Expression of a Drosophila mRNA is under circadian clock control during pupation

LORI J. LORENZ\textsuperscript{1,2}, JEFFREY C. HALL\textsuperscript{1} and MICHAEL ROSBASH\textsuperscript{1,2}

\textsuperscript{1}Department of Biology and \textsuperscript{2}Howard Hughes Medical Institute, Brandeis University, Waltham, Massachusetts 02254, USA

Summary

Rhythmic eclosion of Drosophila adults requires per gene function. We have found that a previously identified 0.9 kb RNA transcribed from DNA adjacent to per becomes abundantly expressed during pupation, just prior to eclosion. The daily synchronized emergence of young adults, coupled with a subsequent rapid decay of the transcript, is responsible for what previously appeared to be cycling of the 0.9 kb RNA in adults. \textit{In situ} hybridization analyses localize the 0.9 kb transcript to the epidermis of newly eclosed adults. Conceptual translation of genomic DNA and cDNA sequences predicts that the 0.9 kb transcript produces a 261 amino acid protein containing a putative signal sequence for membrane transport at its amino terminus. Pupae that reach the same stage of development at slightly different times of day show a subsequent synchronized rise in 0.9 kb RNA levels, indicating that the expression of this transcript is under circadian clock control.

Key words: Drosophila melanogaster, eclosion rhythm, per, developmental gating, \textit{in situ} hybridization, epidermis, signal sequence.

Introduction

Homometabolous insects such as Drosophila manifest dramatic changes throughout development. They pass through an embryonic stage, undergo rapid growth during multiple larval instars, experience extensive metamorphosis during pupation, and finally eclose from the pupal case as adults. The timing of eclosion follows the circadian dictates of an endogenous biological clock which is entrained by environmental cues and which continues to function in the absence of persistent external input (reviewed by Saunders, 1982). During light:dark conditions, adult emergence is 'gated' or largely restricted to the early part of the light phase of the circadian cycle; the resulting daily peak in eclosion constitutes the \textit{Drosophila} eclosion rhythm.

This eclosion rhythm, as well as the circadian activity rhythm and a short-period rhythm of the male courtship song, is influenced by the \textit{period (per)} gene. Mutations at this locus cause virtual arrhythmicity or aberrant timing of these behaviors (Konopka and Benzer, 1971; Kyriacou and Hall, 1980; Smith and Konopka, 1981; reviewed by Hall and Rosbash, 1988).

We and others have cloned \textit{Drosophila} DNA in the vicinity of the \textit{per} gene (Reddy \textit{et al.} 1984; Bargiello and Young, 1984) and have assigned \textit{per} biological activity to DNA that encodes a 4.5 kb transcript (Zehringer \textit{et al.} 1984; Bargiello \textit{et al.} 1984; Yu \textit{et al.} 1987; Baylies \textit{et al.} 1987). In our initial study, we also focused our attention on a 0.9 kb RNA which is transcribed from an immediately adjacent region of the genome and which appeared to fluctuate in level in a circadian manner. The rhythmic oscillations in RNA level were reported to continue in constant dark conditions (as would be expected of a rhythm under circadian clock control). Furthermore, flies which were arrhythmic due to a mutation in the \textit{per} gene (\textit{per}\textsuperscript{01} flies) were found to have relatively low levels of the 0.9 kb RNA. These observations suggested that the \textit{per} product might contribute to the cycling of the 0.9 kb RNA.

We have since carried out experiments which address the connection between the \textit{per} gene and the changes in the levels of the 0.9 kb transcript. During the course of these experiments, we found that the amplitude of this cycling was quite variable. We subsequently found that the 0.9 kb RNA is present at extremely high levels in late pupae and disappears rapidly after the emergence (or eclosion) of adult flies from their pupal cases. It is now clear that the level of this RNA does not undergo circadian cycling, but rather that the daily and synchronized emergence of young adults, coupled with the rapid decay of the transcript, is responsible for what previously appeared to be an adult rhythm in the 0.9 kb RNA levels (Reddy \textit{et al.} 1984). In this paper, we present the evidence for this conclusion. It is likely that \textit{per}'s influence on 0.9 kb RNA expression is indirect and is manifested only through \textit{per}'s ability to regulate the eclosion rhythm.

