Hastings: If we have a good SCN lesion that doesn't impinge deep into the chiasm, animals such as this will still mask their behaviour on a LD schedule. So there are routes avoiding the SCN that can control masking behaviour.

Menaker: I gather that no one has managed to lesion the SCN with kainic acid to spare the fibres that pass through it. It is highly resistant.

Hastings: Paradoxically, the whole photic cascade is dependent on NMDA and AMPA signalling, yet none of us have been able to use neurotoxins to kill it off.

Young: Is the same interdependence of CRY and PER stability that Steve Reppert described for the liver seen in the SCN?

Hastings: In the *Cry* double knockouts we have done immunostaining for PER2 and it is not present. Equally, in the *Per1* and *Per2* mutants, in the absence of PER1 and PER2 there are half-maximal levels of CRY immunostaining (Bae et al 2001). Part of this is because in the *Per2* mutant there is no *Cry* mRNA cycling, and in the *Per1* mutant rhythmic gene expression occurs but the protein is unstable in the absence of PER1.

References

Abe M, Herzog ED, Yamazaki S et al 2002 Circadian rhythms in isolated brain regions. J Neurosci 22:350–356

Bae K, Jin X, Maywood ES, Hastings MH, Reppert SM, Weaver DR 2001 Differential functions of mPer1, mPer2, and mPer3 in the SCN circadian clock. Neuron 30:525–536

Lee C, Etchegaray JP, Cagampang FR, Loudon AS, Reppert SM 2001 Posttranslational mechanisms regulate the mammalian circadian clock. Cell 107:855–867

Reddy AB, Field MD, Maywood ES, Hastings MH 2002 Differential resynchronisation of circadian clock gene expression within the suprachiasmatic nuclei of mice subjected to experimental jet lag. J Neurosci 22:7326–7330

Circadian rhythms in *Drosophila*

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Abstract. We discuss some historical features of the circadian field in *Drosophila melanogaster*. We then describe some recent progress from our laboratory in three different areas. First, we discuss the regulation of circadian gene expression as assayed with microarrays. Results are discussed that verify and extend published data, both with respect to the previously identified cycling mRNAs as well as some clustering within the genome of some of the genes that give rise to these circadian transcripts. Also discussed are experiments that attempt to identify transcripts that are enriched in lateral neurons, the key circadian pacemaker cells in the *Drosophila* brain. Second, the issue of damping within the brain is addressed, by assaying molecular oscillations after many days in constant darkness. Third, the identification of a new circadian mutant is described, which is a fully recessive allele of the gene *Clock*. The previous allele in flies, as well as the single mutant allele in mice, is a dominant allele. This limits the conclusions that can be drawn from the genetic and molecular analyses in these mutant strains. Results with the new recessive allele not only support the notion that *Clock* is an important clock gene but also indicate that it contributes more to the amplitude of the rhythm rather than the period.

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The modern molecular genetic era of circadian rhythms arguably began more than thirty years ago when Konopka and Benzer published the results of a genetic screen, describing their three identified alleles of the period gene in *Drosophila melanogaster* (Konopka & Benzer 1971). This was not only a landmark achievement, which kick-started the molecular-circadian field, but it was also unusually prescient. This is because it was still several years before the first recombinant DNA technology was published and almost a decade before it became practical, even in the most sophisticated of laboratories. In other words,

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the period mutants were identified and characterized well before a molecular vision could be conceptualized let alone realized.

