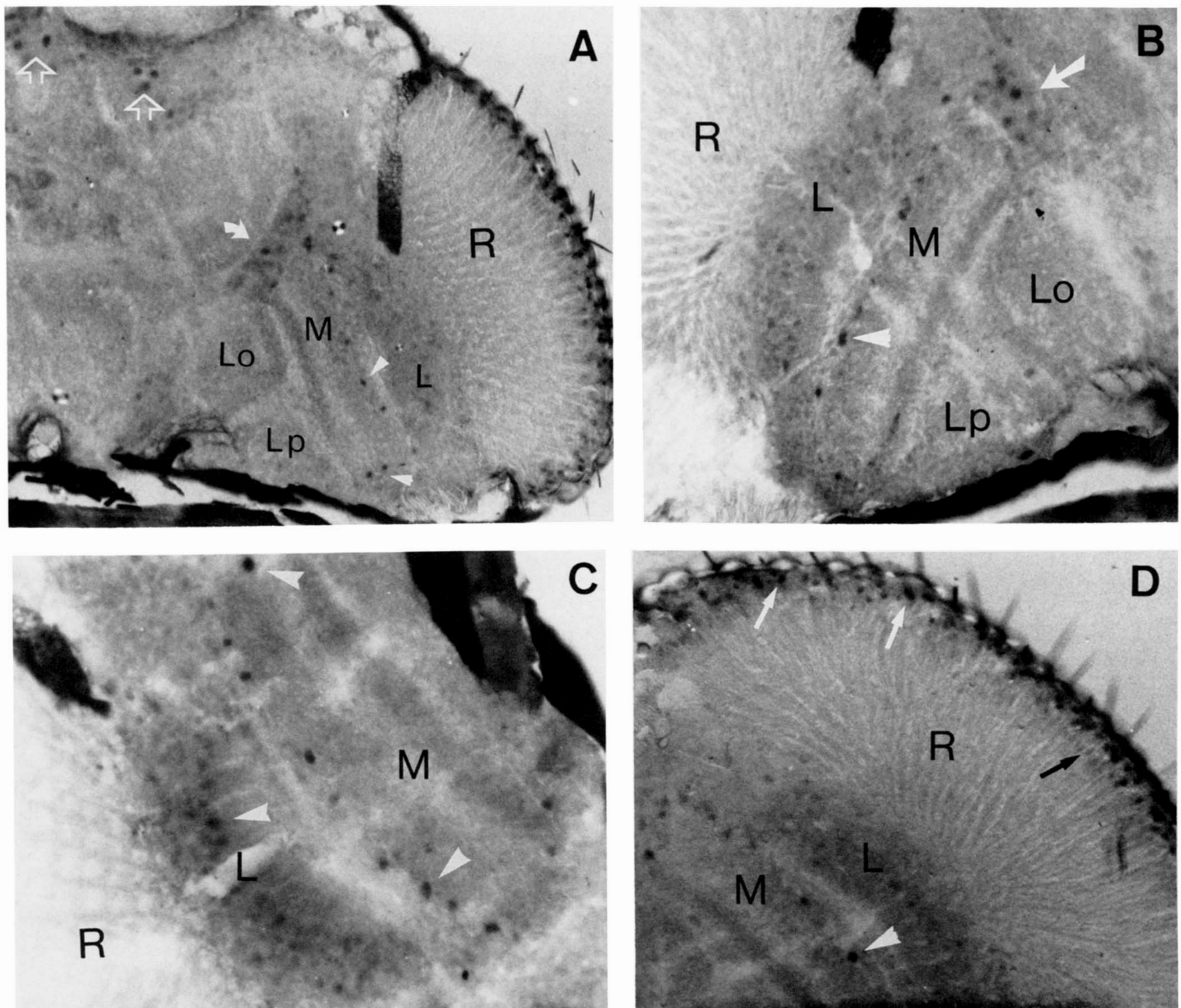


FIGURE 9.—Localization of the transposase-β-galactosidase fusion protein within the nervous system of pharate adults. All panels depict horizontal sections through heads of approximately 96-hr pupae. (A) Locations of immunoreactive cells in the optic lobes and central brain; although only the right half of the head is shown, immunostained cells are present bilaterally in the brain. (B) Higher magnification view of the left side of the head showing the optic lobes and lateral brain region. (C) Section in which stained cells can be observed in the optic lamina. (D) Staining of sensory neurons within the retina. L, lamina; M, medulla; Lo, lobula; Lp, lobula plate; R, retina. Open white arrows, cells in the ventral medial region of the central nervous system; solid white arrows, cells in the lateral nervous system (panels A and B) or in the retina (panel D); white arrow heads, cells in the medulla and lamina; black arrow, approximate position of photoreceptor 1–6 nuclei.

