D (Table 2 and Fig. 2). The expression of Ich-1 in those cells was comparable to that in 2fTGH cells, but the amount of Cpp32 was reduced (Fig. 1B). Therefore, p84 and S727A have different apoptotic profiles. We conclude that the constitutive expression of Ich-1 and Cpp32 does not require formation of STAT1 homodimers. However, STAT1 might form complex transcription factors by interacting with other proteins (*24*).

STAT1-null mice are defective in all responses to IFN-α or IFN-γ (*25*, *26*), as expected from the properties of STAT1-null human cells (*27*). However, in contrast to Cpp32-null mice (*28*), STAT1-null mice show no gross developmental abnormalities (*25*, *26*). If, like human cells, mouse cells require STAT1 for efficient expression of Cpp32, the reduced levels of this protease in STAT1-null mouse cells must still be sufficient to support the apoptosis required for nearly normal development. Apoptosis of underlying keratocytes that follows the ablation of corneal epithelial cells is grossly defective in STAT1-null mice (*29*), revealing that at least one form of stress-induced apoptosis is defective in the absence of STAT1.

STAT1 is also required for constitutive expression of LMP2 and LMP7 (low molecular mass polypeptides 2 and 7) (*30*), in addition to Ice, Ich-1, Cpp32, and IRF-1. The finding that STAT1 is required for efficient constitutive expression of several genes reveals a more general role for this ubiquitous transcription factor.

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Independent Photoreceptive Circadian Clocks Throughout *Drosophila*

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Transgenic *Drosophila* that expressed either luciferase or green fluorescent protein driven from the promoter of the clock gene *period* were used to monitor the circadian clock in explanted head, thorax, and abdominal tissues. The tissues (including sensory bristles in the leg and wing) showed rhythmic bioluminescence, and the rhythms could be reset by light. The photoreceptive properties of the explanted tissues indicate that unidentified photoreceptors are likely to contribute to photic signal transduction to the clock. These results show that autonomous circadian oscillators are present throughout the body, and they suggest that individual cells in *Drosophila* are capable of supporting their own independent clocks.

Circadian oscillators have been localized in several organisms. For example, the suprachiasmatic nucleus (SCN) is important for mammalian rhythms (*1*), whereas *Iguana iguana* has at least three independent oscillators: the retina, parietal eye, and pineal gland (*2*). Sparrows show activity rhythms that can be altered by lesioning the pineal gland (*3*); this operation reveals the influence of other oscillators on the bird's behavior. The brain controls behavioral rhythms in moth (*4*) and *Drosophila* (*5*, *6*), whereas sperm release in the moth is controlled by an independent oscillator (*7*). Recently, free-

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running rhythms have been demonstrated in the *Drosophila* ring gland (*8*) and Malpighian tubules (*9*). At a molecular level, two different *Drosophila* clock genes, *period* (*per*) (*10*) and *timeless* (*tim*) (*11*), have been identified. The transcripts and proteins from both of these genes cycle daily in abundance, and both genes are needed to maintain a biological clock (*12*).

In mammals, all demonstrated clock input comes from the eye (*13*), although the exact photoreceptor is unknown (*14*). Photoreceptors within the brain can mediate rhythms in birds (*15*) and insects (*4*, *16*). *Drosophila* that lack all known photoreceptive organs can still transmit light information to the clock (*17*), indicating the existence of unidentified circadian photoreceptors within the animal.

Functional *per* in the head of the fly has been explicitly shown to be essential for one output: behavior (*6*). Eclosion (*10*) and transcriptional control (*18*) have been shown to require *per*, but without any specific localization of the message or its protein. PER protein has been detected in many tissues throughout the animal (*19*). Although specific functions outside the head are unknown, *per* in these tissues may be needed for localized clock function. These *per*-dependent oscillators could be targets for signals from the head; however, light sensitivity is a characteristic of a fully autonomous oscillator that can receive stimuli from the environment, transmit this information to the oscillator, then use the oscillator to affect downstream clock-controlled functions.

per-driven bioluminescent oscillations occur in living *per-luc Drosophila* (*per* is fused to the luciferase gene *luc*) (*20*–*22*). These rhythms are entrainable by light and free-run in constant darkness (*20*). To examine the circadian autonomy of *Drosophila* tissues, we monitored rhythmic bioluminescence from cultured dissociated body segments (head, thorax, or abdomen) from *per-luc* animals (*23*, *24*); *per*-driven green fluorescent protein (GFP) was used concurrently as a bright spatial expression marker.

