J. Membrane Biol. 136, 333–342 (1993)

The Journal of
Membrane
Biology

Transfer of Dye among Salivary Gland Cells Is Not Affected by Genetic Variations of the *period* Clock Gene in *Drosophila melanogaster*

Kimberly K. Flint[1]*, Michael Rosbash[2], Jeffrey C. Hall[1]

[1]Department of Biology, Brandeis University, Waltham, Massachusetts 02254
[2]Howard Hughes Medical Institute, Brandeis University, Waltham, Massachusetts 02254

Received: 22 April 1993/Revised: 27 July 1993

Abstract. Larval salivary gland cells of *Drosophila melanogaster* were injected with a fluorescent dye to assess strengths of intercellular communication among such cells, as influenced by mutations at the *period* locus and by a *per* transgene. This clock gene had been reported to increase the extent of dye transfer when mutated such that it shortens the period of biological rhythms; the previous study also showed that a *per*-null mutant decreased the strength of transfer among salivary gland cells. Our re-examination of this feature of larval physiology—in observer-blind analyses, using the per^s and per^0 mutants as well as two *per*-normal strains—revealed no appreciable differences in extents of dye transfer among these four genotypes. These results are discussed in the context of emerging findings which suggest that the *period* gene's product controls pacemaker functioning as an intracellularly acting entity.

Key words: *Drosophila* — *per* mutants — *per* transgenic — Lucifer Yellow injections — Gap junctions

Introduction

The *period* gene of *D. melanogaster* was originally identified by mutations that cause defects in circadian rhythms (*reviewed by* Konopka, 1987, the discoverer of these mutants). Subsequently, several other phenotypic defects have been reported to be exhibited by one or more of the *per*-mutant-types, expressing either the short-period per^S, long-period per^L, or arrhythmic per^0 mutations (*reviews:* Hall & Kyriacou, 1990, Baylies et al., 1993). Only some of these abnormalities were overtly connected to temporal phenomena; some of these reported defects did not stand up to subsequent study (*reviews:* Hall, 1990; Jackson, 1993; *also see* Gailey, Villella & Tully, 1991).

One kind of *per*-mutant abnormality did not involve circadian rhythms per se; but it was uniquely provocative, because it suggested a cellular function for the clock-gene's product. The defects in question were altered levels of intercellular coupling among larval salivary gland cells, caused by the per^S and per^0 mutations (Bargiello et al., 1987). These were demonstrated by dye-injection experiments, as well as by measurements of electrical coupling and electrotonic spread. Such results led to the hypotheses that the circadian clock in *Drosophila* at other stages of its life cycle—pupae and adults—is comprised of several cells that would need to be in communication for the clock to run at all, and that there should be a particular "coupling strength" if the pace of the clock is to be correct, i.e., about 24 hr (Bargiello et al., 1987). Thus, if intercellular coupling were too strong, which was indeed reported for salivary glands taken from per^S larvae, this could make the overall clock run too fast; and a near absence of communication, as reported for per^0 glands, would disallow the separate cellular components of the pacemaker to have a circadian function.

This hypothesis about *per*'s action would fit in with certain kinds of "clock theories" (summarized recently by Klevecz & Bolen, 1993). These have in general proposed that circadian pacemaking in-

* *Present address:* Medical Research Facility, Department of Physiology and Biophysics, University of Vermont, Colchester, Vermont 05446

Correspondence to: J.C. Hall

volves separate component oscillators, each of which is not a 24-hr clock; but that coupling among the individual components forms the circadian structure. More specifically, high-resolution analyses of locomotor activity in *Drosophila* showed that *per*-null mutants exhibit ultradian, though usually "hidden," rhythmicities (*reviews:* Dowse & Ringo, 1992, 1993); as if the existence of high-frequency oscillators in the fly were revealed by eliminating coupling among such hypothetical entities. However, these putative ultradian oscillators were never explicitly hypothesized to reside in separate cells (Dowse and Ringo, 1992, 1993).

Our investigations of the *period* gene's action, which in relatively recent years have concentrated on molecularly monitored expression of, and the informational content encoded within, the *per* locus, have led us to a quite different model about what this gene product is doing (most recently articulated in Hardin, Hall & Rosbash, 1992; *see* Takahashi, 1992, for review). We view PER (the *per*-encoded protein) to be a transcription factor, which would act only in an intracellular manner. Some pieces of evidence related to this notion are that PER influences circadian fluctuations of *per* mRNA levels by a feedback loop (Hardin, Hall & Rosbash, 1990, 1992); and that PER is a predominantly nuclear protein in the adult brain (Liu et al., 1992), which houses the circadian clock controlling locomotor-activity rhythms in *Drosophila* at that stage of its life cycle (Ewer et al., 1992). In contrast, PER was reported to be a cell-boundary protein (possibly a membranous one, or an "extracellular matrix" factor) in larval salivary glands (Bargiello et al., 1987).

The "intercellular-coupler" *vs.* "transcription-factor" hypotheses on PER function are at such variance that we thought it worthwhile to re-examine the putative effects of *per* mutations on salivary gland physiology (*cf.* Bargiello et al., 1987). Another reason for looking into this matter once more is that we have never been able to observe *per* expression in larval salivary glands, either by in situ hybridization (Liu et al., 1988), immunohistochemistry (Siwicki et al., 1988), or by monitoring a β-galactosidase activity in larvae carrying a *per-lacZ* (fusion) transgene (Liu et al., 1988).