test of allelism on the basis of the behavioral phenotype. In contrast to *psi-3*, however, the *lark* mutation causes a recessive embryonic lethal phenotype in addition to its dominant effect on the daily timing of eclosion events. Thus, it is of interest that *lark*/*psi-3* transheterozygotes are completely viable (L. M. Newby, unpublished data). Although this result does not absolutely disprove allelism, the simplest interpretation is that the two mutations affect different genes. In either case, the *lark* mutation identifies an essential gene, and in this regard is unique among the rhythm mutations that have been characterized in four different organisms (Drosophila, Neurospora, Chlamydomonas and the hamster).

The behavioral phenotype of the *lark* mutant is consistent with a lesion affecting the endocrine regulation of eclosion. This idea is based in large part on the work of TRUMAN and his colleagues (see introductory section). As described earlier, it is known that both eclosion hormone and ecdysteroids are involved in the regulation of eclosion. Moreover, there are circadian rhythms in ecdysteroid synthesis and hemolymph titer during adult development of the insect *Rhodnius prolixus* (AMPLEFORD and STEEL 1985; VAFOPOULOU and STEEL 1991). Furthermore, TRUMAN

et al. (1983) have shown that injecting ecdysteroid into *Manduca sexta* pupae can shift eclosion to a later time of day; *i.e.*, an increase in endogenous ecdysteroid titers delays the eclosion gate. Based on these results, it is possible that a reduced response to hormone might lead to earlier eclosion, a phenotype characteristic of *lark* and other "early" Drosophila strains (PITTENDRIGH 1967; JACKSON 1983). This could occur because of a defect in hormone synthesis/release or because of an alteration in the cellular response to hormone. Perhaps certain components of the pupal β-gal expression pattern in *lark* pharate adults are neurosecretory cells or neural targets of an endocrine factor. The defects of *lark* mutants, however, need not necessarily involve the hormone ecdysteroid or its receptor. Additional members of the steroid receptor superfamily have been identified in Drosophila (reviewed in ORO *et al.* 1992), and some of these might bind to ecdysteroid derivatives or other novel ligands. Alternatively, the *lark* lesion could involve a completely different endocrine system (such as eclosion hormone) that is required for the regulation of eclosion.

Although the eclosion-rhythm phenotype of *lark* heterozygotes could be due to a dominant effect on embryonic neural development (see discussion of embryonic lethality below), we think it more likely that *lark* mutations perturb the neuroendocrine machinery of pupae which regulates adult eclosion. Consistent with this idea, the pupal distribution of β-gal in *lark*/+ pharate adults (late-stage pupae) suggests that the LARK product is present within cells of the central nervous system (CNS) as well as in the optic lobes and retina. Of particular interest are clusters of β-gal immunoreactive cells that were observed within the CNS in ventral medial and ventral lateral locations. Although the identity of these CNS cells has not been determined, they might correspond to neurosecretory cells, as such cell types are known to occur in large numbers in the insect brain (reviewed in RAABE 1982; see ZITNAN, SEHNAL and BRYANT 1993 for a recent description of Drosophila neurosecretory cells). The neurosecretory cells that produce eclosion hormone, for example, are also within the ventral medial CNS (HORODYSKI, RIDDIFORD and TRUMAN 1989; J. TRUMAN, unpublished data), in a location close to or coincident with cells expressing Lark protein. Additional immunolocalization experiments will be required to know if Lark protein is expressed in this neurosecretory cell type.