It was therefore not until 1984, 13 years later, that the period gene was cloned and used in what were arguably the first gene rescue experiments of behavioural import (Bargiello et al 1984, Zehring et al 1984). Yet even this achievement was not illuminating from the circadian point of view, because the function of the period protein (PER) was still unknown. During the next few years, we at Brandeis (Rosbash and Hall labs) and the Young laboratory at Rockefeller worked on the relationship of this gene to circadian rhythms, which resulted in several significant advances. Among these were the sequence of the complete protein and the location of the precise nucleotide changes responsible for the slow, fast and arrhythmic alleles (Baylies et al 1987, Yu et al 1987). However, not even the sequence was particularly clarifying. This is because it was a pioneer protein, with no known relatives. In those early days of DNA sequencing, it was much more frequent that a sequence did not reveal a protein's secrets. This was the situation until 1988, when there appeared the sequence of a *Drosophila* transcription factor with a clear relationship to the period protein (Crews et al 1988). Although the two proteins were not close relatives and the single motif in common was of uncertain function, the similarity was unambiguous and inspired us at Brandeis to pursue this hypothesis, namely, that PER was a transcription factor and that the regulation of transcription was central to circadian rhythms. In 1990, almost 20 years after the landmark Konopka and Benzer publication, we published the finding that period mRNA levels undergo circadian oscillations and that PER regulates the period and phase of its own mRNA cycling. In other words, the mRNA cycling was sensitive to the Konopka and Benzer mutations and paralleled the previously described changes in the behavioural cycling (Hardin et al 1990). We expanded on this observation over the next couple of years and showed that the regulation was transcriptional and almost certainly reflected a negative feedback loop, in which PER inhibits its own transcription (Hardin et al 1992, Zeng et al 1994). This feedback loop and transcriptional regulation have been cornerstones of the mammalian as well as the *Drosophila* circadian system, since the discovery in 1997 of the mammalian *period* genes.

In the decade or so since the publication of the second *Drosophila* circadian rhythm gene *timeless* by the Young laboratory, many fruit fly pacemaker components have been discovered. Like *period*, most of these are conserved in mammals, with similar if not identical functions (Allada et al 2001). Moreover, studies on the biochemistry of the timekeeping mechanism have continued to focus on transcriptional regulation. It is believed that the basic helix-loop-helix (bHLH) transcription factors CLOCK (CLK) and CYCLE (CYC) bind to upstream E-boxes (CACGTG) and directly activate transcription of the period (*per*) and timeless (*tim*) gene (Allada et al 1998, Darlington et al 1998, Hao et al

1997, Rutila et al 1998, Wang et al 2001). This view is based on strong biochemical evidence in both systems: in the *Drosophila* system, PER and TIM proteins subsequently feed back and inhibit transcriptional activation by CLOCK and CYCLE (Darlington et al 1998, Lee et al 1998, 1999). A similar focus on transcription and feedback loops exists in mammals, including humans. Of note, circadian transcription studies *in vivo* have relied heavily on two dominant negative (antimorphic) alleles of *Clock*, one in *Drosophila* (Clk^{Jrk}) and one in mouse (Allada et al 1998, King et al 1997a,b). In the fly system, transcription of the *per* and *tim* genes is incredibly low in the Clk^{Jrk} background.

Further studies have implicated a second feedback loop in circadian timing. Like *per* and *tim*, *Clk* and *cry* RNAs also oscillate with respect to time of day (Bae et al 1998, Darlington et al 1998, Emery et al 1998). However, these oscillations are antiphase to those of *per* and *tim*, suggesting that they are indirect targets of the *Clk–cyc* system. This is consistent with the levels of the *Clk* and *cry* RNAs in Clk^{Jrk} and cyc^{0} mutants; they are high, whereas the levels of *per* and *tim* RNAs are low (Emery et al 1998, Glossop et al 1999). It has been proposed that these genes, *per* and *tim* on the one hand and *Clk* on the other, define two interdependent transcriptional feedback loops. Transcriptional oscillations are thought to emerge from the dynamic interplay of these feedback loops, leading to behavioural and physiological rhythms.

Several aspects of circadian gene expression are also subject to post-transcriptional control, including RNA and protein stability as well as protein phosphorylation (Dembinska et al 1997, Kim et al 2002, So & Rosbash 1997). Protein levels and phosphorylation states of PER and TIM oscillate with time of day (reviewed in Allada et al 2001). Doubletime, a casein kinase I epsilon homologue; shaggy, a glycogen synthase kinase 3 homologue; and casein kinase 2, appear to phosphorylate PER and TIM (Kloss et al 1998, Kloss et al 2001, Lin et al 2002a, Atken et al 2003, Martinek et al 2001, Price et al 1998). These additional layers of feedback make it difficult to untangle the roles of different mechanisms in determining rhythm period, phase, and amplitude.