Our experiments aimed at the re-examination in question involved dye injections, followed by monitoring the extent (and, in effect, the rapidity) of the dye's spread. By subjecting larvae expressing four separate *per* genotypes to these tests, we were unable to observe or quantify any systematic differences in extents of dye transfer among the cells of this larval organ. Some of these findings were included in a recent abstract (Siwicki et al., 1992).

Materials and Methods

Drosophila Strains

The three elementary *per* genotypes used were per^{+}, per^{S}, and per^{01} (hereafter called per^{0}); these stocks were the same ones that had been made isogenic among each other by Gailey, Villella and Tully (1991). One additional strain was not isogenic with the other three: a transformed line called "13.2 : 2" (Citri et al., 1987), which carries 13.2 kb of genomic per^{+} DNA, inserted in chromosome *3*; the X chromosome in this strain carries per^{0} and the third chromosome $rosy^{506}$ (an eye-color mutation, used in the recovery of a fly carrying the transgene, whose per^{+} DNA is physically linked to cloned ry^{+} material). We used larvae homozygous for this autosomal insert, because circadian behavioral rhythms of adults expressing this genotype are almost identical to wild-type (i.e., 24 hr, instead of 24.5–25 hr, for "13.2 : 2" individuals carrying only one copy of the transgene; Dushay, Rosbash & Hall, 1992; M.K. Cooper, M.J. Hamblen-Coyle, J.E. Rutila, X. Liu, M. Rosbash, J.C. Hall, *submitted*). These four strains were maintained on the same medium used for rearing larvae (*see below*).

Larval Salivary-Gland Physiology

To obtain *Drosophila* larvae, mature (nonvirgin) adults emerging from a culture of per^{+}, "13.2 : 2," per^{0} or per^{S} were placed overnight (at 25°C, *ca.* 70% relative humidity), on a medium containing sucrose, cornmeal, yeast, agar, and Tegosept (a mold inhibitor) and allowed to lay eggs for 24 hr. The cultures thus initiated were maintained for 5–6 days at 25°C; then, late third-instar larvae (at a stage when they were still crawling in the food) were picked to initiate a given physiological experiment.

The genotypes of all larvae in these experiments were unknown to the investigator. Moreover, the four strains were recoded frequently (over the course of this study), to eliminate the possibility that physiological trends associated with a given (one-time-coded) strain might be subtly discerned.

The dye-fill and spread-monitoring procedures were carried out at room temperatures, which varied from 19 to 25°C; 94% of the tests were done in the 20-24°C range. These experiments were carried out between 10 a.m. and 7 p.m. The temperature and time accompanying a given injection were noted.

To obtain a larva's salivary gland, the animal was placed in a drop of modified Shen's solution (Loewenstein & Kanno, 1964) on a depression slide and dissected; for this, one pair of forceps was maneuvered to grasp the midsection of the larvae; another pair grasped and pulled the mouth hooks as they were extended, allowing the salivary glands to be extracted from the animal. Only glands that had been isolated from the larva within one hour (after removal from a culture) were used in these experiments. Once the salivary glands were isolated, the attached fat bodies were removed so that they would not interfere with the injection or observation of the gland. The tissue was then placed in a drop of Shen's solution on a Petri dish that had been coated with poly-L-lysine, which caused the gland to stick to the bottom of the dish. Once the gland was firmly adhered, the dish was filled with Shen's.

The microelectrodes (1.2 mm OD, 0.6 mm ID) used in these experiments were filled with 4% Lucifer Yellow (in 0.1% LiCl), via capillary action, and back-filled with 3 M LiCl. The electrode was manipulated to impale individual cells, which were deliberately chosen to be either within the posterior one-third of the gland

or in its anterior one-third (*see* Results). The resting potential for each impaled cell was noted. We regarded "robust" resting potentials for cells of this type to be −28 mV or below (*cf.* Kislov & Veprintsev, 1971). We also filled and monitored dye-spread for several cells whose resting potentials ranged from −27 to −12 mV. The data from these cells are tabulated separately from those whose resting potentials were more robust.

Once a stable resting potential was reached (*see below*), the dye was iontophoretically injected into the cell for 30 sec, at 10 nA per 300-msec pulse (1 Hz). Observations were made through an inverted fluorescence microscope: 1 or 2 min after the start of dye injection, then again at 8 min from that start. At these time points, photographs were taken (as backups to the cell counts described below), with Ilford XP1 and Ilford XP2 iso400 black-and-white film (exposure times, 30 sec).

The "extent of dye spread" was quantified by looking through the microscope and counting the numbers of cells that contained detectable fluorescence; 1 was then subtracted from a given number so counted, to yield the "extent-of-spread" figure. This simple subtraction was performed because the injected cell per se was usually not identifiable, especially in an instance where several cells contained a signal. A preliminary statistic was applied to the dye-spread values from all experiments (*see* Table 3), i.e., a Shapiro-Wilk W test; it showed that the distribution of these scores was not "normal"; therefore, subsequent statistical comparisons were performed using Wilcoxon/Kuskal-Wallis rank-sums (nonparametric) tests.

Results

It is well established that salivary gland cells of *Drosophila* larvae are dye-spread and electrically coupled (*review:* Berendes & Ashburner, 1978). The study by Bargiello et al. (1987) augmented earlier findings by analyzing three *per* genotypes. The seminal observations that apparently prompted the electrophysiological studies accompanying this study (Bargiello et al., 1987) involved extents of intercellular Lucifer Yellow (LY) spread; that dye had been injected into individual cells of salivary glands dissected from 3rd-instar larvae, which expressed either of two *per*-mutations or the normal allele of this gene. We attempted to repeat these experiments by performing a series of dye-fills and visually monitoring the extents of LY spread.

