

# In Situ Localization of the *per* Clock Protein during Development of *Drosophila melanogaster*

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**The *per* locus influences biological rhythms in *Drosophila melanogaster*. In this study, *per* transcripts and proteins were localized in situ in pupae and adults. Earlier genetic studies have demonstrated that *per* expression is required in the brain for circadian locomotor activity rhythms and in the thorax for ultradian rhythmicity of the *Drosophila* courtship song. *per* RNA and proteins were detected in a restricted group of cells in the eyes and optic lobes of the adult brain and in many cell bodies in the adult and pupal thoracic ganglia. *per* products were also found in the pupal ring gland complex, a tissue involved in rhythmic aspects of *Drosophila* development. Abundant expression was seen in gonadal tissue. No biological clock phenotypes have been reported for this tissue in any of the *per* mutants. *per* protein mapped to different subcellular locations in different tissues. The protein accumulated in or around nuclei in some cells and appeared to be cytoplasmic in others.**

Circadian rhythms in animals are driven by endogenous pacemakers located in discrete areas of the central nervous system (6). In certain insects, tissues controlling circadian rhythms of locomotor activity have been identified. The optic lobes play a central role in cockroaches (17-19). The lamina-medulla and the brain are essential for production of circadian rhythms in grylls (5, 24) and *Drosophila melanogaster* (10, 15), respectively.

In *D. melanogaster*, *per* mutations affect both circadian and ultradian behavioral rhythms (9). The tissue focus for control by *per* of the circadian locomotor activity pattern has been mapped to the head by genetic tissue mosaic studies (15) and to the brain by organ transplantation (10). Production of certain ultradian rhythms (for example, the ca. 55-s rhythms of the *Drosophila* courtship song) is dependent of *per* expression in thoracic tissues (8). There is also a requirement for *per* protein in the establishment of periodic larval heartbeat (M. S. Livingstone, Soc. Neurosci. Abstr. 8:384, 1981), for the synchronous rhythmic fluctuation of membrane potential in *Drosophila* salivary glands (26), and for wild-type levels of gap junctional communication among cells of *Drosophila* salivary glands (2).

Transcription of the *per* gene is first detected by Northern (RNA) blot hybridization during midembryogenesis (14, 28). After that time, the gene is continuously expressed, with highest levels found in mid- to late-stage embryos and in pupae and adults (28). Much lower levels of *per* RNA are found throughout the larval instars (2, 14, 28). In an effort to determine whether expression of the *per* gene can be directly observed in tissues having phenotypically measurable requirements for *per* gene activity and to define the spatial and temporal production of *per* RNA and protein, we have undertaken a study of *per* gene action by in situ hybridization and by immunocytochemistry with use of affinity-purified anti-*per* antibodies. Previous studies located *per* transcripts and proteins in embryonic and larval salivary glands (2) and in the embryonic nervous system (14, 16, 23). No consistent localization of RNA or protein has been

detected in other embryonic or larval tissues (2, 14, 16, 23; L. Saez, unpublished data). In the present study, *per* transcripts and proteins are localized in pupal and adult tissues.

## MATERIALS AND METHODS

**Recombinant expression vectors.** Construction of the pBH8 expression vector is shown in Fig. 1. A 1.3-kilobase (kb) *Bam*HI/*Hind*III fragment isolated from *per* cDNA (13) was inserted, in frame, into the polylinker sequence of the *trpE* expression vector pATH3 (22). A derivative of pBH8 was constructed in which the bulk of the *trpE* sequence was deleted by digestion with *Bam*HI, *S*1 nuclease, and *Nru*I. Religation was performed with T4 DNA ligase. The resulting gene fusion, pNBH19, consists of the first 18 amino acids of *trpE* followed by an open reading frame encoding 350 amino acids of *per*.

**Preparation of fusion proteins and antisera.** Cultures were grown to stationary phase in supplemented M9 medium, diluted 1:10 into medium without tryptophan, and grown for 1 h at 37°C. Expression of the fusion gene was induced with indoleacrylic acid (5 µg/ml), and cultures were grown for an additional 3 h. Cells were harvested and lysed in sodium dodecyl sulfate-gel loading buffer. The mixture was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and the fusion protein was isolated by electroelution. Fusion protein was used to immunize rabbits by standard protocols. Antibodies directed against *per* epitopes were purified by passing rabbit sera over an affinity column containing pNBH19 fusion protein covalently linked to Affigel 10 resin (Bio-Rad Laboratories, Richmond, Calif.). Bound antibodies were isolated as described previously (2).

