Cell, Vol. 101, 647–656, June 9, 2000, Copyright ©2000 by Cell Press

The *Drosophila takeout* Gene Is a Novel Molecular Link between Circadian Rhythms and Feeding Behavior

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Summary

We report the characterization of a novel *Drosophila* circadian clock-regulated output gene, *takeout* (*to*). The *to* amino acid sequence shows similarity to two ligand binding proteins, including juvenile hormone binding protein. *to* mRNA is expressed in the head and the cardia, crop, and antennae—structures related to feeding. *to* expression is induced by starvation, which is blocked in all arrhythmic central clock mutants, suggesting a direct molecular link between the circadian clock and the feeding/starvation response. A *to* mutant has aberrant locomotor activity and dies rapidly in response to starvation, indicating a link between locomotor activity, survival, and food status. We propose that *to* participates in a novel circadian output pathway that conveys temporal and food status information to feeding-relevant metabolisms and activities.

Introduction

Circadian (24 hr) rhythms are characteristic features of almost all life forms, from prokaryotes to humans. The circadian clock consists of a feedback loop in which clock genes are rhythmically expressed, giving rise to cycling levels of mRNA and protein. In *Drosophila*, there are five well-characterized genes that are believed to participate in central pacemaker control: *period* (*per*) (Konopka and Benzer, 1971; Bargiello and Young, 1984; Reddy et al., 1984), *timeless* (*tim*) (Sehgal et al., 1994; Gekakis et al., 1995; Sehgal et al., 1995; Zeng et al., 1996), *Clock* (*clk*) (Allada et al., 1998; Darlington et al., 1998), *cycle* (*cyc*) (Rutila et al., 1998), and *doubletime* (*dbt*) (Kloss et al., 1998; Price et al., 1998). Although their precise biochemical functions are not completely certain, PER and TIM probably function directly in the negative regulation of transcription (Darlington et al., 1998; Lee et al., 1998). CLK and CYC are bHLH-PAS transcription factors that activate transcription of *period* and *timeless* via the E box motifs in the promoters of these genes (Allada et al., 1998; Darlington et al., 1998; Lee et al., 1998; Rutila et al., 1998). *dbt* encodes a casein–kinase I homolog that has been suggested to regulate *per* phosphorylation and accumulation (Kloss et al., 1998; Price et al., 1998). *Drosophila* clocks display conservation with mammalian clocks, even at the sequence level: many *Drosophila* clock components have one or more mammalian homologs that are suggested to play similar roles in mammalian rhythms (Dunlap, 1999; Lowrey et al., 2000) .

In addition to remarkable progress made in understanding pacemaker molecular mechanisms, a molecular understanding of circadian outputs is also rapidly advancing. For example, rhythmic expression of RNAs or proteins has been characterized in a variety of species (e.g., Loros et al., 1989; Green and Besharse, 1996; Van Gelder and Krasnow, 1996; Rouyer et al., 1997; Blau and Young, 1999). Although the function of most of these output rhythm genes is unknown, the *Drosophila lark* gene encodes an RNA binding protein involved in the regulation of eclosion (McNeil et al., 1999), and the mammalian transcription factor DBP exhibits remarkable circadian oscillations and has been shown to control the circadian expression of known downstream genes (Lavery et al., 1999). However, the mechanism(s) by which the central clock conveys temporal information to downstream behavioral and physiological processes is largely unknown. Both direct neural connections and humoral control have been proposed (Stehle et al., 1993; Silver et al., 1996). Recent experiments have emphasized potent circadian activities in serum, suggesting that cycling humoral molecules constitute the connection between the brain and the peripheral pacemakers in mammals (Balsalobre et al., 1998).

There are now several hormones known to be involved in circadian rhythms. Arguably, the best known example is melatonin (Cassone, 1998). Others include vasopressin (Reppert et al., 1987), leptin (Ahima et al., 1998), and hypocretin (Faraco et al., 1998). These hormones are involved in controlling different behavioral and physiological output activities. Vasopressin, an antidiuretic neuropeptide involved in salt and water balance, has been suggested to be directly controlled by the central clock transcription factor heterodimer CLK-BMAL1, the mammalian homolog of CLK-CYC (Jin et al., 1998). A similar picture has recently emerged for DBP (Ripperger et al., 2000).

In *Drosophila*, the humoral control of circadian behavior is indicated by a classic transplantation experiment (Handler and Konopka, 1979). A first candidate peptide factor has been proposed (Renn et al., 2000), and very recent results indicate that this putative hormone (PDF) is necessary for free-running locomotor activity rhythms (Renn et al., 2000). Recent evidence suggests that *pdf* expression is controlled by the central pacemaker (Blau and Young, 1999; Park et al., 2000). It would not be surprising if other hormones important for other specific circadian output pathways were found.

The sequence of a novel clock-regulated gene, *takeout* (*to*), is suggestive of a second hormone involved in a circadian output pathway. *to* was found in a PCR-based cDNA subtraction screen. *to* mRNA levels undergo daily cycling with a novel phase, and they are downregulated or undetectable in all circadian mutants

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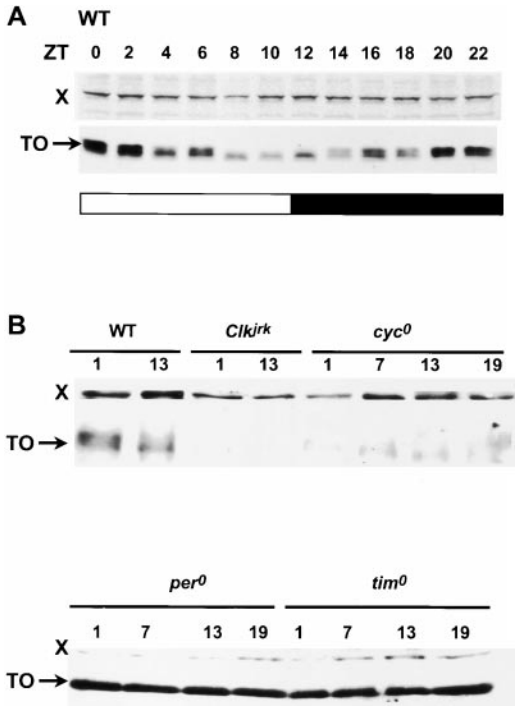

Figure 1. TO Expression Profiles

(A) Western blot showing cycling of TO. Open bar represents the time when lights were on (ZT0–12) and off (ZT12–24), respectively. "X" refers to a cross-reacting band.