Dye Spread Among *per*-Normal Salivary Gland Cells

When the results of observer-blind experiments were decoded with regard to genotypes analyzed (*see* Materials and Methods), the numbers of dye-containing cells that were counted—after injecting individual cells near the posterior ends of glands taken from either of the two "*per* normal" strains—revealed the following: (i) most of the fills did lead to dye spread within 1–2 min of injecting the cells (1st *vs.* 3rd data column, tops of Tables 1 and 2); (ii) there was considerable variability in the extents of spread from gland to gland within a given genotype; this usually ranged from one cell to a half-dozen or so (quantified as "number of filled cells minus one," the latter being the initially injected cell); and (iii) there were no systematic differences between the data from the per^+ wild-type *vs.* the transgenic *per*-normal glands, notwithstanding the nonisogenicity of these two *per*-normal strains. "No difference" refers to experiments involving cells with membrane potentials ≤ -28 mV (Table 1) and others falling in the −27 to −12 mV range (Table 2).

Observations were also made 8 min post-injection, to see if a genotypic difference (*see below*) would manifest itself when the glands were given more time to permit putatively maximal dye spreads. These data are in the bottom halves of Tables 1 and 2. There, it can be seen that "extents of spread" did not increase with time (which would have been the case if a "None−−>spread" category were to have been tabulated). Instead, they decreased, with appreciable numbers of glands that had revealed dye spread at 1 or 2 min dropping down to categories in which a signal was seen in but one cell or even in none of them; another category (appearing at the 8-min time points) was one in which even the fluorescence within the originally injected cell had faded by that time. The overall decreases in number of fluorescing cells at 8 min post-injection are probably due to fading of the dye (as just implied)—a minor contribution to this decrease—and to the dye spreading so extensively that its concentration in a given cell was too low to yield a signal. In any event, the high degrees of variability in the data from these 8-min time points (*see* the profusion of columns at the bottoms of Tables 1 and 2) suggest to us that the results from the two earlier time points are most meaningful, in terms of comparing *per*-mutant to the normal genotypes (*see below*).

Salivary gland cells of *Drosophila* larvae have different electrical properties in anterior compared to posterior regions of the organ (van Venrooij et al., 1974). Therefore, a different set of dye injections was performed for these two regions of the glands, and the data are presented separately in Tables 1 and 2. Anterior injections tended to lead to less spread, evident as a decrease in the average numbers of cells filled and an increase in the proportions of glands in which no detectable dye-transfer occurred; once again, there was a great deal of variability in both parameters (Tables 1 and 2). That dye spread is less extensive between anterior cells is consistent with one element of the data collected by K.K. Siwicki and D.C. Spray (*see* Siwicki et al., 1992), whereby "small" salivary glands yielded relatively less extensive spreading after LY injection.

Table 1. Intercellular spread of Lucifer Yellow observed after injecting individual salivary gland cells that had robust membrane potentials

1–2 min post-injection

Genotype	Glands with dye spread (*n*) [avg. extent]	Range (no. cells)	Glands with no spread (*n*)
Posterior			
per^+	5 [4.2]	2–6	1
per^0; 13.2	5 [3.4]	1–8	1
per^0	10 [3.5]	1–8	1
per^S	4 [2.0]	2	1
Anterior			
per^+	3 [1.0]	1	1
per^0; 13.2	4 [1.8]	1–2	10
per^0	3 [5.0]	4–6	3
per^S	5 [3.0]	1–5	4

8 min post-injection

Genotype	Spread	Range	Spread−−> 1 cell	Spread−−> no signal	No spread	None−−> fade
Posterior						
per^+	1 [4.0]	4	2	2	0	1
per^0; 13.2	1 [2.0]	2	2	2	0	1
per^0	4 [2.8]	1–5	2	4	0	1
per^S	0	0	1	3	0	1
Anterior						
per^+	1 [1.0]	1	1	1	0	1
per^0; 13.2	0	0	1	3	4	6
per^0	2 [5.5]	5–6	0	1	2	1
per^S	1 [1.0]	1	1	3	3	1

These cells within salivary glands dissected from 3rd-instar larvae had membrane potentials of ≤ -28 mV, prior to LY injection. The genotypes are normal (+) or mutant (*0*, *S*) alleles of the *period* (*per*) gene; *13.2* designates a DNA fragment cloned from a normal version of the *per* locus and transformed into a mutant per^0 strain. The numbers under "Glands with dye spread" indicate the number of glands in which, after injecting one cell per dissected organ, such spread occurred; then [in brackets] is given the average number of cells *to which* the dye was transferred. This information is followed by the ranges of numbers of cells to which dye was transferred. For the observations made 1–2 min after initiating the injections, the last column lists the numbers of glands in which only the injected cell contained a signal. For the 8-min observations, headers for the first two data columns are shorthand versions of the first two in the top half of the table; the other headers involve the complicated dynamics of how signal spreading and detection were broken down: into instances of glands in which dye spread had been observed at 1–2 min, but only one cell contained a signal 6 min later ["Spread−−>1 cell"]; or, transfer was observed at 1–2 min, followed by no fluorescing cells detectable later on ["Spread−−>no signal"]; or, there was still no spread observed (i.e., none at 1–2 or at 8 min post-injection), but the injected cell still fluoresced ["No spread"]; or, finally, a no-spread instance faded to a case in which no signal at all remained in the gland by 8 min ["None−−>fade"]. Within the top and the bottom halves of the table, the data are broken down as to whether a cell in the posterior one-third of the larval gland was injected, or whether a cell in the anterior one-third was so chosen.