**Western blot (immunoblot) analysis.** Induced bacterial extract was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred electrophoretically to nitrocellulose paper. The filter was first incubated with 1% milk in 10 mM Tris (pH 8.0)-150 mM NaCl-0.05% Tween 20 (TBST) for 30 min at room temperature. This procedure was followed by a 6-h incubation with a 1,000-fold dilution of rabbit anti-*per* antibody in the same blocking solution. After incubation with primary antibody, the filter was washed for

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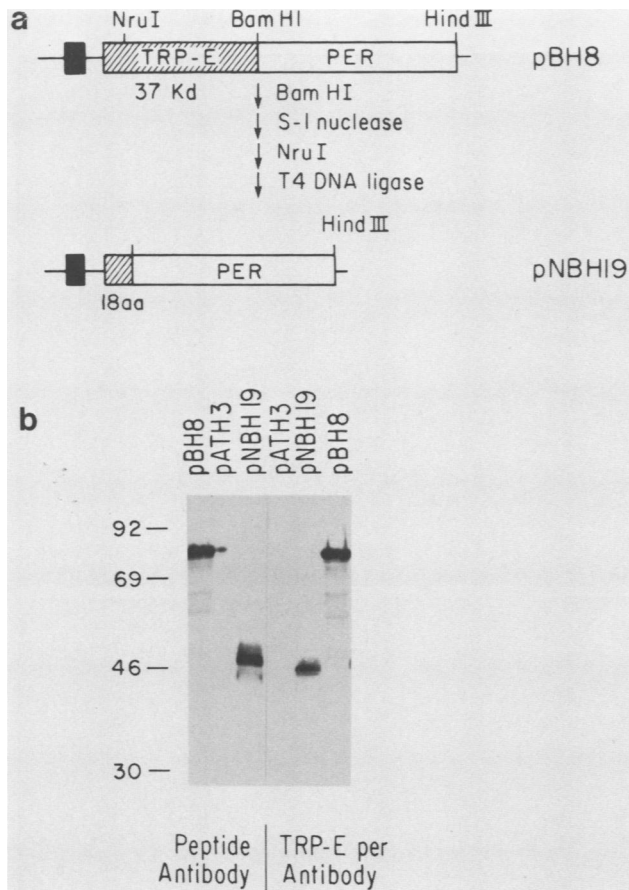


FIG. 1. *trpE-per* fusion proteins. (a) Construction of expression plasmid pBH8. A 1.3-kb *Bam*HI-*Hind*III fragment isolated from *per* cDNA (13) was inserted into the polylinker sequence of the *trpE* expression vector pATH3. The resulting plasmid, pBH8, expressed a 75-kd fusion protein. In a second step, the bulk of the *trpE* sequences were deleted. The resulting gene fusion, designated pNBH19, consists of the first 18 amino acids of the *trpE* gene followed by an open reading frame encoding 350 amino acids of *per* protein. (b) Specificity of the *trpE-per* fusion protein. Immunoblots of total cell protein lysates from bacterial culture were probed with affinity-purified *per* antibodies raised against a synthetic peptide (2) and against *trpE-per* fusion protein. Antibodies fail to react with the *trpE* protein as expressed by pATH3.

30 min at room temperature, followed by incubation with goat anti-rabbit immunoglobulin G conjugated to alkaline phosphatase (Promega) diluted 5,000-fold in TBST. After a 1-h incubation, the filter was subjected to three 15-min washes and the color was developed by using 5-bromo-4-chloro-3-indolyl phosphate-Nitro Blue Tetrazolium (Promega).

**In situ hybridization and immunocytochemistry.** *D. melanogaster* were collected at the indicated stages of development and processed for cryostat sectioning as follows. Pupal cases were removed from staged pupae in freshly prepared 4% paraformaldehyde, and pupae were washed in phosphate-buffered saline and embedded in O.C.T. (Miles Scientific, Div. Miles Laboratories, Inc., Naperville, Ill.). Adults were frozen, and heads were separated from bodies mechanically and embedded in O.C.T. on dry ice. Poly-L-lysine-coated slides were used to lift 10- to 15- $\mu$ m cryostat sections that were postfixed in 2% paraformaldehyde. In situ hybridization and immunocytochemistry in crystal sections were

carried out as described previously (2) with the following modifications: sections were blocked in 5% goat serum-0.1% saponin and incubated with *per* antibodies in the same solution.

**RNA extraction and hybridization probes.** Ovaries from 2-week-old female flies were hand-dissected, and total RNA was isolated by using guanidinium isothiocyanate and centrifugation through CsCl cushions. Poly(A)<sup>+</sup> RNA was selected on oligo(dT)-cellulose. Gel electrophoresis, blotting, and hybridization procedures were performed as described previously (2). Single-stranded RNA probes were prepared by in vitro transcription of DNA templates in Riboprobe vectors (Promega) described previously (2) in addition to a 1.3-kb *Bam*HI/*Hind*III fragment from a *per* cDNA (13) (also used to express *per* protein; Fig. 1). <sup>35</sup>S-labeled RNA probes, including either the sense or antisense strands of *per*, were used for in situ hybridization as previously described (2). Comparable <sup>32</sup>P-labeled probes were used for RNA blot hybridizations.