(B) Western blot showing the downregulation of TO in various circadian mutants compared to wild-type flies (WT).

tested. This reflects unusual circadian transcriptional regulation, which takes place principally in most clock cells within the head (W. V. So et al., submitted). In this paper, we report several observations that link *to* to feeding. In the body, *to* is localized to structures related to feeding and olfaction: the alimentary canal in the cardia, the crop, and the antennae. The *to* protein (TO) sequence resembles a group of lipophilic ligand binding proteins. It is circadianly regulated, and its expression is induced by starvation, especially in organs related to feeding. Although there is no effect of a *to* mutant on locomotor activity rhythms, there are mutant phenotypes, which indicates a relationship between starvation, locomotor activity, and survival. We propose a novel circadian regulatory pathway in which *to* conveys temporal and food status information to feeding activities, including locomotor activity.

Results

TO Shows Daily Cycling Expression

to mRNA levels oscillate during a circadian cycle (W. V. So et al., submitted). To confirm that TO protein levels also oscillate, a TO-specific antibody was generated. Immunoblotting results are consistent with the mRNA data (Figure 1). TO levels exhibit daily oscillations and peak at around ZT21 to ZT2, a 3 to 4 hr delay from the mRNA peak (W. V. So et al., submitted). The effects of central clock mutations on TO were similar to the effects on *to* RNA: in all four arrhythmic clock mutations, the protein did not cycle. Among the different clock mutations, the highest levels were in per^{01} and tim^{01} and lowest in cyc^{01} and Clk^{jrk} (Figure 1B). The weak signal in cyc^{01} and Clk^{jrk} may be due to a basal level of noncycling TO expression. Alternatively, the residual signal may reflect a cross-reacting protein. The precise relationship of the two closely spaced bands (three or four in some experiments) has not been resolved (data not shown).

The Localization of TO

TO was found to be a member of a novel family of insect proteins. All members share sequence similarity throughout their entire lengths, and they are all about 250 amino acids in length. However, sequence analysis with an iterated BLAST search (Altschul et al., 1990) suggests that TO is also similar to two hydrophobic ligand binding proteins: hemolymph juvenile hormone binding protein JHBP (Touhara et al., 1993; Du et al., 1994) and JP29 (Wojtasek and Prestwich, 1995) from moths (Figure 2A). These two ligand binding proteins form a superfamily with TO and the 0.9 protein. The latter was previously studied in this laboratory, because it is encoded adjacent to the *period* gene and is expressed at eclosion, which is under clock control (Lorenz et al., 1989).

The sequence similarity between TO and the known ligand binding proteins extends throughout the complete sequence. Pairwise alignment of TO and the hemolymph JHBP from *Manduca sexta* shows an overall 24% identity and 54% similarity (data not shown). Interestingly, both categories of ligand binding proteins are small like the TO family members, with about 250 amino acids in all three proteins. In addition, some other TO family members show even higher similarity with the ligand binding proteins. For example, a BLAST search against a nonredundant database using family member AI142207 (W. V. So et al., submitted) also recognizes JP29, with an expect value of 2×10^{-13} (data not shown). Such transition homology further strengthens the evolutionary relationship between TO and these known ligand binding proteins.

Moreover, the N-terminal ligand binding fragment defined in JHBP is the best conserved region within the TO family (Touhara et al., 1993; Figure 2A). It also has an almost identical secondary structure prediction profile with that of *to* (data not shown). Strikingly, the two cysteines implicated in disulfide bond formation and ligand binding are absolutely conserved throughout the family (Wojtasek and Prestwich, 1995), suggesting that TO also has a ligand binding property.

Phylogenetic analysis using both maximum likelihood and distance matrix methods showed that TO is more closely related to hemolymph juvenile hormone binding proteins than to the nuclear JP29 (data not shown). In addition, the TO N-terminal 18 amino acids are predicted to be a signal peptide (SignalP V2.0, Nielsen et al., 1997). Therefore, TO may be a secretory protein. To address this possibility, *to* RNA and protein levels were measured in both bodies and heads (Figures 2B–2D). Consistent with the secretion hypothesis, *to* body mRNA levels are very low despite significant body protein levels (Figure 2).