However, cycling RNAs are generally considered to be under transcriptional regulation. This is due in part to the fact that all RNA cycling is apparently eliminated in the *Clock* mutant Clk^{Jrk} (McDonald & Rosbash 2001). Of course many of these mRNAs could be regulated post-transcriptionally and only indirectly by the circadian transcription machinery, for example through the transcriptional regulation of a splicing factor. But the current view of the field is that most cycling mRNAs are regulated at the transcriptional level. This is also because in addition to *period* and *timeless*, the CLK–CYC heterodimer directly activates at least three additional transcription factor-encoding genes. Direct target genes of CLK–CYC have been defined in a microarray experiment with S2 tissue culture cells, in which the CLK–CYC heterodimer is able to activate target

gene expression in the presence of cycloheximide. The activation takes place with dexamethasone and a CLK–GR (clock–glucocorticoid) fusion gene in the cells, so that the S2 cell-expressed endogenous CYC protein and the CLK–GR protein is activated by the addition of glucocorticoid and without a transcription or protein synthesis requirement. Only a handful of genes are activated in this system, including at least three transcription factors (McDonald & Rosbash 2001). These include VRI as well as PDP1, both of which have been subsequently shown to participate in clock gene regulation (Cyran et al 2003, Glossop et al 2003). Our current view is that the large number of cycling genes is the product of a transcriptional cascade and that the CLK–CYC heterodimer sits at the top of the pyramid.

At the base of that pyramid sit 134 mRNAs, which undergo circadian oscillations. Based on sequence criteria, these genes are grouped into pathways with different functions, many of which had already been discovered to have roles in circadian rhythms. There were also several novel pathways, suggesting that many different physiological systems are under clock control. Although the number and identity of cycling mRNAs was very different in the different studies published to date (Ceriani et al 2002, Claridge-Chang et al 2001, Lin et al 2002b, McDonald & Rosbash 2001, Ueda et al 2002), they identified many genes in related biochemical and metabolic pathways. A large fraction of the variation may come from biological differences; that is how the samples were collected and RNA harvested. Perhaps even more important is the fact that all groups used different methods of analysis and different thresholds for significance. In fact, we could never recapitulate any results with another method of analysis, either our results with another methodology or other results with our methods. In any case, it is presently uncertain whether the rather small overlap (for example, about 25% between McDonald & Rosbash 2001, Claridge-Chang et al 2001 and Etter & Ramaswami 2002) is due to a high fraction of false-positives in the various studies or a high fraction of false negatives. Although it has been suggested that small overlap is due to false positives, we prefer the false negative explanation. This fits with the fact that all groups used different methods and criteria to define their cycling mRNA subpopulations. Also, it should be easier to disprove the 'high fraction of false positives' hypothesis.

To begin an examination of the fraction of false positives, we chose 14 of our cycling mRNAs at random and examined their cycling by real-time PCR. We could clearly confirm the cycling and microarray patterns for 10 of the 14 mRNAs, and the cycling was likely positive for two more. Only in two of the 14 cases was circadian cycling unlikely, based on the real-time results. We conclude that most of our 134 mRNAs are real cyclers and that false positives constitute only a minority of the 134 mRNAs. The number 134 is probably a gross underestimate.

Another finding from our microarray paper was the fact that there are clusters of cycling genes, closely spaced within a single chromosomal region. To verify and extend this observation, we took the same real-time PCR approach and examined every open reading frame within the takeout-Duf 227 cluster. The results verified the cycling mRNAs originally identified and also identified several new cycling genes within this cluster. Moreover, expression of many more genes from this region were identifiable by real-time criteria than by microarray criteria. It is unclear at present why this is the case, i.e. whether these genes were just expressed at levels too low to detect or whether there is some other obstacle that limits the sensitivity or the generality of the microarray approach.

A third approach we are taking with microarrays is to identify mRNAs that are highly expressed in the brain neurons most important for locomotor activity rhythms in flies. Because there are no available techniques for sorting or enriching adult brain neurons from *Drosophila*, we have taken an ablation approach and eliminated brain neurons by specifically expressing cell-death genes in these cells. This approach has been previously used in behavioural studies, and these neurons, the sLN_vs and the lLN_vs, can be killed without any adverse effects other than a loss of circadian rhythms (Renn et al 1999). Head microarrays from these strains identify a number of genes that are low in the ablation strains compared to a wild-type strain. Because the neuropeptide PDF is specifically expressed in the LN_vs, this gene serves as a positive control; *pdf* mRNA is indeed present only at low levels in these strains. These studies identify a number of mRNAs that behave like *pdf* and are present at low levels in the cell-ablated strain. We have tested three of them by *in situ* hybridization, and all three give strong signals in both groups of LN_vs; this is consistent with the notion that they are highly expressed in those cells. More work needs to be done on these genes to verify that they make a contribution to circadian rhythms.