## RESULTS

**Construction of a *per* fusion protein and generation of specific antibodies.** To generate large quantities of *per* gene product for use as an immunogen, we expressed portions of the gene as a *trpE* fusion protein in *Escherichia coli*. A fragment from a cDNA (13) encoding the last four exons of *per* was inserted into the polylinker site of the *trpE* expression vector, pATH3. The resulting plasmid, pBH8, was designed to express a large fusion protein of 75 kd (Fig. 1a). A second plasmid was constructed by removal of the bulk of the *trpE* coding sequence from plasmid pBH8 (see Materials and Methods). This plasmid, pNBH19, encodes a fusion protein of 47 kd consisting of 18 amino acids of *trpE* and 350 amino acids from the C terminus of *per* protein. Western blot analysis of these fusion proteins by using a *per* antibody generated against a synthetic peptide (2) showed that both plasmids abundantly expressed a *per* fusion protein (Fig. 1b). Polyclonal antisera to pBH8 fusion protein were raised in New Zealand White Rabbits, and specific antibodies to *per* protein were purified by immunoabsorbing the serum to pNBH19 fusion protein that lacks most of the *trpE* protein. The affinity-purified antibody reacted only with *per* and not with the *trpE* domain of the fusion protein (Fig. 1b). Each of the protein localizations described below was also observed when a previously described (2) peptide-derived antibody preparation was used as probe.

**Localization of *per* RNA and proteins in pupae.** Figure 2 shows the results obtained when *per* RNA expression was monitored in developing pupae by using <sup>35</sup>S-labeled RNA probes (for description of probes, see legend to Fig. 2 and Materials and Methods). In mid- to late-stage pupae, *per* RNA was consistently detected in the pupal brain (Fig. 2a and b). The signal was localized in the ganglion cells, which surround a dense mass of fiber nerves, as well as in the cortical cells of the optic lobe. The most intense transcription at this stage corresponded to the ring gland complex (Fig. 2c and d; labeling of the ring gland complex and brain can be compared in Fig. 2b). We cannot distinguish whether one or both of two different cell types (prothoracic ganglia cells and corpus allatum cells) were labeled in these preparations.

Labeling in the cell bodies of pupal thoracic ganglia was also detected with *per* RNA probes (Fig. 2e and f). Ganglion cells of the three thoracic segment centers, which constitute the greater part of the ganglion, showed *per* expression.