We considered that *to* body mRNA levels might be

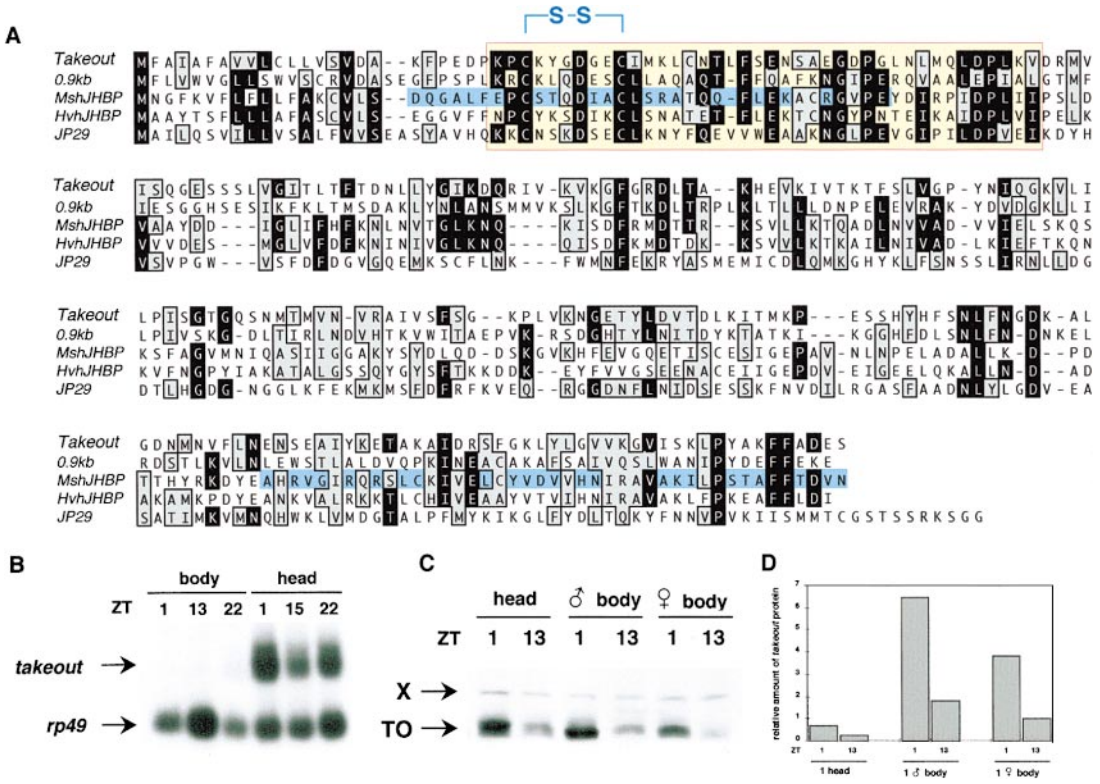

Figure 2. TO Is a Secretory Protein Expressed Mainly in the Head

(A) Sequence alignment of the *takeout* family and with the ligand binding proteins. Multiple sequence alignment of *to*, *0.9kb*, the hemolymph juvenile hormone binding protein from *Manduca sexta* (MshJHBP, GenBank accession number A40668), the hemolymph juvenile hormone binding protein from *Heliothis virescens* (HvhJHBP, GenBank accession number AAA68242), and JP29 (GenBank accession number AAA21588). The hormone binding fragments identified by peptide mapping in MshJHBP (Touhara and Prestwich, 1992) are highlighted in blue. The disulphide bond suggested in HvhJHBP (Wojtasek and Prestwich, 1995) is indicated. The box indicates the region that has the highest sequence identity between *to* and the ligand binding proteins. The alignment was prepared with MacVector (Oxford Molecular Group). A further description of homologies to *to* is in W. V. So et al. (submitted).

(B) Northern blot on polyA mRNA (10 μg/lane) showing *to* mRNA is very low in the body. *rp49* is the internal control.

(C) Western blot showing TO protein in the head (unsexed) compared to the male and female bodies. Flies were entrained and collected at the times shown. Three heads and one quarter of a body-worth of proteins were used in each lane. "X" is a cross-reacting band.

(D) Quantification of TO expression from (B), showing the total amount of TO in one head and one body at the time specified.

low because of a very restricted tissue distribution. Indeed, in situ hybridization revealed that *to* mRNA was localized to just two areas of the alimentary canal: an inner part of the cardia and the crop (Figure 3). The cardia is a highly folded epithelial structure at the anterior end of the stomach in the thorax (Demerec, 1950). Its function has not been well defined in fruit flies. The crop is a saccular structure for storing and passing liquid food to the stomach (Demerec, 1950). There was also intense staining of *to* mRNA in the antennae (data not shown), which is an olfactory organ in insects. Therefore, *to* mRNA is present in structures related to feeding and smelling, in addition to being in the brain.

takeout Is Induced by Starvation

Encouraged by this localized expression pattern, we tested *to* involvement in feeding by measuring expression levels under starvation conditions. This is because starvation effects are common for genes involved in lipid and glucose metabolism and even in the regulation of appetite (North, 1999). Indeed, *to* mRNA levels were increased after 9 to 10 hr of food deprivation, and a 2 hr refeeding reversed the starvation effects (Figure 4A). Induction was most striking in the adjacent gastrointestinal tract regions where *to* expression was not detectable under normal conditions (Figure 4A). *tim* RNA expression in the gut was more widespread than that of *to* under normal conditions, and *to* expression appeared to expand and coincide with the *tim* pattern only when the flies were starved (see below; Figure 3). Induction was also observed in the head (Figures 4B and 4C). The starvation-induced enhanced expression was also detectable at the protein level (Figure 4B). Note that the rapid reversal by refeeding was much less obvious at the protein level, most likely because of a delayed effect on protein by mRNA changes. No enhanced expression was observed with heat shock or oxidative stress (paraquat treatment; data not shown).

Interestingly, an increase in *to* gene expression was most prominent when the flies were deprived of food at the peak of the mRNA cycle (W. V. So et al., submitted; Figure 4), suggesting that functional clock machinery may be required for this starvation-driven increase in expression. Consistent with this notion, induction was