We have also been interested in the function of these few clusters of brain neurons, i.e. how they contribute to circadian gene expression. The issue at hand is damping, the fact that the amplitude of gene expression oscillations decreases as a function of the time that the animals are in constant conditions (constant darkness). Damping in the fly system was observed in the original cycling gene expression observations from the early 90s: after several days in constant darkness, the amplitude of gene expression cycling is very modest compared to LD (light–dark) conditions or compared to the first day in constant darkness. More recently, immunohistochemical experiments suggest that molecular rhythms in the eyes and even in some of the circadian brain neurons undergo dramatic damping in constant darkness. Importantly, locomotor activity rhythms persist in constant conditions for at least two weeks with no detectable damping. Because of this conflict (molecular damping vs. no behavioural damping), we re-examined molecular cycling in the brain neurons by *in situ* hybridization with a

tim antisense probe. Although this assay has been used often to examine brain neurons, it has not been applied to flies maintained for a long time in constant darkness. We examined flies after four and eight days in constant darkness and observed robust transcriptional oscillations, undiminished from what is observed in a light–dark cycle. We conclude that there is no conflict for the brain, in which the robust molecular oscillations match the robust behavioural oscillations. The damping of molecular rhythms must come principally from other head tissues like the eye, or the molecular damping that has been observed is probably due to the short-term adjustment in going from LD to constant darkness conditions.

I now want to return to the transcriptional cascade and the *Clk* gene. As mentioned previously, *Drosophila* circadian transcription studies *in vivo* have relied heavily on a single dominant negative allele of *Clock*, (Clk^{Jrk}) and one in mouse (Allada et al 1998). This is a precarious situation for the field, because some of the mutant phenotypes could be due to effects on other transcription factors and systems rather than just to low activity of the CLK–CYC complex. For this reason, we characterized a second allele of the *Clk* gene, which turned out to be a real recessive allele.

The mutant gene was found in our search for novel genes involved in circadian rhythmicity. We were screening ethyl methane-sulfonate (EMS) mutagenized flies for alterations in circadian locomotor activity (Rutila et al 1996). One line homozygous for a mutagenized third chromosome was arrhythmic. The phenotype mapped to the third chromosome, and homozygotes do not exhibit robust rhythms; in contrast, heterozygotes are virtually indistinguishable from wild-type. All other genetic and phenotypic characterization also indicated that the mutant, called Clk^{ar}, is fully recessive (Allada et al 2003).

Expression of *Clk* by *pdfgal4* in a Clk^{ar} background did not result in significant rescue of rhythmicity. On the other hand, *crygal4*-driven expression of *Clk* resulted in rescue in the rhythmicity of a majority of these flies. The rescued flies exhibited a slightly short period, similar to periods in flies with *crygal4*-driven expression of *Clk* in a wild-type background. The period shortening with increased *Clk* expression is consistent with the long periods of flies with only a single dose of *Clk*. BAC transgenic mice containing extra copies of *Clock* also exhibit short periods (Antoch et al 1997). We obtained similar results in a Clk^{Jrk} background: *crygal4*-driven *Clk* expression was able to rescue the rhythmicity of Clk^{Jrk} (18% rhythmic), although more weakly than Clk^{ar} (60% rhythmic), consistent with the antimorphic effects of Clk^{Jrk}.