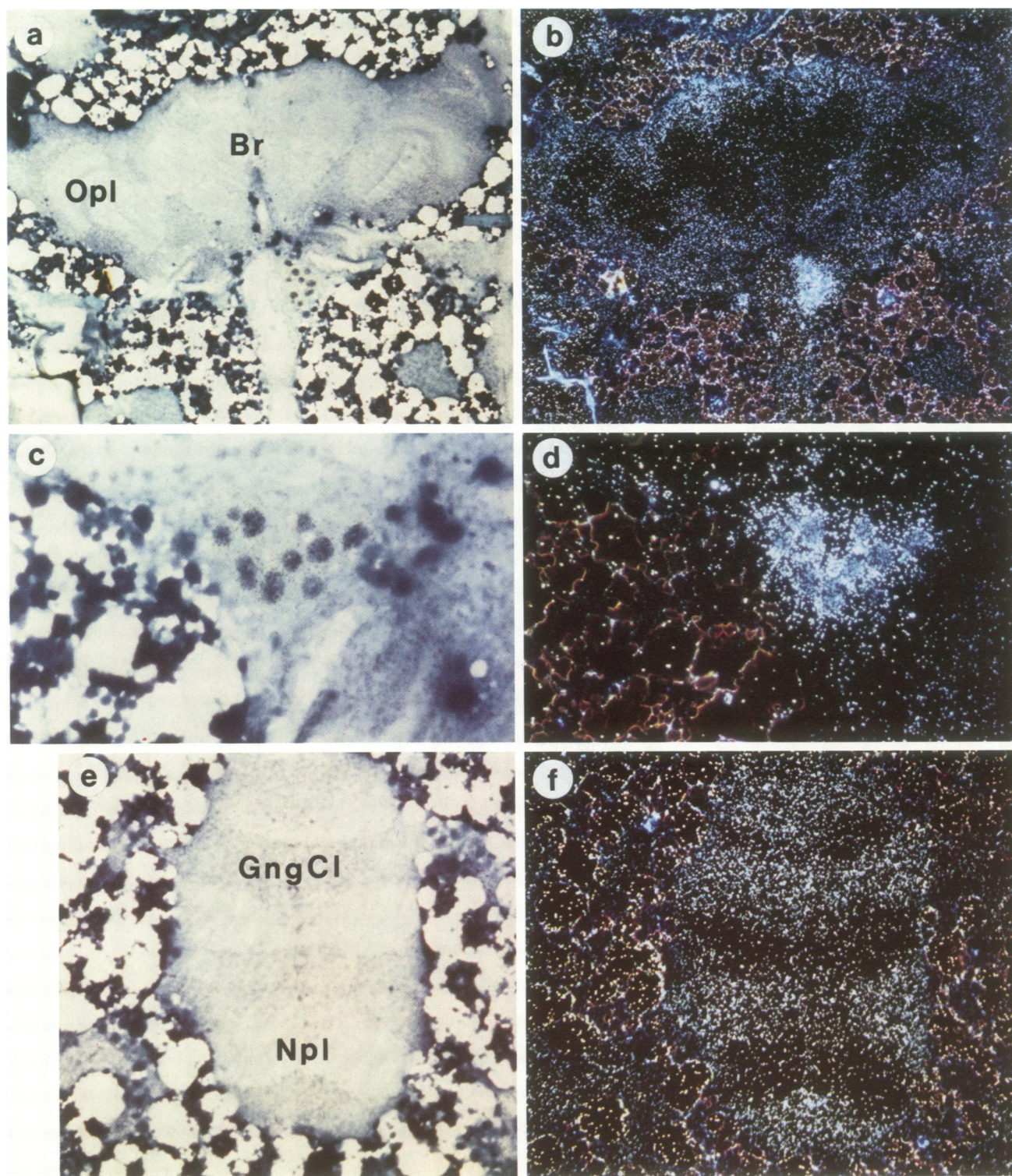


FIG. 2. Distribution of *per* RNA in pupae. *per* transcripts in mid- to late-stage pupae were detected by in situ hybridization, using cryostat sections of wild-type individuals hybridized with  $^{35}\text{S}$ -labeled single-stranded RNA probes specific for *per* protein (see Materials and Methods). Shown are bright-field (a, c, and e) and dark-field (b, d, and f) pictures of pupal sections stained with Giemsa. (a and b) Horizontal section of mid-stage pupal head; (c and d) high magnification of pupal ring gland complex; (e and f) horizontal section of thoracic ganglia of mid-stage pupa. *per* transcripts are expressed in the ganglion cells (neurocytes) of cerebral and thoracic ganglia as well as in the cytoplasm of cells of the ring gland complex. Abbreviations: Br, brain; GngCl, ganglion cells; Opl, optic lobe; Npl, neuropile.



Thus, for both pupal brain and thoracic ganglia, it seems likely that many neurocytes express *per* RNA to some extent. Each of these hybridizations involved a single-stranded RNA probe complementary to *per* RNA. No hybridization was detected in nervous tissue when a sense-strand probe was used as a control (data not shown).

A comparable staining of cell bodies was seen in the thoracic ganglia and brain when antibodies directed against the *per* fusion protein (Fig. 3) were applied to pupal sections. In these experiments, the distribution of the primary antibody raised to a *per* fusion protein was visualized by using horseradish peroxidase-conjugated second antibody. The distributions of horseradish peroxidase staining in both brain and thoracic ganglia corresponded to those seen when RNA probes were used (Fig. 3a through d), namely, cell bodies of the pupal central nervous system and cells of the ring gland complex. *per* protein expression was rather diffuse in pupal brain and in thoracic ganglia. The subcellular localization of much of the antigen may have been cytoplasmic. In contrast, the protein appeared to be associated with the nuclei of cells in the ring gland complex. Figure 3c suggests a perinuclear location for the antigen, possibly associated with the nuclear membrane, the nucleus, or both (also compare with Giemsa staining of these nuclei in Fig. 2c). The same antibody preparation has also been applied to pupal tissues derived from *per* mutants. DNA sequencing studies have shown that the *per*<sup>o</sup> allele contains a nonsense mutation (4, 9). *per*<sup>o</sup> mutants are expected to produce a truncated protein that does not contain the fusion protein sequence used to generate our antibodies. No reaction of the antibody preparation was seen with any of the tissues described above in *per*<sup>o</sup> material (Fig. 4f).

**Mapping *per* products in adults.** The distribution of *per* transcripts and proteins in the heads of adult flies is illustrated in Fig. 4a through d. In contrast to the patterns of expression observed in the pupal nervous system, *per* transcripts and proteins were expressed in discrete regions of the adult brain. Figure 4a shows the result of in situ hybridization to a horizontal section of adult head. Specific hybridization was seen in the eyes and optic lobes; no reactivity was observed in these tissues with probes composing the sense strand or in *Df(1)TEM202/Df(1)64j4* mutants, which carry a homozygous deletion of the *per* locus (3, 21). A strong response was also visible in regions of the central brain. However, comparable labeling of these latter regions was obtained with the sense-strand probe and in *per* mutants, indicating that much of this response was nonspecific.