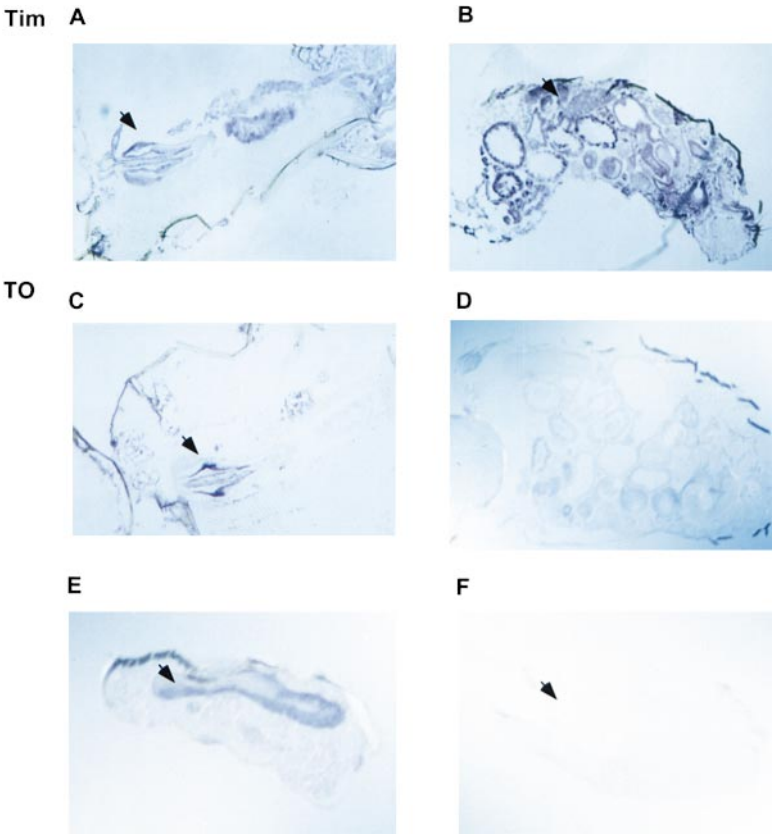

Figure 3. *to* mRNA Is Localized in Distinct Areas Related to Feeding

In situ hybridization of antisense dioxigenin RNA probes for *to* and *tim* was performed for whole body sections at the high time point ZT23 for *to* and for *tim* at ZT13.
(A) Horizontal section of a male thorax showing staining of the cardia (indicated by arrows) and staining of the gastrointestinal track in *tim*.
(B) Sagittal section of a male abdomen showing a broad staining of *tim*.
(C) Horizontal section of a male thorax showing restricted staining of *to* in the inner layer of the cardia.
(D) Sagittal section of a male abdomen showing no specific staining with a *to* probe.
(E) Sagittal section of a male abdomen showing *to* staining in the crop.
(F) In situ hybridizations using sense RNA probes did not show signals.

not detectable in arrhythmic clock mutants: per^{01}, tim^{01}, and cyc^{01} flies (Figure 5A). The starvation-induced expression of *to* could therefore be secondary to an effect on a central clock component. To address this possibility, protein levels of PER and TIM were measured under the same starvation protocol. The results showed that they were not enhanced, unlike *to* (Figure 5B and data not shown). Therefore, starvation-induction of *to* expression is probably not a secondary consequence of general clock gene induction. Rather, it requires and perhaps works through the clock machinery.

A *takeout* Mutant Exhibits an Aberrant Starvation Response

A *to* deletion mutant was identified during the analysis of *to* expression levels in various genetic backgrounds. *to* mRNA levels are low in the common lab strain ry^{506}. In addition, TO protein levels in ry^{506} were noncycling and reduced 3-fold compared to trough levels of a wild-type (wt) strain (Figure 6B). TO levels in ry^{506} were also not increased in response to starvation (data not shown). These effects were not due to the ry^{506} mutation itself, because other lab strains bearing the same *ry* mutation showed normal *to* expression (Figure 6A and data not shown). *to* maps far from *ry*: *to* is located at 96B19–96C6 (data not shown), whereas *ry* is located at 87D11. The ry^{506} mutation was generated by γ-ray mutagenesis, suggesting that a significant DNA lesion might be present at the *to* locus. To test this directly, a PCR analysis across the *to* genomic area was performed in the ry^{506} strain. To facilitate the analysis, the genomic sequence of *to* was obtained (Experimental Procedures). The PCR results showed that the 3′ end region of *to* is missing in ry^{506} flies: fragment b could not be obtained, and the EcoRI site within the *to* 3′ UTR is absent (Figures 6C–6E). Because PCR using another pair of primers revealed the presence of the normal stop codon (data not shown), the genomic deletion in this ry^{506} strain probably removes the polyA cleavage site or a substantial portion of the 3′ UTR, resulting in an unstable transcript.

Because of the *to* response to starvation, we decided to analyze locomotor activity patterns without food. Flies were transferred to locomotor activity monitors under starvation conditions. Over the subsequent two days, average activity events and the percentage of active flies were monitored as a function of time in LD. The latter parameter approximates survival, as the inactive flies are dead or nearly so. Wild-type flies manifest at least three activity peaks, similar to the morning and evening peaks observed under standard LD conditions (Figure 7A). The percentage of active flies slowly decreases during the two days without food (Figure 7C). By both criteria, *ry506* flies become less active more quickly, i.e., exhibit defects in both circadian activity and survival under these conditions. They showed only two peaks of activity, the second significantly smaller than the first, and the average death time (50% inactive flies) was 30 hr for wt flies and 20 hr for the ry^{506} strain (Figures 7A and 7C). However, the ry^{506} were more active than wt flies during the activity decline that followed the first activity peak.

To confirm that the differences between ry^{506} and the wt strain are the result of the *to* mutation, we introduced two transgenes into a *to* mutant background: a *tim* GAL4

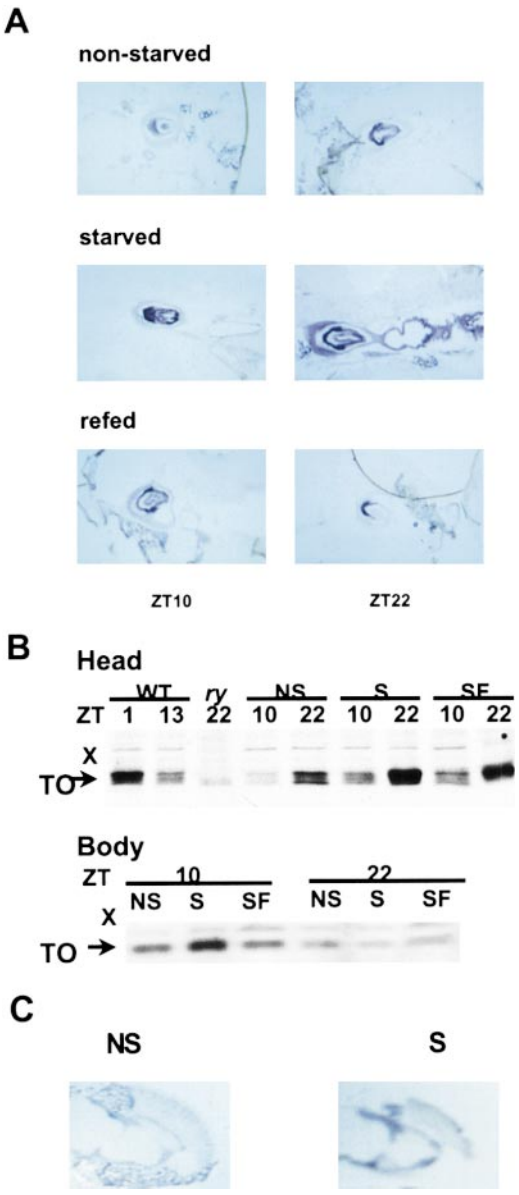

Figure 4. *to* Is Induced by Starvation

(A) In situ hybridization of antisense dioxigenin RNA probes for *to* was performed for whole body sections of male flies collected at the time points indicated at the bottom of each column and under the conditions indicated above each row. The starved flies were food deprived for 9 to 10 hr before being collected, and the refed flies were starved for 7 to 8 hr and then put back onto food for 2 hr before being collected. The horizontal sections show the staining of the inner layer of the cardia before food deprivation and an induction of the *to* staining in the gastrointestinal tract, in addition to a more intense staining of the cardia after food deprivation, especially at ZT22. Refeeding the starved flies for 2 hr reversed the induction effect of starvation.