We searched coding exons and exon–intron boundaries for EMS-induced base changes, comparing Clk^{ar} with sibs. We identified a single mutation at the 5′ splice site of the second intron, destroying the GT dinucleotide required for efficient splicing. The mutation is a G to A transition classically found in EMS-induced alleles. We examined *Clk* splice forms across the second intron in the Clk^{ar} mutant using reverse transcriptase-polymerase chain reaction (RT-PCR). RT-PCR across this intron identified a single band of the appropriate size in wild-type flies. In Clk^{ar}, multiple bands are observed, none of which correspond by electrophoretic migration to that seen in wild-type, consistent with the observed splice site mutation. Splice junctions between other coding exons were not grossly perturbed as assayed by RT-PCR. Exon 2 encodes for the N-terminal 13 amino acids, including the first two amino acids of the basic region. The exons beyond exon 2 encode the remainder of the CLK protein, including most of the basic region, the PAS dimerization motif and the glutamine-rich activation domain (Allada et al 1998). To determine whether these altered Clk^{ar} transcripts can produce functional CLK protein, we sequenced Clk^{ar} cDNAs. In only 4/22 clones, an upstream methionine codon is in frame with the remainder of the *Clk* gene. Assuming initiation from this methionine, translation of these transcripts would result in a CLK protein with novel N-termini: two of 15 amino acids and two of 28 amino acids. In all four cases, only the first two amino acids of the basic DNA binding domain are altered. Based on this analysis, we believe that there is a low level of CLK activity in the Clk^{ar} strain, which comes from a small fraction of aberrantly spliced mRNAs.

The molecular assays in Clk^{ar} indicate *bona fide* rhythms with a predominant effect on circadian rhythm amplitude and no more than a modest effect on phase or period. With circadian *per* and *tim* enhancers, we observed reduced enhancer activity and a reduced cycling amplitude in a Clk^{ar} background, consistent with the role of *Clk* in regulating these enhancers. Nonetheless, the phase of oscillating bioluminescence is similar to that of wild-type flies. The presence of molecular rhythms contrasts with the absence of detectable behavioural rhythms. We favour the notion that this reflects a level or amplitude reduction below a critical threshold for behavioural rhythmicity. The absence of anticipation of light–dark transitions makes it very unlikely that an effect restricted to the lateral neurons — the absence of the neuropeptide PDF, for example — is primarily responsible for the behavioural phenotypes. This is also because LD behavioural rhythms are largely normal in flies devoid of PDF or the pacemaker lateral neurons (Renn et al 1999). However, we cannot exclude the possibility of selective effects of Clk^{ar} on other behaviourally relevant neurons.

Previous results with Clk^{Jrk} also support a role for *Clk* in defining rhythmic amplitude. Clk^{Jrk} heterozygotes reveal a dominant reduction in the amplitude of molecular rhythms with little apparent change in phase (Allada et al 1998). These heterozygotes also exhibit reductions in rhythmic behaviour with only slightly long periods. Indeed, *Clk* over-expression results in a selective increase in the amplitude of *per* RNA oscillations (Kim et al 2002). This modest effect of varying *Clk* activity on period is similar to the phenotype of transgenic strains missing the *per* promoter or expressing *per* and *tim* from constitutive promoters

(Frisch et al 1994, Yang & Sehgal 2001). These strains also have reasonable periods (22–26 h) with poor rhythm amplitudes, as evidenced by the poor penetrance of rhythmicity. Taken together, these data suggest that changes in clock gene transcription have limited effects on circadian period. Separate control of circadian rhythm amplitude on the one hand and period (or phase) on the other is also consistent with anatomical experiments in both the fly and mammalian system (Liu et al 1991, Low-Zeddies & Takahashi 2001).

We propose that the post-transcriptional phosphorylation turnover feedback loop involving several clock components (e.g. *per*, *tim* and the protein kinase *Dbt*) is predominantly responsible for period determination. Excluding null alleles that are either arrhythmic or lethal, Flybase lists mutant alleles of *per*, *tim* and *Dbt* which exhibit period alterations ranging from 16–30 h for *per* (8 mutant alleles), 21–33 h for *tim* (8 mutant alleles) and 18–29 h for *Dbt* (5 mutant alleles; Flybase 2002). Indeed, the only *Dbt* allele that fails to exhibit rhythmicity as a homozygote, displays a potent period-altering phenotype as a heterozygote (Rothenfluh et al 2000). More recent additions to this list are the protein kinases *shaggy* (Martinek et al 2001) and *CK2*. Indeed, one mutant allele of *CK2*, $CK2^{Tik}$, exhibits one of the strongest dominant period effects of any rhythm mutant (Lin et al 2002a). These large period effects contrast with the transcriptional factor mutants of *Clk* and *cyc*. Their phenotypes indicate that near-normal periods are maintained despite large protein level changes.