Each of the three segments are capable of rhythmic bioluminescence (Fig. 1) in light-dark (LD) conditions. Changing the conditions to constant darkness (DD) resulted in a gradual decrease in amplitude. The cultures were able to reentrain to a new LD cycle, where the new onset of light occurred 6 hours later than the free-running subjective dawn. Reentrainment occurred within one cycle, with the main bioluminescent peak falling about 20 hours after lights-on, just as it did in the initial LD cycle. The waveform and phase of the rhythms from all three segments were nearly identical, and there was very little noise in the individual traces, especially when compared with whole-animal records (*20*–*22*). Also, there was no evidence of the second peak of bioluminescence that was previously reported in whole-animal studies (*20*, *21*), indicating that this feature likely arises from a whole-animal physiological-bioluminescent phenomenon rather than as a direct feature of *per* transcription. The proboscis and antenna (Fig. 2A) expressed *per*-

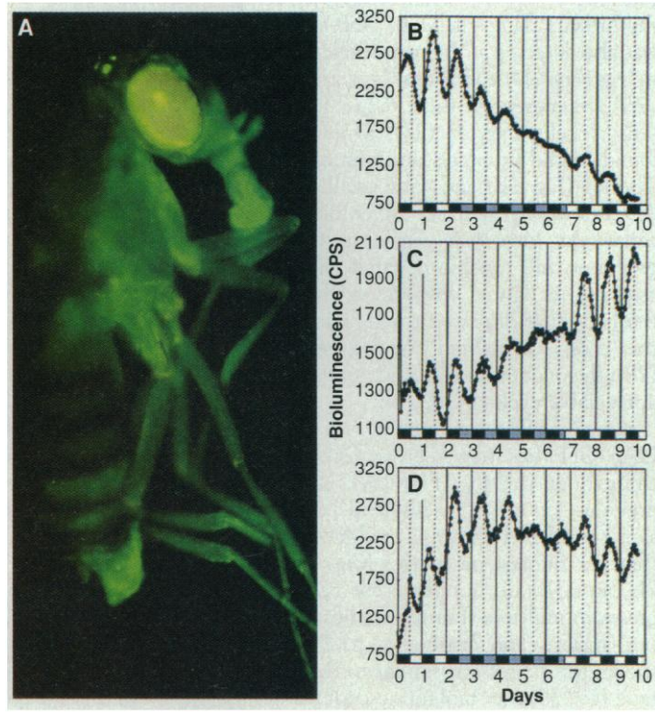

Fig. 1. Bioluminescence rhythms in cultured body segments. *per*-driven GFP expression can be seen throughout the whole fly (**A**). Individual (**B**) heads, (**C**) thoraxes, and (**D**) abdomens were individually cultured in LD and DD and monitored for bioluminescence expression levels. Of the cultured segments 79% (221/279) demonstrated rhythms in LD; 59% (130/222) demonstrated at least two cycles in DD, and 82% (182/222) became arrhythmic within four cycles in DD. Filled bars, darkness; open bars, light; gray bars subjective light. CPS, counts per second.