(B) Western blot showing the starvation-induced expression of TO in the head and body. The same samples were used as for the in situ hybridization in (A). Shown are the TO levels in nonstarved (NS), starved (S) and refed (SF) fly heads. The experiment was reproduced four times with identical results. The wild-type (WT), peak (ZT1), and trough (ZT13) levels of TO expression are shown for comparison. ry^{506} served as a negative control in this experiment.

(C) In situ hybridization of antisense digoxigenin RNA probes for *to* was performed for head sections of male flies collected at ZT22; nonstarved (NS), starved (S).

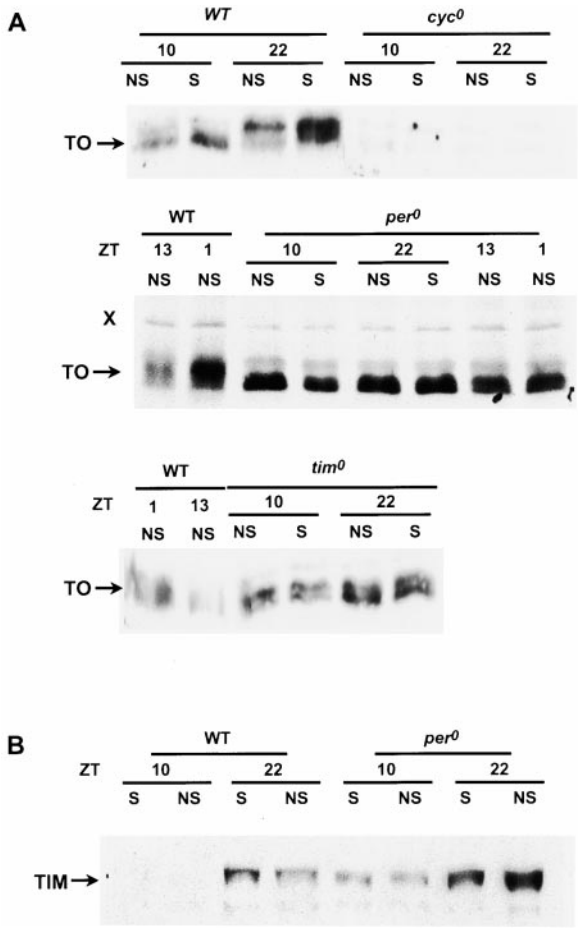

Figure 5. Starvation-Induced *to* Expression Is Blocked in *Drosophila* Circadian Mutants

(A) Western blots showing no obvious change of the TO level in per^{01}, tim^{01}, and cyc*01* flies before and after starvation. The Wild type (WT), peak, and trough levels of TO expression are shown for comparison. A different antibody bleed was used in this experiment. This antibody does not produce the cross-reacting band detected with the bleed used in other figures.

(B) Western blot showing no obvious change of TIM in the starved (S) and nonstarved (NS) wild-type (WT) and per^{01} flies.

gene and a UAS-*to* gene. This combination leads to potent *to* expression in all *tim*-expressing cells (data not shown). We examined two different UAS-*to* transgenes, and in both cases there was a significant increase in the second activity peak, a small third peaklet, and a dramatically improved survival by about 10 hr of starvation. The transgenic rescue strains also showed decreased locomotor activity relative to *to* after the first peak (Figure 7B), even when only active flies were analyzed (Figure 7E). No differences in survival rate were observed under heat shock and oxidative stress (paraquat) conditions; in these cases, the *to* mutant was identical to the wild-type strain (Figures 7F–7G). The strain differences indicate that *to* contributes to wild-type activity patterns and survival under starvation conditions.

Discussion

Only under starvation conditions were we able to detect a survival and locomotor activity phenotype of ry^{506} flies