In an attempt to explore further the relationship of this RNA to eclosion, we have examined its expression in pupae by Northern blot hybridization. The results show that there is a rapid elevation in transcript level
within a few hours of eclosion; high levels are seen in pupae which show a high 'readiness' to emerge. Furthermore, expression of the 0.9 kb RNA appears to be under the control of the pupal (eclosion) circadian clock, since its appearance in late stage pupae is gated, or restricted to a particular time of day. These and other results show that this transcript is indeed under clock regulation, but in a manner quite different from what was initially envisioned.

**Materials and methods**

**Fly stocks**

All stocks were reared at 25°C on cornmeal–molasses–agar medium in a 12:12 h light:dark environment. Canton-S is a wild-type strain of *Drosophila melanogaster*. The per [9] stock was induced in a Canton-S background by Konopka and Benzer (1971), and the per [9] stock (of either Oregon-R or Hikone ancestry) has been described by Hamblen-Coyle et al. (1989). Df(1)TEM202/Df(1)64j4 flies, which are deficient for per and the 0.9 kb RNA (as well as at least two additional transcripts), were generated by a genetic cross (see, for example, Smith and Konopka, 1981; Hamblen-Coyle et al. (1989). In general, flies were raised and entrained in a 12:12 h light:dark environment. Adults were placed in fresh food vials for aging. Pupae and adults were collected for RNA analyses by freezing on dry ice, and were stored at -80°C until use.

**Recombinant DNA clones**

The 2.1 kb HindIII–EcoRI genomic DNA fragment encoding the 0.9 kb transcript (Fig. 1; Reddy et al. 1984) was subcloned into the HindIII–EcoRI sites of pSP64 (Promega Biotech) to create the plasmid p64HR2.1. pE49 was constructed from pHR0.6 (O’Connell and Rosbash, 1984) by subcloning a 640 bp genomic DNA EcoRI–HindIII fragment, which codes part of the coding sequence for the *Drosophila* ribosomal protein rp49, into the vector pEMBL18+.

**RNA preparation**

RNA was prepared from frozen flies by homogenization in RNA extraction buffer (150 mM-NaOAc, 50 mM-Tris pH 9, 5 mM-EDTA, 1% SDS plus fresh diethyl pyrocarbonate to 0.2%) on ice. RNA was extracted multiple times with phenol, once with phenol:chloroform (1:1), and then precipitated.

**Northern blot hybridization**

RNA was fractionated on 1 or 1.5% agarose formaldehyde gels according to Maniatis et al. (1982) and blotted to nitrocellulose as described by Thomas (1980). A single-stranded 32P-RNA probe specific for the 0.9 kb RNA was generated by in vitro transcription by SP6 polymerase of p64HR2.1, which had first been linearized with EcoRI. Approximately one µg of linear DNA was transcribed in 50 µl transcription buffer (40 mM-Tris pH 7.5, 6 mM-MgCl2, 2 mM-sterimidine, 20 mM-NaCl, 10 mM-DTT) with 20 units RNAase (Promega), 0.5 µm each of ATP, CTP, and GTP, 50 µM-UTP, 50 µCi α-35S-UTP (approximately 800 Ci mmol⁻¹) and 15 units SP6 polymerase (Promega). The ratio of cold UTP and α-35S-UTP in the transcription mixture was set to obtain a probe of about 2.8×10⁸ cts min⁻¹ µg⁻¹. Hybridizations with the RNA probe were done as reported by Zinn et al. (1983), except that the prehybridization and hybridization steps were done at 55°C and the blots were washed at 68°C. Blots were reprobed for ribosomal protein rp49 by preparing a double-stranded 32P-DNA probe from pE49 via random primer labelling (Feinberg and Vogelstein, 1983) and by reducing the prehybridization and hybridization temperatures to 42°C, and the washing temperature to 55°C.