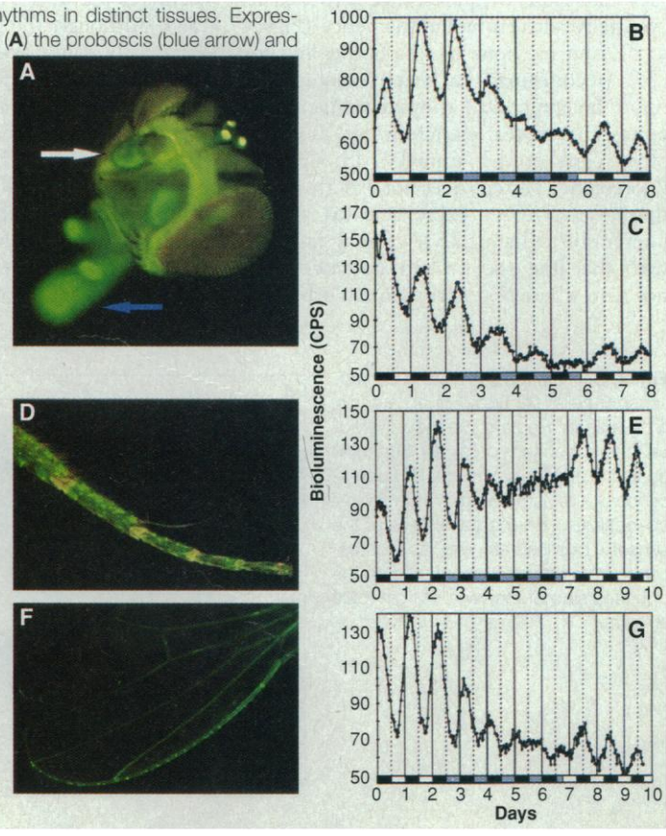

Fig. 2. Bioluminescent rhythms in distinct tissues. Expression of *per*-driven GFP by (**A**) the proboscis (blue arrow) and antennae (white arrow). (**B**) Single probosci and (**C**) antennae were cultured individually and were rhythmic in LD and DD. (**D**) Expression of *per*-driven GFP in the leg. (**E**) Rhythmic *per*-driven bioluminescence in the leg. (**F**) Expression of *per*-driven GFP in the whole wing. (**G**) Rhythmic *per*-driven bioluminescence in the wing: 94% (159/169) of the cultures exhibited rhythms in LD; 82% (137/167) persisted for at least two cycles in DD; and 18% (30/166) became arrhythmic within four cycles in DD. Filled bars, darkness; open bars, light; gray bars subjective light.

driven GFP (*23*, *25*); the bright green signal was easily distinguishable from the yellow autofluorescence [compare (*26*)]. Like the whole-body segments, the proboscis (Fig. 2B) and antennae (Fig. 2C) are also capable of maintaining oscillations in LD and DD (although, as in whole-body segments, rhythmicity damps in DD). GFP expression was also detected at the near-single-cell level in legs (Fig. 2D) and wings (Figs. 2F and 3B). GFP was present in these tissues in chemosensory cells (Fig. 3) (*27*), which are also found in the proboscis and antennae (*27*). The leg (Fig. 2E) and wing oscillators (Fig. 2F) showed rhythmic *per*-driven bioluminescence. As with body segments, the tissue oscillators are capable of rapid resetting in response to light. Other tissues throughout the body that displayed rhythmicity include the eyes, Malpighian tubules, and testes (*28*); the ovaries, though, did not display any appreciable cycling (*28*, *29*).

Our imaging and analysis of *per-gal4*; UAS-GFP flies showed broad *per* expression throughout the fly (Fig. 1A), supporting previous reports of widespread *per* expression (*19*). Several lines of evidence confirm that our fluorescent images (Figs. 1A, 2A, 2D, and 3B) do not represent ectopic expression: multiple staining studies with *per-lacZ* fusions and antibodies to PER have shown *per* expression similar to that shown here (*19*, *30*); our bioluminescent studies in culture show that all of the examined tissues that express fluorescence also express cycling *per*-driven bioluminescence (Figs. 1 to 3); and previous single-photon imaging (*20*) [reconfirmed in recent bioluminescence imaging (*28*)] showed *per-luc* bioluminescence in the legs and wings, as well as other parts of the animal. Although the fluorescence pattern does not exactly reflect that of native *per* expression (as assayed immunohistochemically), inconsistencies with this line occur when fluorescence is not seen where *per* expression has otherwise been detected (notably the eye; see Fig. 1A). We have at no time observed external GFP fluorescence where *per* has not been otherwise detected.

Because there are numerous oscillators in the fly, each of these oscillators are photoreceptive, and *per* is expressed in single cells, we hypothesize that the *Drosophila* clock can operate at a cell-autonomous level. Each clock cell may be capable of photoreceptivity and endogenous rhythm maintenance to some extent. Independent clock cells within a circadian tissue can also explain the overall arrhythmicity of a tissue after several days in DD. Whole animals free-run with a range of near-circadian, but different, periods [as monitored by bioluminescence (*21*)]. In LD conditions, single cells are resynchronized every 12 hours by light transitions; however, cells free-running with a range of periods similar to whole animals will mathematically yield net arrhythmicity in a matter of days. Single-cell monitoring of cells in a tissue as it becomes arrhythmic will be necessary to tell whether the arrhythmicity is due to cell asynchronicity [compare (*31*)], or the gradual "winding-down" of the clock itself at a cellular level.