Figure 1 exemplifies the kinds of results described above (with reference to their tabulated form), depicting instances of dye transfer and no dye transfer for LY injections into both the posterior and the anterior regions of salivary glands taken from the per^+ wild-type strain.

per-Mutant Cells

The effects of a "fast-clock" and a "no-clock" *per* mutation were compared to the normal larval salivary gland physiology in a series of blind experiments. The numerical data are once more in Tables 1 and 2; and examples of dye spreading, under the influence of per^S and per^0 (compared to the per^+ transgene), are shown in Fig. 2.

Although the intra-genotype variability for the mutants was similar to that of the two *per*-normal types, no systematic difference was discernible among the four strains for the 1–2-min or 8-min (post-injection) time points. An example of these mutant-based findings is that there was nominally more extensive spreading from injections of per^0

Table 2. Intercellular spread of Lucifer Yellow observed after injecting individual salivary gland cells with less robust membrane potentials

1–2 min post-injection

Genotype	Glands with dye spread (*n*) [avg. extent]	Range (no. cells)	Glands with no spread (*n*)
Posterior			
per^+	5 [4.0]	1–6	2
per^0; 13.2	7 [3.5]	1–8	0
per^0	12 [4.1]	2–7	0
per^S	2 [5.5]	2–9	3
Anterior			
per^+	2 [3.0]	1–5	0
per^0; 13.2	0	0	2
per^0	5 [1.4]	1–2	5
per^S	1 [1.0]	1	0

8 min post-injection

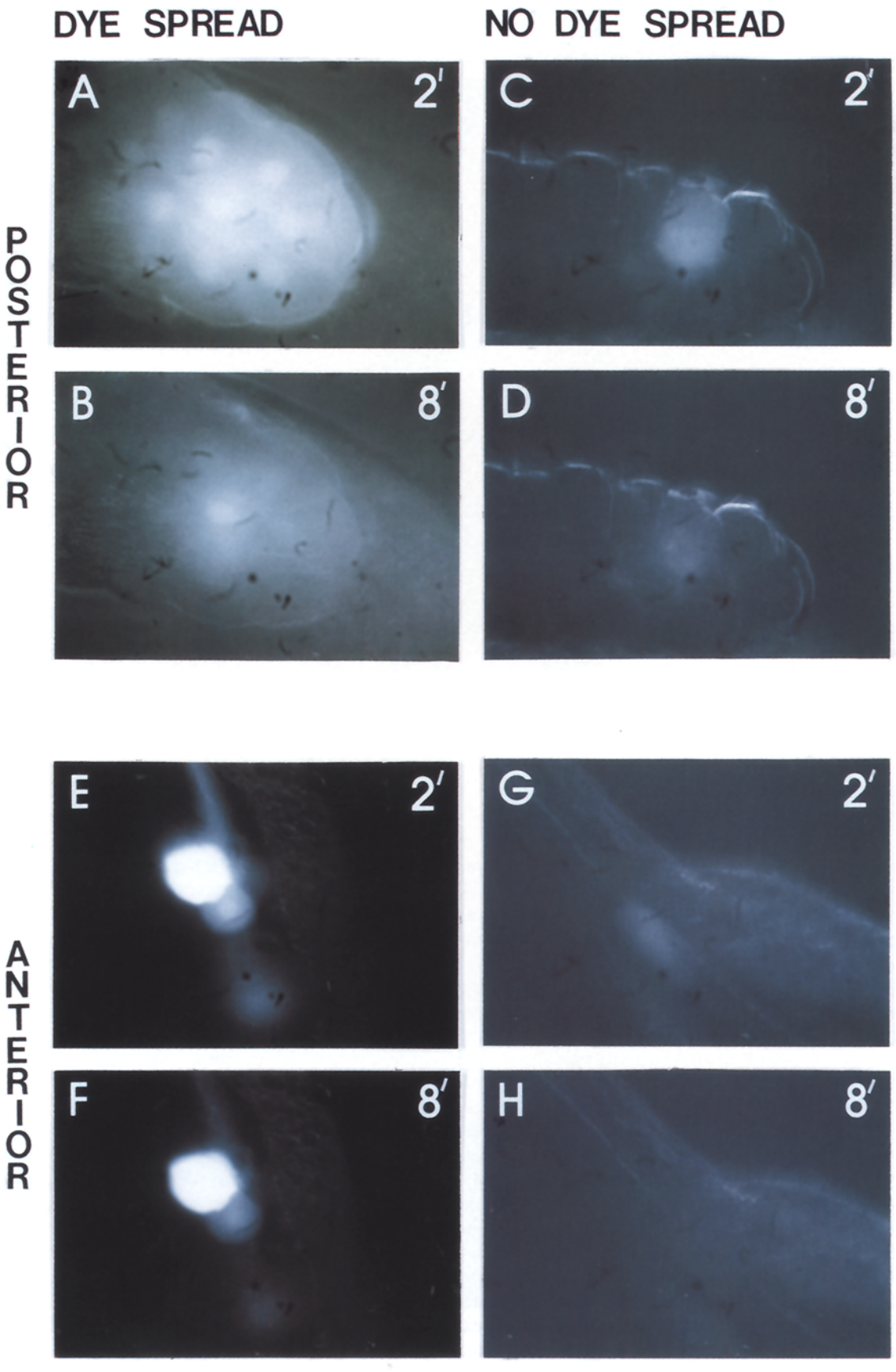
Genotype	Spread	Range	Spread–> 1 cell	Spread–> no signal	No spread	None–> fade
Posterior						
per^+	3 [5.0]	5	1	1	1	1
per^0; 13.2	2 [2.3]	2–3	1	0	0	0
per^0	5 [2.8]	1–5	5	2	1	0
per^S	1 [9.0]	9	0	1	2	1
Anterior						
per^+	1 [1.0]	1	1	0	0	0
per^0; 13.2	0	0	0	0	1	1
per^0	4 [1.3]	1–2	0	1	4	1
per^S	1 [1.0]	1	0	0	0	0