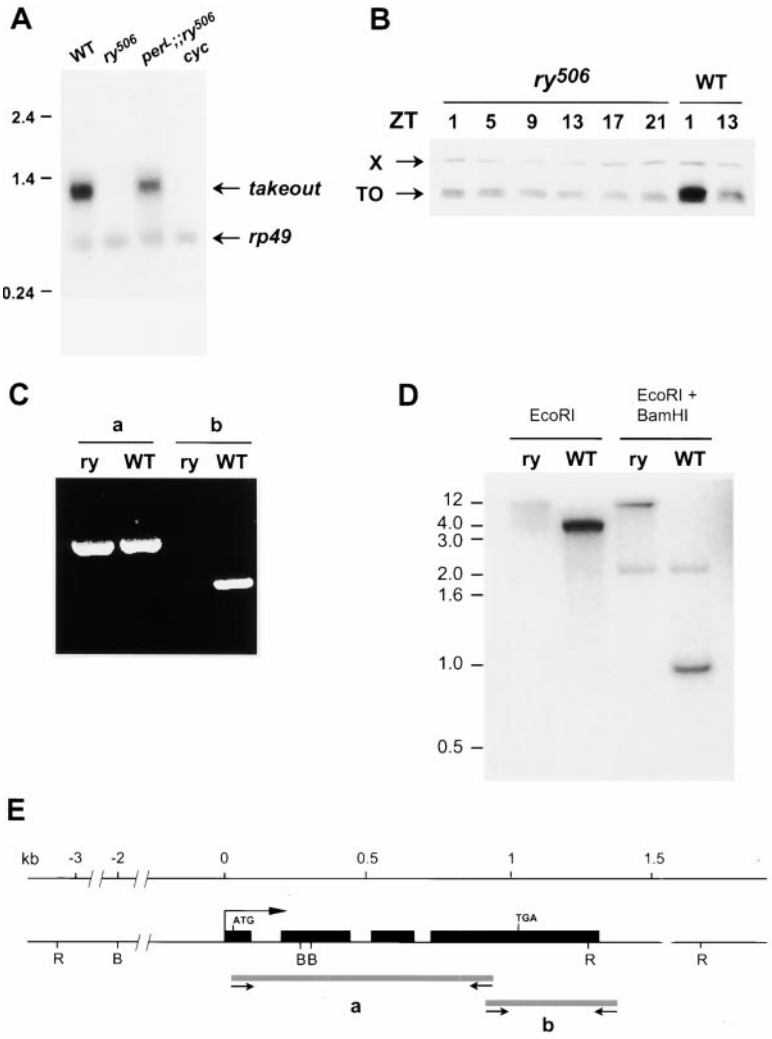

Figure 6. A Deletion at the *to* Locus in ry^{506}

(A) Northern blot showing *to* mRNA is very low or undetectable in ry^{506}. One microgram of polyA mRNA from flies of the genotypes indicated was used. *rp49* is the internal control. Flies were entrained and collected at ZT17.

(B) Western blot showing the low level and noncycling TO protein signal from ry^{506} compared to that in wild type. Flies were entrained and collected at the time shown. "X" is the cross-reacting band.

(C) PCR product amplified from ry^{506} (*ry*) and wild-type (CS) using primer sets "a" and "b" shown in (E).

(D) Southern blot showing the absence of EcoRI restriction sites at the *to* locus. Five micrograms of genomic DNA from ry^{506} (*ry*) and wild-type (WT) was used in each lane and the blot hybridized with a mixture of both "a" and "b" probes shown in (E).

(E) Genomic structure of *to*. Filled box denotes exon. "E" is the abbreviation for EcoRI and "B" for BamHI restriction sites. Probes and primer sets "a" and "b" were used in (C) and (D).

with convincing *to* transgenic rescue. The starvation paradigm was inspired by the *to* expression pattern, which is localized to body tissues that are relevant to food detection and metabolism: the cardia, the crop, and the antennae. mRNA levels are increased by starvation, in the brain as well as these body tissues, and *to* affects survival and locomotor activity patterns under these conditions. On this basis, we suggest that the *to* brain expression is also relevant to feeding.

Before analyzing *to* body expression and the locomotor activity patterns under starvation conditions, we tried to find a *to* behavioral phenotype under normal conditions. This was also based on a prior report that *rosy* alleles have a late eclosion phase, although the free-running period of locomotor activity and eclosion is normal (Newby and Jackson, 1993). We could reproduce this phenotype and also found a reproducible delayed locomotor activity phase in ry^{506} flies. But the eclosion profiles and the delayed locomotor activity phase of the ry^{506} lines were not rescued with a ry^{+} transgene, and they were also unaffected by the presence or absence of the *to* deletion (data not shown).

to gene expression is not only under starvation control but also under clock gene control. It exhibits daily oscillating expression and is downregulated in all the clock mutants tested. Based on run-on experiments, *to* cycling is largely transcriptional, as previously described for *per* and *tim* (So and Rosbash, 1997; W. V. So et al., submitted). But the *to* mRNA cycle peaks several hours after *per* and *tim*, and *to* transcriptional oscillations may not be dependent on a *cis*-acting E box. This issue is addressed elsewhere (W. V. So et al., submitted).

We presume that clock regulation of *to* expression contributes to metabolic and even behavioral fluctuations that are relevant to food and feeding. This relationship is underscored by the *to* starvation response and the lack of a response to starvation in the arrhythmic clock mutant backgrounds. The latter observation implies that the upregulation occurs through a clock mechanism or perhaps requires a functional clock. Consistent with this notion, per^{01} and tim^{01} flies die even more rapidly than *to* flies under starvation conditions (data not shown). As all *to* positive tissues appear to express *per* and *tim*, this relationship between the circadian clock and *to* expression might be intracellular. Based on run-on assays (W. V. So et al., submitted) and previous studies on clock gene function (So and Rosbash, 1997), we assume that *to* transcription rates are changing in response to starvation. A circadian regulation of important output functions is consistent with the proposed