In constant conditions, RNA oscillations in the adult body damp in DD with about the same kinetics reported here, but that rhythmicity persists in the head (*29*). The brain is the source of control for locomotor behavior (*5*, *6*), and such behavior oscillates for weeks in constant conditions (*32*). Although the brain rhythms do not apparently damp over several days in constant conditions, other parts of the head do (the proboscis, eyes, and antennae; Fig. 2, B and C). The asynchrony within these tissues, which are on the surface of the head, most likely masks the synchronized brain rhythmicity deep within the head.

The function or functions of *per* outside the head are unknown (*19*). A particularly interesting feature of the GFP expression pattern is its labeling of chemosensory cells. This pattern specifically identifies structures at the base of chemoreceptor bristles in the proboscis, antennae, anterior wing margins, and legs (Figs. 1A, 2A, 2D, 2F, and 3) (*27*). Moreover, *per*-driven bioluminescence in these tissues is rhythmic (Fig. 2, B, C, E, and G), showing that there is a functional clock in these cells. These cells are spatially independent of each other (on the basis of noncontiguous fluorescence) and are capable of cycling and entraining without an attached head. The presence of a functional clock implies circadian regulation of chemosensory sensitivity, analogous to the circadian regulation of sensitivity thresholds in luminance (*33*) and pain (*34*) reported in mammals. Although the evidence for clock control of sensory thresholds in the fly is still circumstantial, the presence of independent clocks along with examples of similar rhythmic phenomena in other systems indicates a central role for *per*-dependent clock functions in tissues outside the head.

Every known oscillating tissue in the fly has shown the capacity for light perception. Also, each dissociated segment is rhythmic with the same phase and waveform. This raises the possibility that the head, which was previously believed to be the master oscillator in the fly, does not coordinate all rhythms throughout the animal. In this case, light, which has the potential to affect all parts of the fly simultaneously, serves as the master coordination signal. The asynchrony of independent clock cells over several days of free-run should not be of practical concern to the whole animal because flies in the wild almost always have an environmental light cycle. This control mechanism could reasonably be extended to other animals (although it will likely be more complicated in higher eukaryotes). The mouse circadian gene, *Clock*, is expressed throughout the animal (*35*), indicating that mammals may have oscillators throughout the body; also, a mammalian homolog of *Drosophila per* has been recently identified (*36*) and found to be localized throughout the body. Like the fly, much of the evidence for a central oscillator in mammals has come from observing a single output: behavior. Similarly, the simple interpretation of a master controlling oscillator may need to be revised with the closer examination of multiple outputs and isolated multiple oscillators.

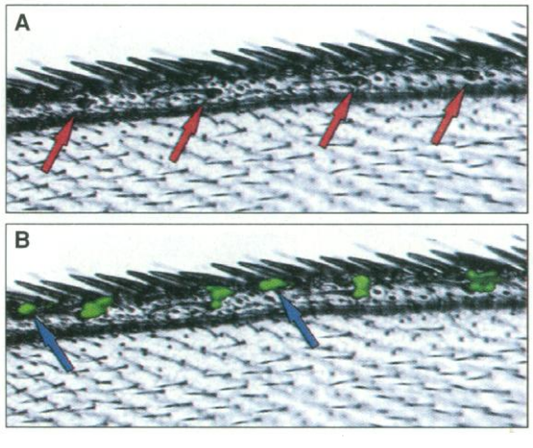

Fig. 3. GFP expression in wing chemosensory cells. (**A**) Detail of an anterior wing margin. Red arrows show individual chemosensory cells. (**B**) Superimposition of *per*-driven GFP fluorescence on the same wing margin. Signal co-localizes with the basal cells of the sensory bristles, as well as fluorescence from chemosensory cells on the opposite side of the wing margin (blue arrows).