**RNase protection assay**

A 2.1 kb 32P-RNA probe was transcribed from p64HR2.1 (linearized with EcoRI) in vitro using SP6 polymerase as described above. Full-length transcripts were isolated from 8 µl-urea, 4% polyacrylamide gels by soaking the gel band in 0.2 M-Tris pH 7.5, 0.3 M-NaCl, 25 mM-EDTA and 2% SDS at 37°C overnight, followed by phenol:chloroform extraction and ethanol precipitation. About 1×10⁶ cts min⁻¹ of purified probe were used for each RNase protection (based on the procedure of Zinn et al. 1983). Total RNA was mixed with the SP6 probe in a 30 µl volume containing 80% formamide, 40 mM-Pipes pH 6.4, 0.4 M-NaCl and 1 mM-EDTA, and the mixture was heated to 85°C for 5 min and allowed to hybridize at 45°C overnight. RNase treatment followed the procedure of Zinn et al. 1983. The unhybridized RNA was digested by adding 270 µl cold TNE (10 mM-Tris pH 7.5, 0.3 M-NaCl and 5 mM-EDTA), 3 units RNase T1 (Calbiochem) and 12 units RNase A (Sigma), followed by a 15 min incubation at 15°C. The reaction was stopped by the addition of 20 µl 10% SDS and 2.5 µl proteinase K (20 µg µl⁻¹) and incubation at 37°C for 15 min. The resulting fragments were extracted with phenol:chloroform, precipitated with ethanol and analyzed on 8%-urea, 10% polyacrylamide gels by autoradiography.

**DNA sequencing**

In situ hybridization RNA probes for hybridization were transcribed in vitro (using SP6 polymerase and 35S-UTP) from p64HR2.1, which had been linearized with EcoRI. Probes were transcribed such that they had a specific activity of approximately 1.8×10⁶ cts min⁻¹ µg⁻¹, and were then hydrolyzed to an average size of 50–100 nucleotides as described previously (Liu et al. 1988). Fixation, embedding in paraffin, sectioning, hybridization and autoradiography were all carried out as in Liu et al. 1988, except that the digestion with RNase A (after hybridization) was done at room temperature. Slides were developed after 1–8 days of exposure.

**Results**

**Genomic location**

The 0.9 kb RNA is transcribed from X-chromosomal
DNA adjacent to \( \text{per} \) (Fig. 1). \( \text{per} \) and the 0.9 kb RNA are transcribed in opposite directions and are both contained within a 13.2 kb \( \text{BamHI}-\text{EcoRI} \) genomic DNA fragment (Fig. 1).

**0.9 kb RNA expression in adult flies**

Because we encountered difficulty in consistently reproducing the apparent circadian cycling of 0.9 kb RNA levels, RNA was assayed in adult flies as a function of age (Fig. 2). The levels of the 0.9 kb RNA are high in newly eclosed adults and decrease rapidly with age. [The levels of RNA encoding the \textit{Drosophila} ribosomal protein rp49 (O'Connell and Rosbash, 1984) are shown as a control for the amount of RNA loaded in each lane.] From these results, we estimate that the effective half-life of this RNA is at most about one hour over the first six hours after eclosion (since the steady-state levels shown in Fig. 2B decrease approximately twofold each hour). The levels are not appreciably different between wild-type (Canton-S) and arrhythmic \( (\text{per}^{01}) \) flies (Fig. 2B).

Despite the similar 0.9 kb RNA profiles during the first few hours of adult life in wild-type and arrhythmic \( (\text{per}^{01}) \) flies (Fig. 2B), a seemingly paradoxical result was consistently obtained when RNA from newly emerged adults, collected during the first few hours of the light phase, was assayed (Fig. 3A). Under these conditions, RNA from arrhythmic \( \text{per}^{01} \) and \( \text{per}^{04} \) (a new mutant isolate of \( \text{per} \), Hamblen-Coyle et al. 1989) flies contained higher levels of the 0.9 kb transcript than RNA from wild-type flies. However, RNA from newly emerged adults of both genotypes, collected only during the first hour of the light phase, contained similar levels of 0.9 kb RNA (Fig. 3B). We suspected that these observations were due to the rhythmic eclosion of wild-type flies, in contrast to the arrhythmic eclosion of \( \text{per}^{01} \) and \( \text{per}^{04} \) flies (Konopka and Benzer, 1971; Bargiello et al. 1984; Hamblen-Coyle et al. 1989). If, under our conditions, the majority of normal animals eclosed just after the lights are turned on, 0–4 hour wild-type flies (e.g. Fig. 3A) would then be older, on average, than 0–4 hour \( \text{per}^{01} \) flies, since \( \text{per}^{01} \) flies eclose arrhythmically even during light:dark conditions (Bargiello et al. 1984).