*per* antibodies labeled photoreceptor cells of the eyes (Fig. 4b through d). Antibody labeling appeared to be perinuclear. The close association of histochemical staining with nuclei was similar to the reaction described above for cells in the ring gland complex (cf. Fig. 3c). Also seen in Fig. 4b and d is a strong reaction with many small cells in the optic lobe. Inspection of additional sections indicated that groups of cells apposed to the lamina, medulla, and lobula were labeled (not shown). This pattern of histochemical staining was similar to that of specific in situ hybridization (Fig. 4a). Usually antibody staining was most intense in layers of cells in the lamina about four or five cells in depth (Fig. 4b and d). Labeling again appeared to be strongest at the borders of nuclei. No labeling of photoreceptor cells or the above-mentioned cells of the optic lobe was seen in head sections of *Df(1)TEM202/Df(1)64j4* flies or in *per*<sup>o</sup> mutants. Some nonspecific staining with our antibody preparation and RNA probes was seen in both classes of mutant flies (Fig. 4a and e). This staining included small regions of the central brain

and may have been due to proteins related to *per* protein that are encoded at other loci (28).

The adult thoracic nervous system was labeled with anti-*per* antibody. In comparison with the labeling of the pupal ganglia, lower levels of *per* RNA and protein were observed (data not shown).

**Expression of *per* in gonadal tissues.** *per* gene products were detected outside the nervous system in both pupae and adults. Pupal ovaries and testes were labeled with our *per* RNA probes, and ovaries and testes reacted with anti-*per* antibody (Fig. 3). In the pupal sections of Fig. 3e, the labeling was associated with nurse cells and the histochemical staining appeared to be dispersed throughout the cytoplasm. Nuclei were not stained. Abdominal sections from adult females (Fig. 3f) also showed abundant labeling, which appeared to correspond to continued synthesis of RNA and protein in ovaries. These in situ localizations were confirmed by recovery of 4.5-kb *per* mRNA from isolated adult ovaries (Fig. 5).

## DISCUSSION

Some of the major sites of *per* RNA and protein synthesis can be correlated with genetically defined *per*-dependent functions, which suggests that control of these functions is autonomously determined by the identified cells. The localization of *per* products in the thoracic ganglia fits well with the observation that *per* must function is required in the thorax (8) for production of ultradian rhythms associated with the male courtship song. *per* RNA and proteins are both detected in the thoracic ganglia during pupation and in the adult. This finding suggests that song rhythm is controlled by this tissue specifically and that the ability to sing rhythmically as an adult may depend on expression of *per* products in the ganglia that is initiated during pupation and maintained in adulthood.

*per* RNA and proteins have been detected in many cells of the developing pupal brain and in neurocytes of the adult optic lobes. This expression may be correlated with transplantation and genetic mosaic mapping; a tissue focus for control by *per* of circadian behavioral rhythms maps to the *Drosophila* brain (10, 15). Mutations affecting the development of the optic lobes in *D. melanogaster* induce complex locomotor rhythmicity, characterized by simultaneous expression of multiple long- and short-period rhythms (11, 12; C. Helfrich, Ph.D. thesis, University of Tübingen, Tübingen, Federal Republic of Germany, 1985). The latter finding indicates that *per* expression observed in the optic lobes may be required but cannot be sufficient for production of wild-type locomotor behavior.

Eclosion is hormonally regulated in insects (27). The ring gland complex in pupal stages controls eclosion through the timed secretion of eclosion hormone. This secretion is known to be controlled by an underlying circadian pacemaker (25). The high level of expression of *per* in the ring gland complex suggests that this organ may itself provide some clock function. Conceivably, physically distinct clocks support circadian locomotor activity and eclosion.

Localization of *per* transcripts and proteins in *Drosophila* gonads was unexpected, as none of the rhythm phenotypes reported for *per* to date include effects on gonad development or function. However, a circadian rhythm of oviposition has been observed in *D. melanogaster* and can be modified by changes in the entrainment regimen (1). Sperm release in certain moths also exhibits a circadian rhythm that is autonomously controlled by a pacemaker in the abdomen,