Acknowledgements

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References

Allada R, White NE, So WV, Hall JC, Rosbash M 1998 A mutant *Drosophila* homolog of mammalian *Clock* disrupts circadian rhythms and transcription of *period* and *timeless*. Cell 93:791–804

Allada R, Emery P, Takahashi JS, Rosbash M 2001 Stopping time: the genetics of fly and mouse circadian clocks. Annu Rev Neurosci 24:1091–1119

Allada R, Kadener S, Nandakumar N, Rosbash M 2003 A recessive mutant of *Drosophila Clock* reveals a role in circadian rhythm amplitude. EMBO J 22:3367–3375

Antoch MP, Song E-J, Chang A-M et al 1997 Functional identification of the mouse circadian *clock* gene by transgenic BAC rescue. Cell 89:655–667

Atken B, Javch E, Genova GK et al 2003 A role for CK2 in the *Drosophila* circadian oscillator. Nat Neurosci 6:208–210

Bae K, Lee C, Sidote D, Chuang KY, Edery I 1998 Circadian regulation of a *Drosophila* homolog of the mammalian Clock gene: PER and TIM function as positive regulators. Mol Cell Biol 18:6142–6151

Bargiello TA, Jackson FR, Young MW 1984 Restoration of circadian behavioural rhythms by gene transfer in *Drosophila*. Nature 312:752–754

Baylies MK, Bargiello TA, Jackson FR, Young MW 1987 Changes in abundance and structure of the *per* gene product can alter periodicity of the *Drosophila* clock. Nature 326:390–392

Ceriani MF, Hogenesch JB, Yanovsky M, Panda S, Straume M, Kay SA 2002 Genome-wide expression analysis in *Drosophila* reveals genes controlling circadian behavior. J Neurosci 22:9305–9319

Claridge-Chang A, Wijnen H, Naef F, Boothroyd C, Rajewsky N, Young MW 2001 Circadian regulation of gene expression systems in the *Drosophila* head. Neuron 32:657–671

Crews ST, Thomas JB, Goodman CS 1988 The *Drosophila single-minded* gene encodes a nuclear protein with sequence similarity to the *per* gene product. Cell 52:143–152

Cyran SA, Buchsbaum AM, Reddy KL et al 2003 vrille, Pdp1, and dClock form a second feedback loop in the *Drosophila* circadian clock. Cell 112:329–341

Darlington TK, Wager-Smith K, Ceriani MF et al 1998 Closing the circadian loop: CLOCK-induced transcription of its own inhibitors *per* and *tim*. Science 280:1599–1603

Dembinska ME, Stanewsky R, Hall JC, Rosbash M 1997 Circadian cycling of a *period-lacZ* fusion protein in *Drosophila*: evidence for an instability cycling element in PER. J Biol Rhythms 12:157–172

Emery P, So WV, Kaneko M, Hall JC, Rosbash M 1998 CRY, a *Drosophila* clock and light-regulated cryptochrome, is a major contributor to circadian rhythm resetting and photosensitivity. Cell 95:669–679

Etter PD, Ramaswami M 2002 The ups and downs of daily life: profiling circadian gene expression in *Drosophila*. BioEssays 24:494–498

Frisch B, Hardin PE, Hamblen-Coyle MJ, Rosbash M, Hall JC 1994 A promoterless DNA fragment from the *period* locus rescues behavioral rhythmicity and mediates cyclical gene expression in a restricted subset of the *Drosophila* nervous system. Neuron 12:555–570

Glossop NR, Houl JH, Zheng H, Ng FS, Dudek SM, Hardin PE 2003 VRILLE feeds back to control circadian transcription of clock in the *Drosophila* circadian oscillator. Neuron 37: 249–261

Glossop NR, Lyons LC, Hardin PE 1999 Interlocked feedback loops within the *Drosophila* circadian pacemaker. Science 286:766–768

Hao H, Allen DL, Hardin PE 1997 A circadian enhancer mediates PER-dependent mRNA cycling in *Drosophila melanogaster*. Mol Cell Biol 17:3687–3693

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

Hardin PE, Hall JC, Rosbash M 1992 Circadian oscillations in *period* gene mRNA levels are transcriptionally regulated. Proc Natl Acad Sci USA 89:11711–11715