We assayed the eclosion profiles (number of adults emerging each hour) of both wild-type and \( \text{per}^{01} \) flies under 12 h:12 h light:dark entrainment conditions. The wild-type profile peaked one hour after the lights went on (lights-on), but the \( \text{per}^{01} \) profile was flat throughout the collection period (Fig. 4). It is therefore likely that arrhythmic adults, collected during a four hour window after lights-on (Fig. 3A), do indeed contain a higher fraction of very young (e.g. 0–1 h) flies than rhythmic adults collected under the same conditions. Because very young adults contribute much of the 0.9 kb RNA in a mixed-age population of flies (Fig. 2), these observations explain the higher levels of the 0.9 kb RNA found in arrhythmic vs. rhythmic 0–4 hour flies (Fig. 3A).

To verify that age, rather than time of day, influences
Fig. 3. Levels of the 0.9 kb RNA in young wild-type (Canton-S, denoted CS) and mutant (per01 and per04) flies. Total RNA from ten flies (approximately 20 μg) was analyzed by an RNase protection assay for the 0.9 kb transcript (see Materials and methods). Population vials were cleared at lights-on during a 12h:12h light:dark regime, and adults were collected either four hours (A) or one hour (B) thereafter. Bands represent exons that encode the 0.9 kb RNA. RNase protection experiments with truncated RNA probes indicate that an approximately 155 nt doublet (common to all of the stocks) and a large, roughly 300 nt band (also in common) represent the first and second exons of the gene (L. Lorenz, data not shown). The third exon of the Canton-S and per01 stocks is represented by an approximately 220 nt fragment and an approximately 90 nt doublet. The fragmentation of the exon in this case is most likely due to sequence heterogeneity between Canton-S (the parental stock of per01) and Oregon-R (the source of the probe; H. V. Colot, unpublished sequence data). A full-length band of approximately 375 nt, representing the entire third exon, is obtained with per01 RNA (of either Oregon-R or Hikone ancestry; see Hamblen et al. 1989). A shorter, approximately 300 nt band also is seen in the case of per04. Since both of these bands are protected by a 472 nt Ball–HindIII RNA probe (derived from Oregon-R genomic DNA; L. Lorenz, data not shown), they both appear to map to the 3'-end of the gene, and may represent alternate splicing or polyadenylation events.

0.9 kb RNA expression in pupae
Given the high levels of the 0.9 kb RNA just after eclosion, we asked when this transcript accumulates during pupal development (Fig. 6A). Because of heterogeneity in the developmental rates of Drosophila pupae (in a light:dark environment, even when egg laying is limited to a two hour period; L. Lorenz, unpublished observations), pupae were marked (by marking the glass directly under the pupae) one day after the beginning of pupation and only marked pupae were subsequently chosen for RNA analysis. These marked pupae continued to show signs of asynchrony as judged by the development of wing colour (cf. Bainbridge and Bownes, 1981). Consequently, pupae were divided into two groups that did or did not display dark-coloured wings at the time of collection. Animals were also collected at two different times of day (at lights-on and at four hours after lights-on) to check for a possible clock (or timing) influence on 0.9 kb RNA levels in pupae. The transcript was first detectable at the dark-winged stage of pupation on day 4 (Fig. 6A). Equally
freezing for Northern blot hybridization analysis. One fly were allowed to eclose over the following hour before Northern blot hybridization. Collection times are designated in hours from the time when the lights were turned on (Fig. 6A).

To pursue these observations further, dark-winged pupae were successively marked and followed with respect to 0.9 kb RNA levels (Fig. 6B) and eclosion behavior (see below). This allowed us to distinguish between pupae that already had acquired dark wings when the experiment began (day 1, lights-on) and those that had just acquired dark wings during the previous four hours [day 1, lights+4 h (marked day 1, lights+4 h)]. The absence of 0.9 kb RNA in pupae whose wings had recently turned dark (Fig. 6B, third and last lanes) can explain the dip in transcript levels in dark-winged pupae at lights+4 h on days 4 and 5 in the previous experiment (Fig. 6A). These populations would have been substantially depleted of older flies because of recent eclosion. Pupae whose wings turned dark during the first four hours of light on day 1 showed high transcript levels by the next day [Fig. 6B, day 2, lights-on (marked day 1, lights+4 h)].