A ventral lateral group of brain cells which is β-gal positive in *lark*/+ pharate adults is close to a recently described set of lateral neurons that contain Period (PER) protein (SIWICKI *et al.* 1988; ZERR *et al.* 1990). As previously indicated, genetic and molecular evidence strongly indicates that the PER protein is a component of the *Drosophila* circadian pacemaker (KONOPKA and BENZER 1971; BAYLIES *et al.* 1993; HARDIN, HALL and ROSBASH 1993; JACKSON 1993). Similar to PER (EWER *et al.* 1992), the LARK product is also located within cells of the optic lamina and medulla. Thus, it will be of interest to determine if PER and LARK proteins are co-localized within certain neural cells.

Our genetic analysis indicates that the *P*-induced *lark* mutation is a loss-of-function allele; it is lethal when homozygous or when in combination with one of six other excision-generated lethals, some of which are probably null alleles. This is not an unexpected result, since the *P* insertion of *lark* mutants is apparently situated within transcribed sequences rather than in an adjacent cis-regulatory element. Thus, the dominant effect on eclosion behavior is probably due to a reduction of the wild-type gene dosage, rather than a hypermorphic or neomorphic mutation. Similar dosage effects are seen at the Drosophila *period* locus (SMITH and KONOPKA 1982) and are consistent with a model wherein the wild-type product becomes limiting even when present at half the normal amount. Although this behavioral phenotype could be due to diminished enzymatic activity, it seems more likely that such an effect of gene dosage is a consequence of reducing the level of a noncatalytic component of the eclosion output pathway. If the *lark* lesion does affect the response to an endocrine signal, then a possible candidate for the encoded product is a hormone receptor or a protein that interacts with such a receptor to determine activity. Members of the nuclear hormone receptor superfamily (which includes the ecdysteroid receptor), for example, are known to interact as heterodimers to regulate transcription (YAO *et al.* 1992; YOSHINAGA *et al.* 1992; CARLBERG *et al.* 1993).

RNA blot analysis indicates that the insertion of the *lark P-lacW* element interrupts a transcription unit which expresses two size classes of message. Furthermore, hybridization studies using multiple, contiguous genomic DNA probes demonstrate that these two classes of mRNA overlap to a great extent, and thus probably arise by alternative RNA splicing or by the use of distinct promoters or polyadenylation signals. We postulate that disruption of this transcription unit causes the mutant phenotypes observed in *lark* flies, although the formal proof of this supposition will require *P* element-mediated DNA transformation and rescue of these phenotypes. However, *in situ* hybridization analyses show that mRNAs from this transcription unit are first detected during embryogenesis just prior to the stage at which homozygous *lark* embryos become developmentally arrested. This result is consistent with a requirement for the product(s) of these mRNAs at this stage in embryonic development, and

suggests that this transcription unit encodes *lark* gene functions. Given the pleiotropic nature of the *lark* mutation, it is possible that the encoded function of each of these mRNA size classes corresponds to one of the two characterized phenotypes (embryonic lethality or eclosion rhythms). Alternatively, both of the transcript classes could mediate related essential functions in different cell types or tissues.

Although as yet we have not defined the encoded products of these transcripts, they are apparently expressed within the nervous systems of embryos and pharate adults. In light of the embryonic expression pattern, it is of interest to speculate about the lethal phenotype of *lark* homozygotes. In such individuals, development is arrested at about stage 12, or approximately 7–9 hr into embryogenesis. At this stage of development, germ band retraction occurs and the ventral nerve cord becomes morphologically distinct from the epidermis (CAMPOS-ORTEGA and HARTENSTEIN 1985). Interestingly, there is also a major peak of ecdysteroid hormone at this stage (ASHBURNER 1989a) and ecdysteroid receptors are present (J. TRUMAN, unpublished data), indicating the probable importance of endocrine regulation in embryonic development. It is also known that a wave of programed cell death begins at embryonic stage 11, and prominent cell death is evident several hours later (stage 15/16) within the developing nervous system (ABRAMS *et al.* 1993). Perhaps this program of cell death is initiated in response to endocrine cues, similar to other types of naturally occurring cell death in insects (reviewed in SCHWARTZ 1992; TRUMAN, THORN and ROBINOW 1992). Thus, it is an intriguing thought that the *lark* gene product is generally important for the cellular response of neural tissue to an endocrine signal (ecdysteroid or perhaps some other hormone). This hypothesis is entirely consistent with the pleiotropic effects of the *lark* mutation.

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