The design of these data listings is the same as in Table 1. Here, however, the resting potentials of the cells to be injected were in the range of −12 to −27 mV.

compared to per^S cells, in the cases involving resting potentials ≤ −28 mV (top half of Table 1); this apparent genotypic difference is the opposite of what was reported by Bargiello et al. (1987).

When the ambient temperatures accompanying the injections and dye monitorings (*see* Materials and Methods) were taken into account, there was no substantial effect of this environmental variable on (i) extents of dye spread, or (ii) the genotypically based conclusions (e.g., neither mutant could be discriminated from the normals in the data collected at relatively low temperatures). There was a slight tendency for increased dye transfer to occur at higher temperature: The overall average number of fluorescing anterior cells (all genotypes), for fills done at 19–20°C (the lower end of the temperature range for those experiments), was 2.6 (including instances of no dye spread, given a score of 1-cell-filled for this purpose); for anterior fills done at 23–24°C, this value was 3.3. The numbers of posterior fills performed at the temperature extremes pertaining to those experiments—21–22°C and 24–25°C—were 4.0 and 4.4, respectively. No particular contribution of any *per* allele to this mild temperature-dependent variation could be discerned.

Whereas the dye injections were not performed across the entire daily cycle, no substantial differences in extents of spread resulted from fills that occurred relatively early in the morning *vs.* the middle of the day *vs.* the evening: After dividing the portion of a day (during which the experiments were performed, *see* Materials and Methods) into three equal segments, the average numbers of posterior cells to which dye did spread were found to be 4.1, 4.1, and 3.4 (early–>late); for the anterior cells, these values were 2.0, 2.4, and 2.6. The different genotypes (though known only by their blind codes) were deliberately intermingled when performing experiments within a given part of the day; taking into account the temporal variation accompanying such experiments did not lead to enhanced discrimination among the three different classes of *per* genotype.