Table 1 shows the results of counting adults marked for the RNA analysis experiments of Fig. 6A and 6B as they eclosed from pupal cases. The number of newly emerged adults one hour after marking dark-winged pupae at lights-on vs. one hour after marking four hours later (Experiment 1; 28 and 26% vs. 6 and 2%) demonstrates that dark-winged pupae of mixed ages show a higher probability of emergence (or eclosion 'readiness') at lights-on than they do at lights+4 h. Experiment 2 shows that dark-winged pupae of mixed ages show a higher probability of emergence during the first four hours of light (by lights+4 h) than do pupae whose wings became pigmented only within the previous 20 h (46 vs. 19%). These results indicate that older pupae (as judged by when wing pigmentation occurred) show a higher probability of eclosion with age. By four hours after lights-on (lights+4 h), the population of dark-winged pupae is substantially depleted of older animals, leaving a younger population with lower average 0.9 kb transcript levels.

To obtain a more accurate picture of when pupae begin to express the 0.9 kb RNA, we analyzed its levels in dark-winged pupae (which had acquired dark wings during the first four hours after lights-on) over a subsequent 24 hour period (Fig. 7). High levels of 0.9 kb RNA were first detected 16 h later, four hours before lights-on (day 1, dark+8 h). Since essentially all dark-winged pupae (whose wings turn dark during the first four hours of light) eclose the day after the wings underwent a colour change (Table 1), the onset of 0.9 kb RNA accumulation is quite near (within a few hours of) the population's eclosion peak.

To test directly for a gating effect on 0.9 kb expression, we analyzed RNA from pupae which had acquired dark-coloured wings at different times of day (Fig. 8). Pupae whose wings turned dark during the first, third and fifth hours of the light phase all showed a burst of 0.9 kb RNA accumulation at the same time of day. Importantly, pupae whose wings turned dark during the first hour of light did not show substantial levels of 0.9 kb RNA expression two hours before pupae whose wings turned dark during the third hour of light. The opposite result should have obtained were a strictly developmental event at issue. These results suggest that the expression of the 0.9 kb transcript is under clock control in pupae. We note that pupae...
whose wings turned dark during the fifth hour of light showed lower overall expression of the 0.9 kb transcript. Although we do not fully understand the implications of this observation (seen in two successive experiments), it may reflect the presence of pupae that would not have eclosed during the following light phase.

**Tissue distribution of the 0.9 kb RNA in newly emerged adults**

To define the tissue or tissues that express the 0.9 kb RNA near the time of eclosion, we probed tissue sections of newly emerged (0–1 h) adults by in situ hybridization (Fig. 9). The most prominent expression was seen in the epidermis of the entire fly (Fig. 9A, 9B and 9C). Because cuticular stickiness has been problematic in terms of nonspecific binding of probes (L. Lorenz, unpublished observations), we compared these in situ hybridization results to those obtained with Df(1)TEM202/Df(1)64j4 flies, which are deficient for DNA that encodes the 0.9 kb transcript (Reddy et al. 1984; Bargiello et al. 1984; Fig. 9D and data not shown). A decided and reproducible difference between wildtype and control [Df(1)TEM202/Df(1)64j4] flies indicates that the 0.9 kb RNA is specifically expressed in the epidermis of young adults.

**Sequence analysis of the DNA encoding the 0.9 kb RNA and of its predicted encoded protein**

We have used a 2.1 kb HindIII–EcoRI subclone from an Oregon-R wild-type stock (the right-most restriction fragment in Fig. 1; see Reddy et al. 1984) to map the exon/intron structure of the gene that encodes the 0.9 kb RNA by RNase protection of poly(A) RNA from wild-type Canton-S and Oregon-R flies (L. Lorenz, data not shown). We also sequenced this restriction fragment, as well as cDNA clones of the 0.9 kb RNA (Fig. 10). The gene consists of at least three exons (approximately 155, 497 and 373 bp) and two introns (84 and 58 bp). RNase mapping data and primer extension analyses (L. Lorenz, data not shown) indicate that no more than 50 nt are missing from the 5' end of cDNA1. (Note that the nominally 0.9 kb RNA is actually somewhat longer than 1 kb.) Based on the most 3' cDNA clone (cDNA2), which includes a p(A)12 tail, and the previous characterization of per cDNA clones (Citri et al. 1987), the 3' ends of these two transcripts overlap by 42 nucleotides (compare the positions of p(A)12 with p(A)* per in Fig. 10). Within this overlap region is a
Control of Drosophila mRNA expression

Table 1. Pupal eclosion fate. Number of dark-winged pupae that eclose as adults with time