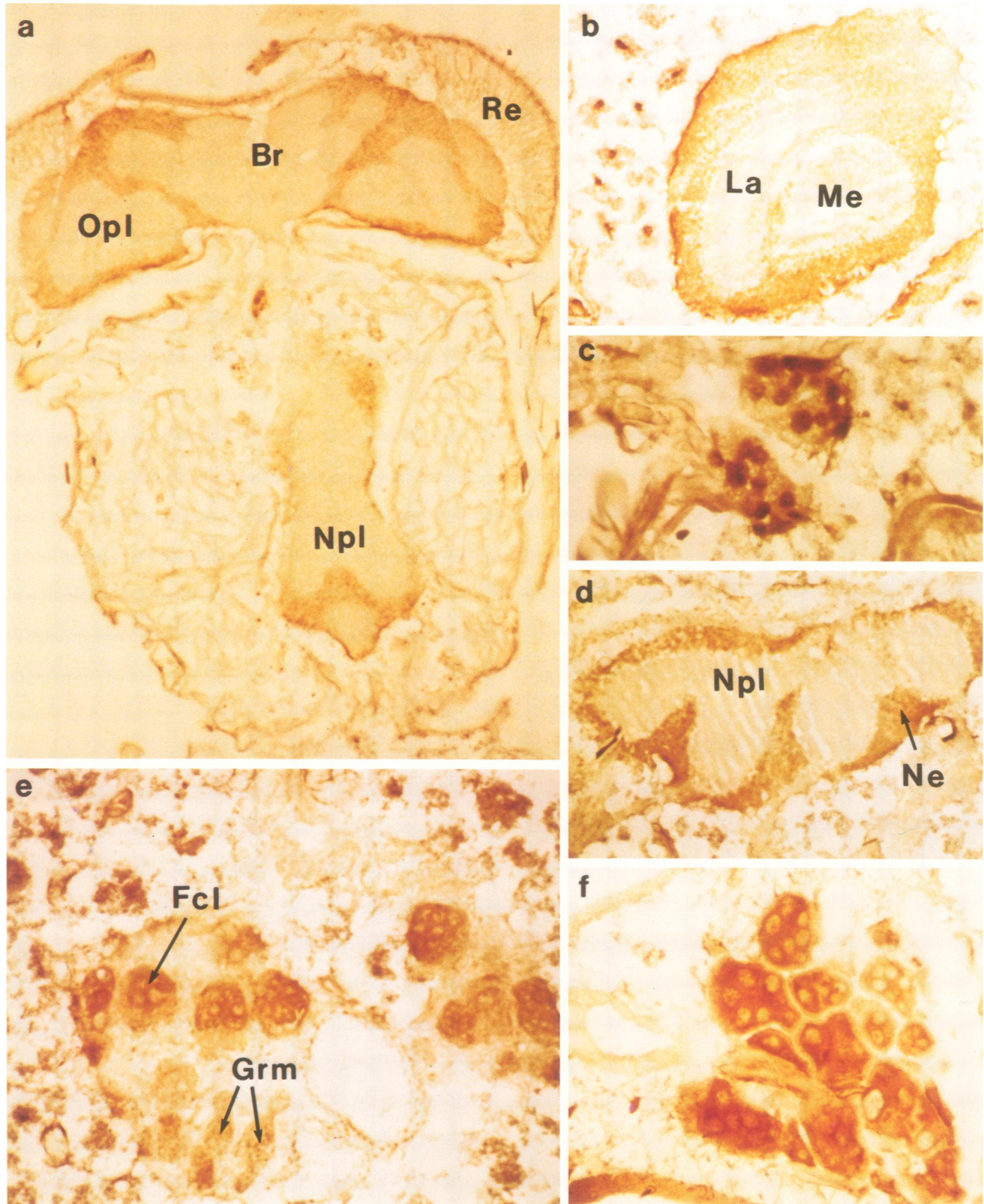


FIG. 3. Expression of *per* proteins in pupae. Localization of *per* proteins in wild-type pupal sections was carried out by using affinity-purified polyclonal antibodies raised against the *per* gene product expressed by pNBH19 (see also legend to Fig. 1 and Materials and Methods). (a) Horizontal section of late-stage pupa; (b) sagittal section through optic lobe of mid-stage pupa; (c) higher magnification of pupal ring gland complex; (d) sagittal section of mid-pupal thoracic ganglia; (e and f) sections of pupal and adult ovaries, respectively. Abbreviations: Br, brain; Fcl, follicle; Grm, germarium; La, lamina; Me, medulla; Ne, neurocytes; Npl, neuropile; Opl, optic lobe; Re, retina.