DYE SPREAD
NO DYE SPREAD
POSTERIOR
ANTERIOR
A
2′
B
8′
C
2′
D
8′
E
2′
F
8′
G
2′
H
8′

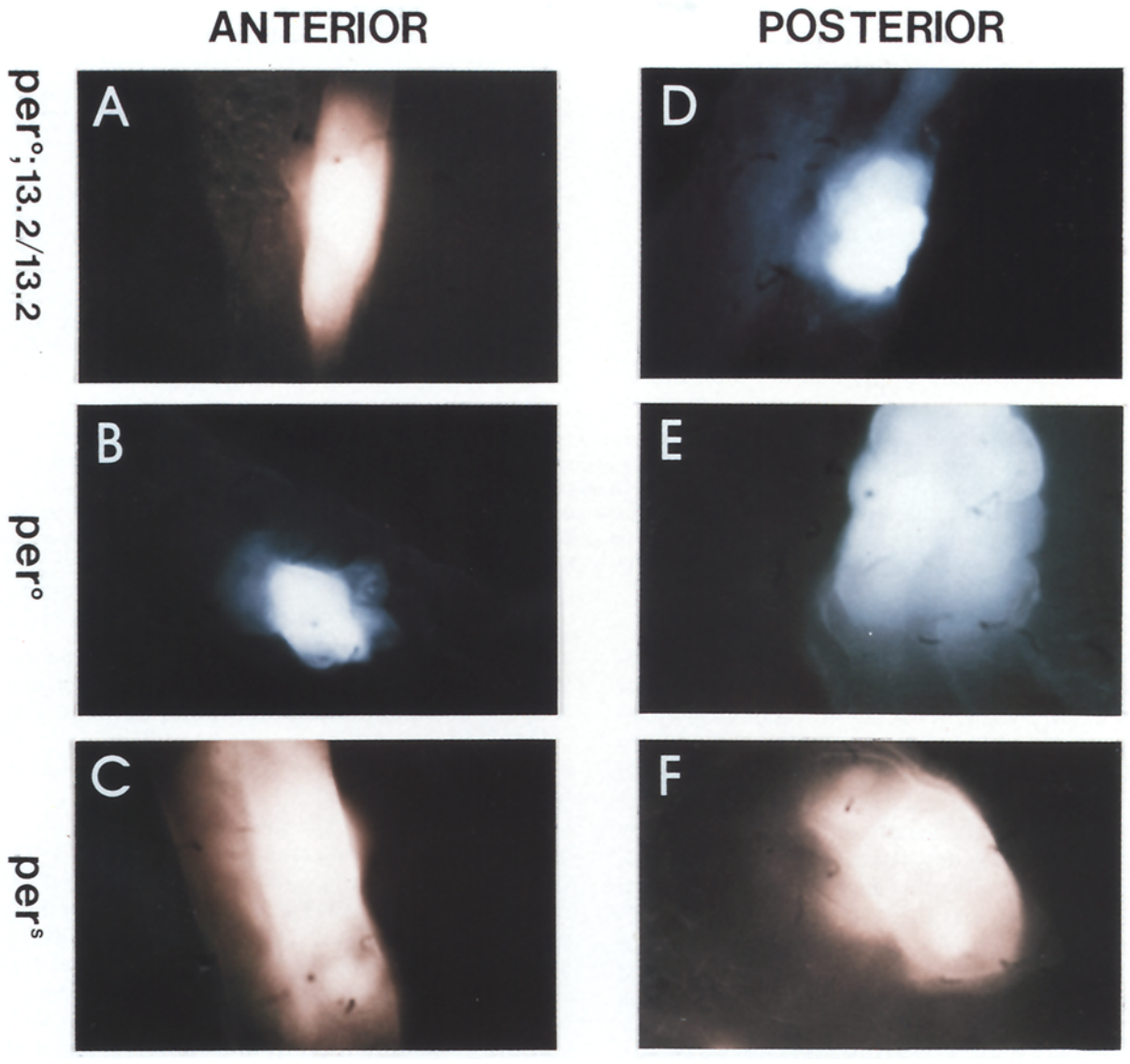


Fig. 2. Lucifer-Yellow transfers among *per* mutant and *per*-transgenic salivary gland cells. These examples show instances in which dye was transferred beyond the injected cell (in all six cases), as photographed 1.5 min after the end of LY injection. It is difficult in these images to resolve the numbers of transferred-to cells, which is why the counts (given in the Tables) were made by viewing these salivary glands through the microscope. Nevertheless, two of these panels reveal that, in glands from the arrhythmic per^0 mutant, a mediocre extent of LY transfer might be observed (*B*, though this is typical of anterior injections involving any of these genotypes); but quite extensive transfer could also occur (*E*).

Fig. 1. Lucifer-Yellow transfers, or the lack thereof, among wild-type salivary gland cells. The dye was injected into one cell of each 3rd-instar gland (dissected from per^+ larvae), either in the *POSTERIOR* one-third of the organ (*A-D*) or the *ANTERIOR* one-third (*E-F*). The left-hand panels depict instances in which detectable dye-transfer occurred, as photographed 2 and 8 min after initiating the 30-sec LY injections. (*A*) A case of transfer to six cells beyond the originally injected one; (*B*) same gland, 6 min later, showing fading or signal-reducing dispersion of the signal. (*E*) and (*F*), similar signal intensities (at both time points), for a case in which dye transfer occurred to only one cell beyond that injected initially. The right-hand panels show examples in which only the posterior or anterior injected cells were detected to contain LY; and in one such instance (*H*), the signal had faded to essentially zero (or dispersed itself into oblivion) by 8 min post-injection. The width of the distal end of the gland (visible in *A*) is about 0.1 mm

Table 3. Summary of dye-spread results

Genotype	Cells injected (*n*)	Cells with no dye spread (*n*)	Average extent of spread (no. cells ± SEM)
Posterior			
per^+	13	3	4.1 ± 0.5
per^0; 13.2	13	1	3.5 ± 0.7
per^0	24	2	3.8 ± 0.4
per^S	10	4	3.2 ± 1.1
Anterior			
per^+	6	1	1.8 ± 0.7
per^0; 13.2	16	12	1.8 ± 0.2
per^0	16	8	2.8 ± 0.7
per^S	10	4	2.7 ± 0.6

These data merge those from the previous two tables, with regard to the most readily interpretable "extent-of-spread" numbers, i.e., from the observations made 1–2 min post-injection. Thus, the dye-spread numbers from injected cells displaying relatively robust membrane potentials (top of Table 1) were pooled with those from the "−27 to −12 mV" cells (top of Table 2). The preliminary statistic applied to these cell counts (*see* Materials and Methods) showed that they were not normally distributed ($P < .001$); the nonparametric testing that was thereby necessitated revealed no significant differences among genotypes for the posterior injections ($\chi^2[3] = 2.72$, $P = 0.44$) and for the anterior ones as well ($\chi^2[3] = 2.05$, $P = 0.56$).

A summary statement about the four *per* genotypes is that the overall dye-spread values did not reveal even a vague tendency for anomalously increased intercellular communication in salivary glands taken from per^S larvae or decreased spread in this tissue as dissected from per^0 (*cf.* Bargiello et al., 1987). This conclusion can be appreciated by examination of the pooled data in Table 3. Note that, for the posterior fills, per^S gave nominally the least extent of spread; and that this extent for per^0 falls between the values for the two *per*-normal types (Table 3). Statistical analysis of these data revealed no difference among genotypes; the same statement pertains to the numbers from the anterior-cell fills (*see legend to* Table 3). Though values such as "1.8" (from wild-type and the transgenic) might look significantly different from the higher mutant-derived numbers (bottom of Table 3), the high degree of intra-genotype variability belied this possible inference. In any case, the previous study (Bargiello et al., 1987) leads to the expectation that our values from per^S should be appreciably higher than those from per^0 glands, which clearly did not obtain.