<table>
<thead>
<tr>
<th>Dark wings marked at</th>
<th>Out next hr</th>
<th>Out by lights+4h same day</th>
<th>Out by lights-on next day</th>
<th>Out by lights+4h next day</th>
</tr>
</thead>
<tbody>
<tr>
<td>(a) Experiment 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>lights-on (mixed ages)</td>
<td>47/167 (28)</td>
<td>71/167 (43)</td>
<td>137/167 (82)</td>
<td></td>
</tr>
<tr>
<td>lights+4h (mixed ages)</td>
<td>6/109 (6)</td>
<td>-</td>
<td>34/109 (31)</td>
<td></td>
</tr>
<tr>
<td>(b) Experiment 2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>lights-on (mixed ages)</td>
<td>85/185 (46)</td>
<td>136/175 (78)</td>
<td>174/175 (99)</td>
<td></td>
</tr>
<tr>
<td>lights-on (dark wings within last 20h)</td>
<td>20/107 (19)</td>
<td>90/107 (84)</td>
<td>106/107 (99)</td>
<td></td>
</tr>
<tr>
<td>lights+4h (dark wings within last 4h)</td>
<td>-</td>
<td>2/23 (9)</td>
<td>23/23 (100)</td>
<td></td>
</tr>
</tbody>
</table>

The results listed under Experiment 1 were obtained as pupae were collected for the RNA analysis presented in Fig. 6A, and those under Experiment 2 while pupae were collected for the analysis of Fig. 6B. The results are shown as the number of marked dark-winged pupae emerging over the total number marked and as corresponding percentages (in parentheses).

---

Fig. 7. Time course of expression of the 0.9 kb RNA in dark-winged pupae. Pupae that acquired dark-coloured wings over a period of four hours (from lights-on of a 12h:12h light:dark schedule through four hours later) were marked, and four pupae were collected then and every four hours thereafter (except at lights-off, which would have corresponded to 12h after lights-on). Collection times are shown first, and the length of time for which the pupae displayed dark wings is shown in parentheses in the first five lanes. The last lane shows, for comparison, the level of 0.9 kb RNA in adults that eclosed from the marked pupae during the first four hours of light the next day. Each lane contained one pupal or fly equivalent of total RNA (~2 μg).

Fig. 8. Gating of the 0.9 kb RNA in late pupae. Pupae that had acquired dark-coloured wings during the first, third and fifth hours of light in a 12h:12h light:dark environment (lights+1h, lights+3h and lights+5h) were collected and aged until six, eight and ten hours (dark+6h, dark+8h and dark+10h) after the lights went off for RNA analysis by Northern blot hybridization. Collection times for aging are shown first; collection times for RNA analyses are shown last. One pupal equivalent of total RNA (~2 μg) was loaded per lane.

In an effort to identify the protein encoded by the 0.9 kb RNA, we used the available cDNA sequences to predict the amino acid sequence of the protein. The existence of a single open reading frame in the cDNA sequence, together with a Drosophila codon bias analysis of the sequence (Pustell Sequence Analysis Pro-
Fig. 9. Tissue localization of the 0.9 kb transcript in newly eclosed flies. Population vials were cleared at lights-on in a 12 h:12 h light:dark environment and freshly emerged adults were collected for in situ hybridization one hour later. Parts A, B and C, respectively, show hybridization of an 35S-RNA probe derived from the HindIII-EcoRI genomic fragment encoding the 0.9 kb transcript (see Materials and methods) to the epidermis of the head, thorax and abdomen of a wild-type (Canton-S) female fly. No hybridization signal is seen over the abdominal epidermis (nor that of part of a thorax) of a female fly which is deficient for DNA that encodes the 0.9 kb RNA (of the Df(1)TEM202/Df(1)64j4 genotype; part D), or over the epidermis of the head of the same fly (data not shown). Only dark-field photographs are shown. Each photo reveals the accumulation of silver grains after a two-day exposure. Magnification: ~650x.
Control of Drosophila mRNA expression

Controlled by Drosophila mRNA expression

Fig. 10. Genomic sequence of the 2.1 kb EcoRI-Hindlll DNA fragment that encodes the 0.9 kb RNA. The derived protein sequence is shown below the DNA sequence; the borders of the amino acid sequence designate the positions of splice junctions as defined by RNase protection assays and cDNA sequencing. The ends of the cDNA clones (cDNA1-4) are delimited by asterisks, and arrows (>) denote the direction (5' to 3') of each clone. A poly(A) tail exists at the 3' end of cDNA2, and a sequence that could serve as a 3' cleavage signal for polyadenylation (AAUAAA) is apparent 15 nucleotides upstream of the poly(A) stretch (solid underline). A bracket encompasses a palindrome, which includes putative 3' cleavage sequences for both the 0.9 kb RNA and for the per transcript (dashed underline). The direction and position of the 3' end of the per RNA is also shown (p(A)*per).