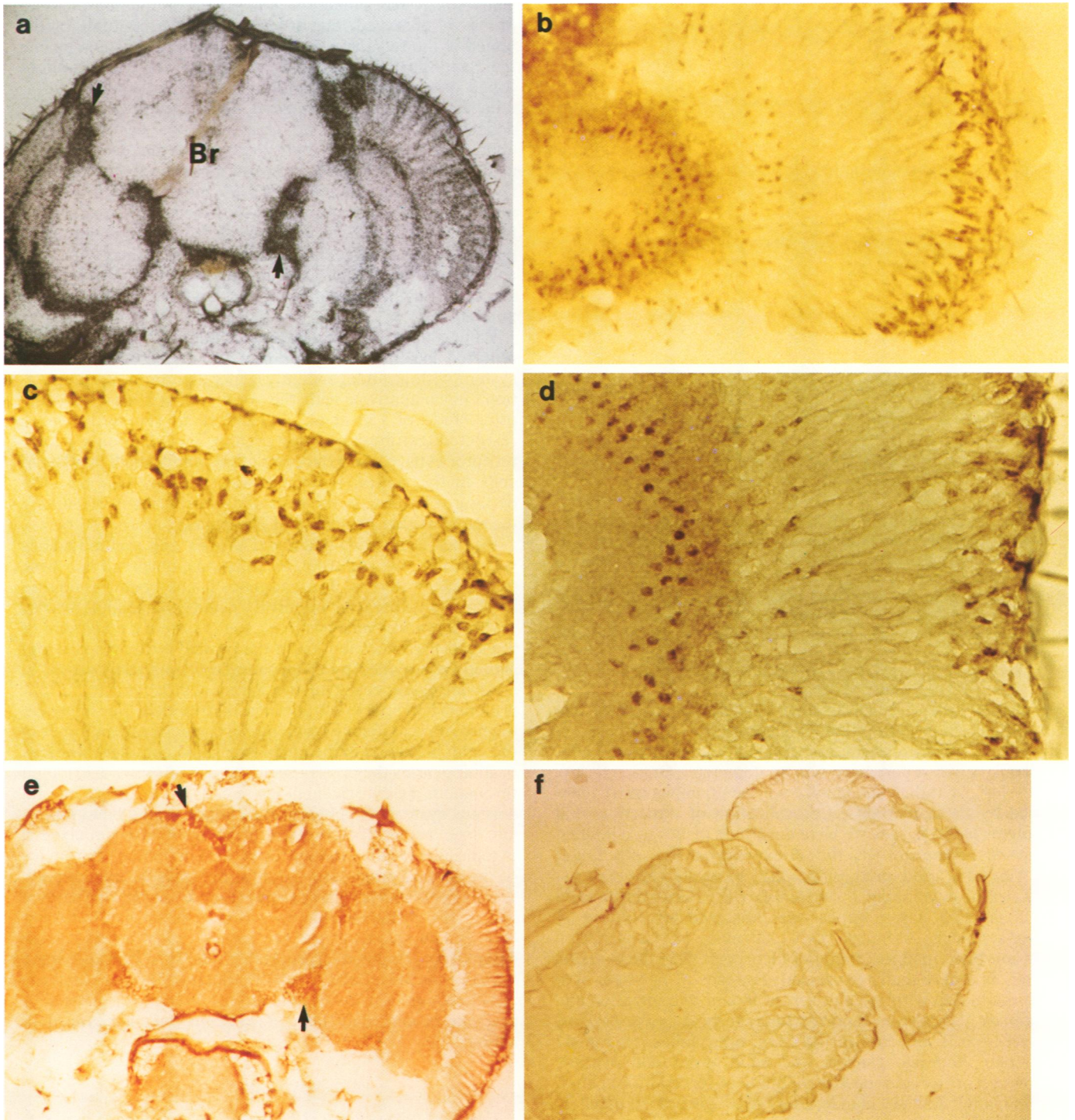


FIG. 4. Expression of *per* RNA and proteins in adult heads. (a) Bright-field in situ localization of *per* transcripts in a wild-type adult fly head. *per* RNA is detected in the eye and in the lamina, medulla, and lobula in the optic lobe. Labeling of the central brain (arrows) is nonspecific (see below); labeling of eyes and optic lobes is absent in *Df(1)TEM202/Df(1)64j4* (*per*) fly heads (data not shown). (b through d) Distribution of *per* proteins in adult heads as determined by using anti-*per* antibodies. (b) Section of eye and optic lobe; (c) higher magnification of distal eye; (d) higher magnification of eye and optic lobe showing label associated with nuclei in optic lobe and photoreceptor cells; (e and f) sections of adult head and pupal *per*<sup>0</sup> flies. Small arrows show nonspecific staining observed in the adult central brain (see also panel a).