Discussion

The *per* Gene and Salivary-Gland Physiology

Our nonconfirmation of the original findings on *per*-related effects on intercellular coupling seems surprising. This is because the earlier study (Bargiello et al., 1987) involved not only dye-spread assessments, but also physiological measurements of electrical coupling and junctional conductance between cell pairs, all of which were reportedly reduced for per^0 cells and increased in per^S salivary glands, relative to wild-type.

Yet, neither our experiments nor another subsequent series of dye injections (Siwicki et al., 1992) could reproduce the results of Lucifer Yellow fills reported previously (Bargiello et al., 1987). For their part, K.K. Siwicki and D.C. Spray filled salivary glands cells from larvae of the same *per*-related strains we investigated (after we sent coded versions of those four *Drosophila* stocks to them), as well as cells of larval glands from a separate group of nonisogenic (wild-type, per^S, and per^0) strains; they found no differences in the extents of dye spread among these genotypes in observer-blind experiments (Siwicki et al., 1992; K.K. Siwicki, *personal communication*). All of these recent investigations showed, upon decoding of the various data sets, that there is a high degree of genotypically independent variability in the extents of dye transfer. The sources of these variations are unknown; they do not seem to involve cell size and/or intra-gland position, temperature, time of day, or larval age (this report, and Siwicki et al., 1992).

Notwithstanding these recent data and their failure to reveal differences in extents of *dye* transfer among *per* genotypes, it seems possible that replication of the electrophysiological *measurements* might confirm that *per* mutations affect intercellular communication within the salivary gland. Nevertheless, the original group of investigators subsequently

abandoned the entire matter of this gene's influence on communication involving cells of this larval organ (Saez et al., 1992).

Implications of the Absence of Evidence for PER Acting Intercellularly

The cell-boundary staining of PER protein in (or among) the cells of this tissue (Bargiello et al., 1987) is quite different from the predominantly nuclear signal seen (immunohistochemically) in the peripheral and central nervous system of adults (Liu et al., 1988, 1992; Saez & Young, 1988; Siwicki et al., 1988). Nuclear localization would be consistent with the notion that PER acts intracellularly as a clock factor (*see discussion in* Liu et al., 1992, and Hardin, Hall & Rosbash, 1992). This model, whose additional components implicate PER as a transcription factor, is buttressed by the nature of a part of the *per* gene sequence: Those nucleotides would translate into a *ca.* 270 amino-acid subset of PER, which is a motif shared by three other gene products known to be involved in transcriptional control (*reviews:* Crews et al., 1992; Takahashi, 1992).

This is not the place to discuss additional features of the model, but if it proves to have force as to how PER acts as a clock factor, a clear implication of these notions will be that *individual per*-expressing cells within the *Drosophila* brain contain *circadian* oscillators. Such cells may need to communicate with one another, but only in order that their individual pacemakers remain in synchrony. Varying degrees of such communication are (in our view) unlikely to be influenced by altered *per* action, although a different etiology for noncommunication among the clock cells could lead to arrhythmicity at the level of the overall pacemaker structure and in terms of something like whole-animal behavior. Moreover, increased intercellular communication levels would not necessarily change the speed at which the daily clock is running, if that pace is, as we believe, not an "emergent" property of coupling among oscillators whose frequencies are far outside the circadian range (*cf.* Klevecz & Bolen, 1993).

If circadian pacemaking in *Drosophila* is a property of individual cells, then this would be consistent with the way that kind of timekeeping almost certainly occurs in unicellular organisms (*review:* Edmunds, 1988). Moreover, a 24-hr clock has recently been shown to be running within individual cells that make up the circadian clock of a metazoan (Michel et al., 1993).

We are extremely grateful for the experimental hospitality provided by D.A. Goodenough, in whose laboratory (at Harvard Medical School)—and as a result of whose training—these experiments were carried out. We thank V.K.L. Merrill and B. Frisch for their "coding and re-coding" of *per* genotypes; T. Tully for helping with statistics; and D.A. Goodenough, K.K. Siwicki, and C.-F. Wu for comments on the manuscript. This work was supported by grants from the National Institutes of Health GM-33205 (to M.R. and J.C.H.) and GM-18974 (to D.A.G.).

References

Bargiello, T.A., Saez, L., Baylies, M.K., Gasic, G., Young, M.W., Spray, D.C. 1987. The *Drosophila* clock gene *per* affects intercellular junctional communication. *Nature* **328**:686–691

Baylies, M.K., Weiner, L., Vosshall, L.B., Saez, L., Young, M.W. 1993. Genetic, molecular, and cellular studies of the *per* locus and its products in *Drosophila melanogaster. In:* Molecular Genetics of Biological Rhythms. M.W. Young, editor. pp. 123–153. Marcel Dekker, New York

Berendes, H.B., Ashburner, M. 1978. The salivary glands. *In:* The Genetics and Biology of Drosophila. M. Ashburner and T.R.F. Wright, editors. Vol. 2b. pp. 453–498. Academic, London

Citri, Y., Colot, H.V., Jacquier, A.C., Yu, Q., Hall, J.C., Baltimore, D., Rosbash, M. 1987. A family of unusually spliced and biologically active transcripts encoded by a *Drosophila* clock gene. *Nature* **326**:42–47

Cooper, M.K., Hamblen-Coyle, M.J., Rutila, J.E., Liu, X., Rosbash, M., Hall, J.C. 1993. Dosage compensation of the *period* gene in *Drosophila melanogaster. (submitted)*