Hydrophobic amino acids of the proposed signal sequence at the amino terminus of the expected protein are underlined.

The predicted protein contains 261 amino acids. Most notably, it contains at its amino terminus an approximately 20 amino acid hydrophobic stretch that probably serves as a leader signal for membrane transport (cf. von Heijne, 1985). We searched the protein sequence database of the Protein Identification Resource (Release 20, June 1989; NBRF) with the FASTA program.
ologies were detected. To other identified proteins, but no significant homologies were detected.

Discussion

Our data show that the 0.9 kb RNA is intensely expressed late in pupal development, in animals that are only hours away from eclosion. Newly eclosed adults contain similarly high levels of the 0.9 kb RNA, but the expression of this transcript falls rapidly during the first few hours of adult life. Constant, low amounts of this RNA are found in flies that are aged for several days (L. Lorenz, data not shown).

We have been unable to reproduce previous work from our laboratories, which claimed the 0.9 kb RNA was expressed in adults in a circadian manner (Reddy et al. 1984). For that study, adults were taken directly from cultures for RNA analyses, as opposed to being aged, post-eclosion, in separate containers (P. Reddy, personal communication). It was erroneously assumed that *Drosophila* develop relatively synchronously from eggs laid over a short (1–2 day) period. Flies that were thought to be about one week old were probably of mixed ages, including some quite young adults (which would have expressed relatively high levels of the 0.9 kb RNA). It is likely that the daily peak of emergence of young adults shortly after lights-on was responsible for an apparently higher level of the 0.9 kb RNA in flies at midday than at midnight. It is also likely that the persistence of the eclosion rhythm under free-running conditions (constant darkness and constant temperature) was responsible for the apparent cycling of the 0.9 kb RNA under those conditions. The relatively low 0.9 kb RNA levels found in *per* flies must then have been due to age differences between stocks. We note, however, that these interpretations cannot account for a previously observed peak of 0.9 kb RNA at midday (Reddy et al. 1984); we have no simple explanation for this finding. Taken together, our current data lead us to conclude that per has little or no direct influence on the expression of the 0.9 kb RNA, and that much, if not all, of its previously observed effect was due to its influence on eclosion timing.

The eclosion of *Drosophila melanogaster* is under a circadian control mechanism known as 'gating' (Pittendrigh, 1966). As pupae of mixed ages near eclosion, they either continue development to adulthood or arrest until the next day. The course of events depends on the time of day at which individual pupae reach this late stage of metamorphosis. Certain hours of the day (around dawn for *Drosophila melanogaster*) constitute the 'allowed' zone during which development can proceed, and this zone has been termed the eclosion 'gate'. Earlier developmental events during *Drosophila* metamorphosis, such as eye and ocellar bristle pigmentation, are not gated, but occur at fixed times after pupariation formation (Pittendrigh and Skopik, 1970).

We have found that pupal expression of the 0.9 kb RNA is under circadian clock control. Pupae that reach the same stage of development (as judged by wing pigmentation) at slightly different times of day begin to express the 0.9 kb RNA in unison just before dawn on the following day. To the best of our knowledge, this is the first demonstration of a gated molecular event during *Drosophila* pupation.

It is not clear when the gating mechanism is first set in place in *Drosophila melanogaster*. In *Drosophila pseudoobscura*, rhythmic eclosion can be induced by light at any stage of larval or pupal development (Bunning, 1935), so the underlying clock that gates eclosion may function as early as the larval stage in this species. Pupation is, itself, a gated event in *Drosophila victoria* (Rensing and Hardeland, 1976), but not in *Drosophila pseudoobscura* or *Drosophila melanogaster* (Pittendrigh and Skopik, 1970). The release of brain hormone (or prothoracotropic hormone) from brain neurosecretory cells of *Manduca sexta* is gated during larval differentiation (Truman, 1972), but we are not aware of any known, gated events before pupation in *Drosophila*. Wing pigmentation, which occurs after eye and ocellar bristle pigmentation but before 0.9 kb RNA expression, appears not to be under clock control; this event occurs asynchronously, at least during the light phase of a 12 h:12 h light:dark cycle (L. Lorenz, unpublished observations). However, light pulses applied very early during pupal development can affect the timing of eclosion in *Drosophila pseudoobscura* (Skopik and Pittendrigh, 1967). It is thus possible that the gating clock is active well before eclosion, but that only a subset of developmental events, including 0.9 kb RNA expression, is under its control.