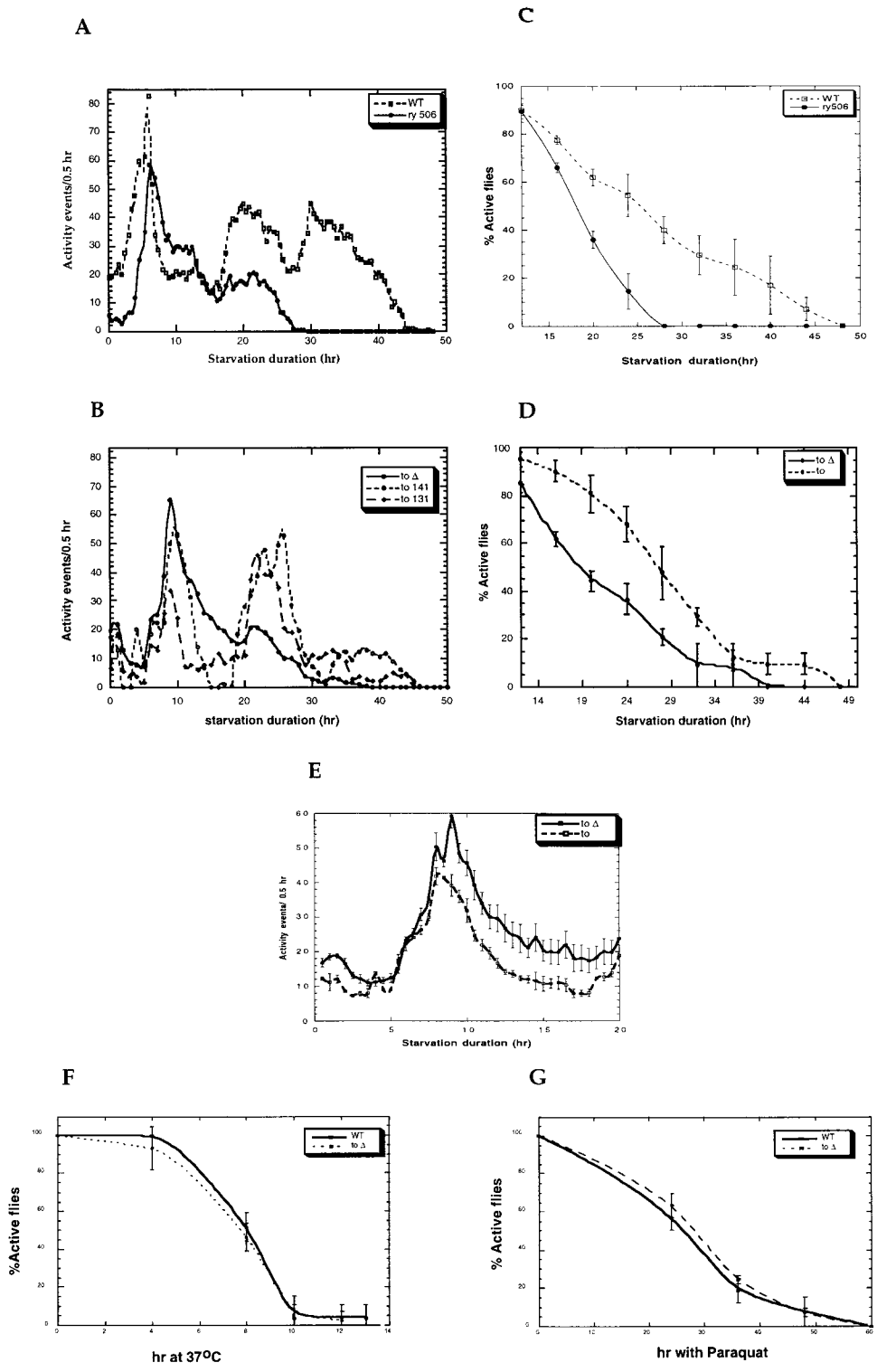

Figure 7. *to* Mutants Exhibit an Aberrant Starvation Response

(A) Activity plot of wt and *ry*^{506} flies in starvation conditions during a 12 hr light:12 hr dark cycle. Activity events in 0.5 hr intervals were averaged for 32 flies from each genotype. Open squares, wt flies; dark circles, *ry*^{506} flies. One representative experiment out of two is shown.

(B) Activity events of *tim*GAL4/CyO; *ry*^{506} (dark circles) compared with two lines of *tim*GAL/UAS*to*; Δ*to ry506* (open circles).

(C) Percent of active flies plotted during starvation. The data from (A) is replotted as a percentage of mobile flies present during the starvation regime.

(*continued*)

circadian regulation of olfactory organ activity in *Drosophila* and in other insects, as recently shown by physiological assays (Van der Goes van Naters et al., 1998; Krishnan et al., 1999). It is also consistent with the regulation of PDF in *Drosophila* (Blau and Young, 1999; Renn et al., 1999; Park et al., 2000).

Many *to* expression features recall leptin, NPY, VGF, and hypocretin—mammalian hormones with proposed behavioral as well as metabolic functions (Zhang et al., 1994; Wisor and Takahashi, 1997; Ahima et al., 1998; Faraco et al., 1998; North, 1999). These shared properties include circadian regulation, a role of peripheral as well as brain tissue, a starvation response, secretion, and a relationship to ligands and receptors (ligand binding proteins). The latter connection comes from sequence analysis, which suggests that the shared property of the *to* superfamily is ligand binding. Since juvenile hormone and the molecules bound by JP29 are lipophilic (Wojtasek and Prestwich, 1995), one can speculate that TO also has an endogenous lipophilic ligand relevant to feeding. The features displayed by the *to* superfamily also resemble the well-characterized lipocalin protein family, present mostly in vertebrates (Flower, 1999). Lipocalins are classified as extracellular transport proteins, typified by the retinol binding protein, RBP (Blomhoff et al., 1990). RBP serves to regulate retinol release from the liver and to transport the insoluble retinol to peripheral target tissues. In general, these small, secreted transporters also serve to protect the ligands from degradation or chemical modification in the circulation. Lipocalin family members display low levels of overall sequence conservation, i.e., pairwise sequence identity is often below 20%, the threshold for reliable alignment. However, after the initial identification of this family (Pervaiz and Brew, 1999), a growing number of crystallographic structures have been solved, and they reveal a compelling structural similarity (Cowan et al., 1999).

The levels of this putative TO ligand might be clock-regulated. In this case, TO would serve to amplify or modulate the signal strength. Alternatively, the ligand concentration might be temporally constant, and the clock would then create the metabolic rhythm by controlling the rhythmic expression of TO—the ligand binding protein. TO might also control the timing or duration of signal activity, resembling a proposed function of some secreted odorant binding proteins (Carlson et al., 1997). Starvation would then further increase the signal.

Based on the circadian as well as starvation regulation, TO may contribute to an anticipation of food availability. Alternatively, it may contribute to a proper response to a change in food status. A specialized version of the second possibility is a response to starvation. This might include behavioral as well as metabolic changes caused by the absence of food. A similar speculation has been made in the case of NPY and mammals (North, 1999). Starvation might elicit an increase in locomotor activity, to stimulate a search for food. A heightened anticipatory event fits with the normal circadian regulation of TO expression, which occurs despite the constant presence of food. Alternatively, starvation might cause a shutdown of activity, to conserve energy, for example. Although we have just begun to examine the behavioral response to starvation, the mutant versus rescued activity patterns suggest that the mutant flies decrease their activity levels less rapidly during the first activity decline after about 10 hr of starvation (Figure 7). This could contribute to the less successful survival of the mutant strain. Alternatively, it could be irrelevant to survival rate and an independent manifestation of TO function under starvation conditions. It could also be a more subtle consequence of the TO-mediated events that also lead to decreased survival. If the latter is the case, however, it is not simply due to an increased percentage of dead (viz. immobile) flies, because the mutant strain is still hyperactive after 10 hr of starvation when immobile flies are excluded from the data (Figure 7E). In any case, the *to* responses appear specific for starvation, because there was no effect of heat shock or paraquat on *to* gene expression (data not shown). Moreover, there was no difference in survival between the *to* and wild-type strains in response to these two other stresses (Figures 7F–7G).