Crews, S., Franks, R., Hu, S., Matthews, B., Nambu, J. 1992. *Drosophila single-minded* gene and the molecular genetics of CNS midline development. *J. Exp. Zool.* **261**:234–244

Dowse, H.B., Ringo, J.M. 1992. Do ultradian oscillators underlie the circadian clock in *Drosophila? In:* Ultradian Rhythms in Life Processes. D. Lloyd and E.L. Rossi, editors. pp. 105–115. Springer-Verlag, London

Dowse, H.B., Ringo, J.M. 1993. Is the circadian clock a "meta-oscillator?" Evidence from studies of ultradian rhythms in *Drosophila. In:* Molecular Genetics of Biological Rhythms, M.W. Young, editor. pp. 195–220. Marcel Dekker, New York

Dushay, M.S., Rosbash, M., Hall, J.C. 1992. Mapping the *Clock* rhythm mutation to the *period* locus of *Drosophila melanogaster* by germline transformation. *J. Neurogenet.* 8:173–179

Edmunds, L.N. Jr. 1988. Cellular and Molecular Bases of Biological Clocks. Springer-Verlag, New York

Ewer, J., Frisch, B., Hamblen-Coyle, M.J., Rosbash, Hall, J.C. 1992. Expression of the *period* clock gene within different cell types in the brain of *Drosophila* adults and mosaic analysis of these cells' influence on circadian behavioral rhythms. *J. Neurosci.* **12**:3321–3349

Gailey, D.A., Villella, A., Tully, T. 1991. Reassessment of the effect of biological rhythm mutations on learning in *Drosophila melanogaster. J. Comp. Physiol.* **169**:685–697

Hall, J.C. 1990. Genetics of circadian rhythms. *Annu. Rev. Genet.* **24**:659–697

Hall, J.C., Kyriacou, C.P. 1990. Genetics of biological rhythms in *Drosophila. Adv. Insect Physiol.* **22**:221–298

Hardin, P.E., Hall, J.C., Rosbash, M. 1990. Feedback of the *Drosophila period* gene product on circadian cycling of its messenger RNA levels. *Nature* **343**:536–540

Hardin, P.E., Hall, J.C., Rosbash, M. 1992. Circadian oscillations of period gene mRNA are transcriptionally regulated. *Proc. Nat. Acad. Sci. USA* **89**:11711–11715

Jackson, F.R. 1993. Circadian rhythm mutants of *Drosophila*. *In:* Molecular Genetics of Biological Rhythms. M.W. Young, editor. pp. 91–121. Marcel Dekker, New York

Kislov, A.N., Veprintsev, B.N. 1971. Electric characteristics of the cellular and nuclear membranes of the salivary gland cells of *Drosophila funebris* larvae. *Comp. Biochem. Physiol.* **39A**:521–529

Klevecz, R.R., Bolen, J.L. Jr. 1993. Dynamic analysis of period mutants in *Drosophila:* how a precise circadian clock might emerge from a tissue composed of chaotic cellular oscillators. *In:* Molecular Genetics of Biological Rhythms. M.W. Young, editor. pp. 221–253. Marcel Dekker, New York.

Konopka, R.J. 1987. Genetics of biological rhythms in *Drosophila*. *Annu. Rev. Genet.* **21**:227–236

Liu, X., Lorenz, L., Yu, Q., Hall, J.C., Rosbash, M. 1988. Spatial and temporal expression of the *period* gene in *Drosophila melanogaster*. *Genes Devel.* **2**:228–238

Liu, X., Zwiebel, L.J., Hinton, D., Benzer, S., Hall, J.C., Rosbash, M. 1992. The *period* gene encodes a predominantly nuclear protein in adult *Drosophila*. *J. Neurosci.* **12**:2735–2744

Loewenstein, W.R., Kanno, Y. 1964. Studies on an epithelial (gland) cell junction. *J. Cell Biol.* **22**:565–586

Michel S., Geusz, M.E., Zaritsky, J.J., Block, G.D. 1993. Circadian rhythm in membrane conductance expressed in isolated neurons. *Science* **259**:239–241

Saez, L., Young, M.W. 1988. In situ localization of the per clock protein during development of *Drosophila melanogaster*. *Mol. Cell. Biol.* **8**:5378–5385

Saez, L., Young, M.W., Baylies, M.K., Gasic, G., Bargiello, T.A., Spray, D.C. 1992. *Per*—no link to gap junctions. *Nature* **360**:542

Siwicki, K.K., Eastman, C., Petersen, G., Rosbash, M., Hall, J.C. 1988. Antibodies to the *period* gene product of *Drosophila melanogaster* reveal diverse tissue distribution and rhythmic changes in the visual system. *Neuron* **1**:141–150

Siwicki, K.K., Flint, K.K., Hall, J.C., Rosbash, M., Spray, D.C. 1992. The *Drosophila period* gene and dye coupling in larval salivary glands: a re-evaluation. *Biol. Bull.* **183**:340–341

Takahashi, J.S. 1992. Circadian clock genes are ticking. *Science* **258**:238–240

van Venrooij, G.E.P.M., Hax, W.M.A., van Dantzig, G.F., Prijs, V., Denier van der Gon, J.J. 1974. Model approaches for the evaluation of electrical cell coupling in the salivary gland of the larva of *Drosophila hydei*. The influence of lysolecithin on the electrical coupling. *J. Membrane Biol.* **19**:229–252