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23. *per-luc* transgenics refer specifically to the *plo1a-1* line described in (*20*). The *per*-promoter–GAL4 fusion gene (*per*-GAL4) was made as has been done with other promoters (*37*). The construct contains the same 4.2-kb genomic fragment used in the *per-luc* fusion (*20*) upstream of the GAL4 gene and the hsp-70 terminator. This fragment was ligated into the P element transformation vector CaspeR4 (*38*). Six transformants with different insertion sites were generated. The spatial expression patterns of *per*-GAL4 in the adult head in these lines were studied by immunohistochemistry in flies carrying these GAL4 elements and either UAS-*lacZ* or UAS-Tau (*39*). Three of the six lines express GAL4 in various ectopic tissue locations as well as in a few of the normal *per*-expressing cells [see for example (*6, 40*)]. The other three lines were relatively normal in their expression patterns and had GAL4-mediated staining in several identified *per* neurons (cf. *6, 40*). However, GAL4 expression in these three lines exhibited some differences from the endogenous *per* expression, such as weak eye expression and ectopic expression in the brain's central complex. The line used in this study is one of the latter three. To generate the actual fluorescent flies, we crossed these *per*-GAL4 flies to another transgenic line that drives GFP from the yeast UAS sequence [UAS-GFP (*41*)].
24. Automated bioluminescent monitoring was carried out as previously described (*22*), with some modifications. Cultured explants were loaded individually into wells of black microtiter plates previously prepared with 100 μl of tissue culture medium (see below). Wells were covered with TopSeal with no ventilation hole. Bioluminescence was automatically counted for 15 s about once per hour. Data were analyzed with the Import and Analysis macro set for Microsoft Excel. Primary explants were removed from whole animals that had been entrained to a 12:12 LD cycle. Flies were briefly anesthetized with CO_2 and immediately separated into heads, thoraxes, and abdomens. Further dissections were performed on the individual body parts. Operations were carried out in a tissue culture medium consisting of (by volume) 85.9% S3 insect tissue culture media, 12% fetal bovine serum (heat inactivated for 30 min at 60°C), 1% penicillin-streptomycin mixture, 1% luciferin solution, and 0.1% insulin (1 mg/ml) solution. Cultures were monitored in the same solution throughout the experiment. The concentration of the luciferin solution varied from experiment to experiment, yielding a final concentration between 0.05 and 0.5 mM. Different concentrations of luciferin did not affect the period or phase of the rhythms, although higher concentrations led to brighter overall bioluminescence.
25. Flies were initially anesthetized with CO_2 followed by a drop of ether. Samples were observed with a long-pass GFP filter cube (Chroma) on an Olympus AX-70 upright microscope; images were collected with a color charge-coupled device camera (Hamamatsu). Images were processed with Adobe Photoshop.
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The Filamentous Phage pIV Multimer Visualized by Scanning Transmission Electron Microscopy

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A family of homomultimeric outer-membrane proteins termed secretins mediates the secretion of large macromolecules such as enzymes and filamentous bacteriophages across bacterial outer membranes to the extracellular milieu. The secretin encoded by filamentous phage f1 was purified. Mass determination of individual molecules by scanning transmission electron microscopy revealed two forms, a unit multimer composed of about 14 subunits and a multimer dimer. The secretin is roughly cylindrical and has an internal diameter of about 80 angstroms, which is large enough to accommodate filamentous phage (diameter of 65 angstroms).

Filamentous phage–encoded pIV is an outer-membrane protein required for phage assembly that is not a part of the virus particle. It has sequence similarity to a family of bacterial proteins that are essential components of the type II and type III protein secretion systems that have been identified in many pathogenic Gram-negative species (*1*, *2*). The members of this protein family (called secretins) are believed to play similar roles in mediating translocation of substrates across the outer membrane. *Gene IV* could be derived from a bacterial gene because some phages contain the gene in different parts of their otherwise colinear genomes and CTX, the lysogenic filamentous phage of *Vibrio cholerae* that encodes cholera toxin (*3*), lacks it entirely; CTX may use a *V. cholerae* secretin for phage assembly. In addition to a secretin, the type II, type III, and phage assembly systems include a protein containing an essential nucleotide-binding motif. The remaining components (~13 for type II secretion, ~20 for type III secretion, and 9 encoded by filamentous phage) are related within, but not between, systems.

Bacteria productively infected by filamentous phage remain viable and can continue to grow and divide indefinitely while producing and releasing phage particles. During assembly, the cytoplasmic single-stranded DNA phage genome is extruded through the cytoplasmic membrane where it becomes coated with the phage-encoded capsid proteins that reside in the cytoplasmic membrane before their incorporation into phage (*4*). Secretion across the outer membrane appears to be concomitant with assembly because periplasmic phage has not been detected. Thus, phage assembly and the type II and III systems all require transport of macromolecules across two bacterial membranes.

The pIV protein exists as a homomultimer that has been previously estimated to consist of 10 to 12 monomers (*5*). Several of the bacterial homologs form mixed multimers with pIV in vivo, implying structural relatedness and suggesting that they them-

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