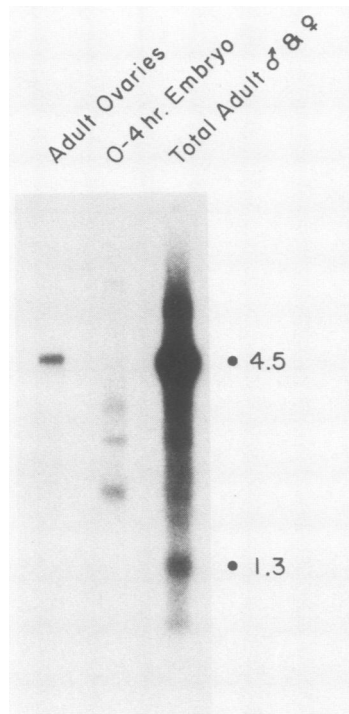


FIG. 5. Expression of *per* RNA in isolated adult ovaries.  $^{32}$ P-labeled riboprobe was produced from *per* restriction fragment a9 (13). The probe detects 4.5-kb *per* mRNA in poly(A)<sup>+</sup> RNA derived from hand-dissected ovaries and from total adult. No transcription is detected in 0- to 4-h embryonic RNA. (PolyA)<sup>+</sup> RNA (5  $\mu$ g) was loaded for total adult and embryonic samples. Total poly(A)<sup>+</sup> RNA from approximately 100 ovaries was loaded for the adult ovary sample.

probably testes (7). A detailed examination of germ line development in *per* mutants is under way.

The in situ localizations of transcripts and immunocytochemistry we report may not be complete. Only cells expressing *per* rather abundantly will be unambiguously identified by these methods. Colocalization of transcripts and proteins in the tissues we have described makes us confident that these are major foci for *per* expression. Recently Liu et al. (16) have used another approach to locate cells expressing *per* in developing *D. melanogaster*. Flies transformed with *per-Bgal* fusion genes were analyzed for patterns of *Bgal* synthesis. Although DNA controlling *per* transcription has not been mapped, material incorporated in these fusion genes is differentially expressed. Patterns of *per-Bgal* synthesis overlap with the patterns of in situ hybridization and antibody localization reported here. In both investigations, expression is detected in the eyes, optic lobes, ring gland complex, thoracic ganglia, and adult testes and ovaries. However, *Bgal* staining was uniquely observed by Liu et al. (16) in adult salivary glands, esophagus, gut, rectal papillae, malpighian tubules, proboscis, ocelli, central brain, and antennae. We uniquely reported *per* RNA and proteins in pupal testes and ovaries. Our inability to detect synthesis in the adult central brain may be due to nonspecific reaction of our probes in *per*-deficient sections of adult heads.

Liu et al. (16) also describe a pattern of embryonic *per-Bgal* expression that differs from that observed in our laboratory (2). Liu et al. detect *per-Bgal* expression in adult but not embryonic salivary glands and find nonspecific labeling of embryonic and adult glands with *per* RNA

probes. In our studies (2), both RNA and antibody probes show a specific reaction with embryonic and larval salivary gland cells. Occasionally a nonspecific interaction of RNA probes is observed, but in such cases the contents of the lumen of the salivary gland, rather than the cells composing the gland, are responsible for the interaction (Saez, unpublished observation). *per* antibodies appear to label embryonic and larval salivary gland cells specifically, as *per*-deficient glands show no reactivity (2). *per* expression appears to be highest in embryonic glands and drops rapidly as development proceeds into and through the larval stages (2). Recently, expression of 4.5-kb *per* mRNA in salivary glands has been confirmed by Northern blot analysis of hand-dissected third-instar glands (L. Vosshall, unpublished observation). Such expression would be consistent with autonomous control of aberrant cell-to-cell communication measured in third-instar salivary glands isolated from *per* mutants (2).

While this paper was in preparation, Siwicki et al. (23) reported localization of *per* proteins in the adult nervous system with an independently generated antibody preparation. In comparison with the results of our work and the prior study of Liu et al., Siwicki et al. reported a more restricted distribution of *per* protein, including only photoreceptor cells, cells in the optic lobes, and a small number of cells in the central brain, thoracic ganglia, and gut. The pattern of labeling in the eyes and optic lobes is essentially the same as that presented in this paper.

Further work with additional probes and methodologies will resolve the different localizations observed in these independent studies. For example, it has been possible to confirm *per* expression in salivary glands and in adult ovaries by Northern blot analysis (e.g., Fig. 5). Better resolution of the subcellular distribution of the protein is also needed and should be provided by electron microscopic analysis.

An intriguing result of the immunocytochemistry of *per* protein is its variable intracellular distribution. For instance, the protein is associated with nuclei or nuclear membranes in the adult brain and in the pupal ring gland complex, whereas in adult ovaries the protein seems to be uniformly distributed throughout the cytoplasm of nurse cells. In the pupal central nervous system, the predominant location may be cytoplasmic (see text). Siwicki et al. (23) have also reported a cytoplasmic localization for the protein in certain neurocytes of the central brain. The location of much of the antibody reactivity in salivary glands appears to be at the cell surface and in the cytoplasm (2; Saez, unpublished data). Earlier biochemical studies of *per* protein have shown that it is an extensively modified glycoprotein (2, 20). Perhaps some variation in subcellular location reflects differences in these modifications. These varied locations do raise the possibility that *per* protein may have more than one mechanism of action.

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