Although a more detailed examination of the behavioral phenotype is required, two molecular issues are now of great interest. It is important to identify the putative TO ligand, as well as its putative intracellular receptor. Identification of both of these molecules will provide important tools and should help deepen our understanding of the relationship between food and circadian rhythms.

Experimental Procedures

Fly Strains

The strain of wild-type flies used was Canton-S. ry^{506} flies were from our laboratory stock, and the same results were obtained from Jeffrey Hall's laboratory stock. per^{L};ry^{506} originated from *perL* (Konopka and Benzer, 1971) and was combined with the ry^{506} eye-color-marker chromosome. cyc^{01};ry^{506}, per^{01}, *y w*;tim^{01}, per^{01};tim^{01};ry^{506}, and *y w*; Clk^{irk} flies were used for analysis. Genomic DNA was tested with PCR for the presence of *to*.

Behavioral Analysis

Behavioral analysis was performed as described by Rutila et al. (1996). Male flies were used for analysis. Starvation behavior: 24–48

(D) Percent of active flies plotted during starvation. The data from (B) is replotted as a percentage of mobile flies present during the starvation regime. The plot represents an average of both the *to 141* and *to 131* lines, analyzed twice each. The P value comparing the 24 hr point for the two genotypes is $P < 0.002$ using a one-tailed T test.

(E) Activity events of *tim*GAL4/CyO; Δto ry^{506} (dark squares) compared with two lines of *tim*GAL/UAS*to*; Δ*to ry506* (open squares). The plot represents an average of three experiments. The P value comparing the 12 hr point in the two genotypes is $P < 0.007$ using a one-tailed T test.

(F and G) *to* mutants are not more sensitive to thermal or oxidative stress.

(F) Thermal stress: wt flies and Δ*to ry506* at age 48 hr were transferred to glass vials containing 2% agarose 5% sucrose and maintained at 37°C. Data from three independent experiments is shown. Fifty to seventy flies of each genotype were used in each experiment.

(G) Oxidative stress: Tolerance to oxidative stress was measured by assaying resistance to paraquat (Lin et al., 1998): 48 hr old wt and Δ*to ry506* flies were transferred to vials containing 20 mM paraquat and 5% sucrose in 2.5% agarose. Data from three independent experiments is shown. Fifty to seventy flies of each genotype were used in each experiment.

hr old males were entrained for 48 hr in 12 hr light:12 hr dark (12:12) LD cycles. At ZT1 (after 1 hr of light exposure), flies were transferred individually to tubes without food and monitored for activity.

RNA Extraction and Analyses

For Northern blots, total and polyA RNA were prepared as described (Sambrook et al., 1989) . One microgram of polyA$^+$ mRNA sample was loaded on formaldehyde gels that were transferred onto nylon membranes as previously described (Sambrook et al., 1989). ^{32}P-labeled probes were prepared by random priming of gel-purified fragments using Prime-It II (Stratagene). Prehybridization (1 hr) and hybridization (≥16 hr) were performed at 65°C in 10 ml Church buffer (0.5 M $NaHPO_4$ [pH 7.2], 7% SDS, 1% BSA, and 1 mM EDTA). The membranes were washed in washing buffer (0.2× SSC and 0.1% SDS) twice briefly at room temperature and then twice at 60°C for 20 min before being exposed to film either at room temperature or at −80°C with an intensifying screen, depending on the strength of the signals. RNase protection assays were performed as described (Marrus et al., 1996). The RNA probe protects nucleotides 529–839 of the cDNA region.

mRNA In Situ Hybridization

Flies were entrained to 12:12 LD. Frozen sections (10 μm) of adult heads and bodies were cut and in situ hybridization was performed as described (Hasan and Rosbash, 1992). Digoxigenin-labeled riboprobes were prepared from the full-length *to* and *period* cDNAs and were hydrolyzed prior to use. All the hybridization and washes were performed at 65°C.

Southern Hybridization

Isolation of genomic DNA from adult flies was performed as described (Bender et al., 1983). ^{32}P-labeled probes were prepared as described above. Five micrograms of genomic DNA was loaded in each lane. Hybridization and washing were performed as described for Northern blots above, except the hybridization temperature was 60°C and the membrane washed at 55°C.

Western Blot

TO antibody was generated in rats, using as antigen recombinant TO without the N terminus (amino acid residues 67 to 249), fused to either a GST or a His tag. The immunoreactivity and specificity of individual antisera were tested on fly head extracts. The antibody with the best specificity was generated using the GST fusion protein as antigen and was used for the subsequent work.

Protein extracts from 20 heads for each sample were prepared in 20 μl of extraction buffer (Zeng et al., 1996) with the addition of 20 mM β-glycerophosphate and 100 mM Na_3VO_4. Twenty microliters of 2× SDS sample buffer was added and the sample boiled. For fly bodies, 2 bodies were prepared in 24 μl of extraction buffer and 24 μl of 2× SDS was added. Six microliters of the denatured product was separated (3-head or 1⁄4-body worth) on 12% SDS-PAGE. Following electrophoresis, gels were electroblotted on to a nitrocellulose membrane for 25 min at 0.12 A using a semidry blotting apparatus according to the manufacturer's instructions (ISS).

Acknowledgments

We thank all the members of the Rosbash laboratory, J. Hall, L. Zwiebel, C. Pikielny, and L. Griffith for helpful discussions and critical readings of the manuscript, and Ed Dougherty for help with the figures. This work was supported by grants from the NSF Center for Biological Timing and the National Institutes of Health.

Received January 7, 2000; revised May 3, 2000.

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GenBank Accession Number

The sequence of *takeout* has been submitted to GenBank with accession number AF261748.