Sequence analysis has yielded little insight into the function of the protein since no significant homologies have been found. This approach may become more successful as additional sequences are added to the current databases.

The 4.5 kb *per* transcript and the 0.9 kb RNA are transcribed from the chromosome in convergent directions such that the last 42 nt of both RNAs are complementary. The shared region of DNA (spanning the two 3' cleavage/polyadenylation signals, AAUAAA) confers potential stem structures and instability sequences (AUUUA) on each transcript. In fact, the polyadenylation signal of each transcript contains most of the potential instability sequence of the other. Although the significance of this sequence awaits further investigation, it might be relevant to the rapid post-eclosion decay of the 0.9 kb transcript.

The abundant, gated expression of the 0.9 kb RNA during late pupal development suggests that this RNA is involved in the terminal stages of pupal metamorphosis and/or eclosion. *In situ* localization of the 0.9 kb RNA to the epidermis of young adults suggests further that this tissue actively transcribes this RNA during pupation. However, since the predicted protein contains a hydrophobic amino terminal sequence, it is possible that the protein is secreted from the epidermal cells and acts on neighboring cells, or is carried by the haemolymph to other target tissues. We do not yet know the extent to which this transcript is expressed.
earlier in development. Although it was not detected before mid/late pupae in our earlier study (Reddy et al. 1984), more recent preliminary data give some indication of 0.9 kb RNA expression in embryos and larvae (L. Lorenz, unpublished). The gated pupal expression and short adult half-life suggest that one should take care to assay narrow developmental stages.

The gene that encodes the 0.9 kb RNA is inessential, i.e. the essentially arrhythmic but viable per– (Df(1)TEM202/Df(1)64j4) genotype contains a deletion of DNA encoding the 4.5 kb per transcript, the 0.9 kb RNA and at least two other transcripts (Reddy et al. 1984; Bargiello and Young, 1984). Restoration of the adult circadian locomotor activity rhythm has been accomplished by P-element mediated transformation of arrhythmic per– flies with per DNA alone (Bargiello et al. 1984; Hamblen et al. 1986), but rescue of the eclosion rhythm without the 0.9 kb transcript has yet to be demonstrated. Experiments aimed at examining the eclosion of flies that express the per gene, but not the 0.9 kb transcript, should allow us to determine whether the 0.9 kb RNA contributes to the circadian eclosion rhythm.

We acknowledge and thank A. C. Jaquier for the initial sequence of the genomic clone encoding the 0.9 kb transcript, sequence of the genomic clone encoding the 0.9 kb transcript, reviewing the text, and T. Tishman for help with word-criticism of the work. We thank H. V. Colot for carefully et al. 1984; Bargiello and Young, 1984). Restoration of the adult circadian locomotor activity rhythm has been accomplished by P-element mediated transformation of arrhythmic per– flies with per DNA alone (Bargiello et al. 1984; Hamblen et al. 1986), but rescue of the eclosion rhythm without the 0.9 kb transcript has yet to be demonstrated. Experiments aimed at examining the eclosion of flies that express the per gene, but not the 0.9 kb transcript, should allow us to determine whether the 0.9 kb RNA contributes to the circadian eclosion rhythm.

References


VON HEUNE, G. (1985). Signal sequences: The limits of variation. Insect Physiol. 31, 1984; Hamblen et al. 1984; Hamblen et al. 1986), but rescue of the eclosion rhythm without the 0.9 kb transcript has yet to be demonstrated. Experiments aimed at examining the eclosion of flies that express the per gene, but not the 0.9 kb transcript, should allow us to determine whether the 0.9 kb RNA contributes to the circadian eclosion rhythm.

We acknowledge and thank A. C. Jaquier for the initial sequence of the genomic clone encoding the 0.9 kb transcript, sequence of the genomic clone encoding the 0.9 kb transcript, reviewing the text, and T. Tishman for help with word-processing. This work was supported by a predoctoral fellowship from the National Institute of Mental Health (MH09751) to L.J.L. and by a grant from the National Institute of Health (GM33205) to M.R. and J.C.H.


(Accepted 24 July 1989)