Kim EY, Bae K, Ng FS, Glossop NR, Hardin PE, Edery I 2002 *Drosophila* CLOCK protein is under posttranscriptional control and influences light-induced activity. Neuron 34:69–81

King DP, Vitaterna MH, Chang A-M et al 1997a The mouse *clock* mutation behaves as an antimorph and maps within the W^{19H} deletion, distal of *kit*. Genetics 146:1049–1060

King DP, Zhao Y, Sangoram AM et al 1997b Positional cloning of the mouse circadian *clock* gene. Cell 89:641–653

Kloss B, Price JL, Saez L et al 1998 The *Drosophila* clock gene *double-time* encodes a protein closely related to human casein kinase Iε. Cell 94:97–107

Kloss B, Rothenfluh A, Young MW, Saez L 2001 Phosphorylation of period is influenced by cycling physical associations of double-time, period, and timeless in the *Drosophila* clock. Neuron 30:699–706

Konopka RJ, Benzer S 1971 Clock mutants of *Drosophila melanogaster*. Proc Natl Acad Sci USA 68:2112–2116

Lee C, Bae K, Edery I 1998 The *Drosophila* CLOCK protein undergoes daily rhythms in abundance, phosphorylation and interactions with the PER-TIM complex. Neuron 4:857–867

Lee C, Bae K, Edery I 1999 PER and TIM inhibit the DNA binding activity of a *Drosophila* CLOCK-CYC/dBMAL1 heterodimer without disrupting formation of the heterodimer: a basis for circadian transcription. Mol Cell Biol 19:5316–5325

Lin JM, Kilman VL, Keegan K et al 2002a A role for casein kinase 2alpha in the *Drosophila* circadian clock. Nature 420:816–820

Lin Y, Han M, Shimada B et al 2002b Influence of the period-dependent circadian clock on diurnal, circadian, and aperiodic gene expression in *Drosophila melanogaster*. Proc Natl Acad Sci USA 99:9562–9567

Liu X, Yu Q, Huang Z, Zwiebel LJ, Hall JC, Rosbash M 1991 The strength and periodicity of *Drosophila melanogaster* circadian rhythms are differentially affected by alterations in period gene expression. Neuron 6:753–766

Low-Zeddies SS, Takahashi JS 2001 Chimera analysis of the Clock mutation in mice shows that complex cellular integration determines circadian behavior. Cell 105:25–42

Martinek S, Inonog S, Manoukian AS, Young MW 2001 A role for the segment polarity gene *shaggy*/GSK-3 in the *Drosophila* circadian clock. Cell 105:769–779

McDonald MJ, Rosbash M 2001 Microarray analysis and organization of circadian gene expression in *Drosophila*. Cell 107:567–578

Price JL, Blau J, Rothenfluh-Hilfiker A, Abodeely M, Kloss B, Young MW 1998 *double-time* is a novel *Drosophila* clock gene that regulates PERIOD protein accumulation. Cell 94:83–95

Renn SC, Park JH, Rosbash M, Hall JC, Taghert PH 1999 A pdf neuropeptide gene mutation and ablation of PDF neurons each cause severe abnormalities of behavioral circadian rhythms in *Drosophila*. Cell 99:791–802

Rothenfluh A, Abodeely M, Young MW 2000 Short-period mutations of *per* affect a *double-time*-dependent step in the *Drosophila* circadian clock. Curr Biol 10:1399–1402

Rutila JE, Zeng H, Le M, Curtin KD, Hall JC, Rosbash M 1996 The tim^{SL} mutant of the *Drosophila* rhythm gene *timeless* manifests allele-specific interactions with *period* gene mutants. Neuron 17:921–929

Rutila JE, Suri V, Le M, So WV, Rosbash M, Hall JC 1998 CYCLE is a second bHLH-PAS protein essential for circadian transcription of *Drosophila period* and *timeless*. Cell 93:805–814

So WV, Rosbash M 1997 Post-transcriptional regulation contributes to *Drosophila* clock gene mRNA cycling. EMBO J 16:7146–7155

Ueda HR, Matsumoto A, Kawamura M, Iino M, Tanimura T, Hashimoto S 2002 Genome-wide transcriptional orchestration of circadian rhythms in *Drosophila*. J Biol Chem 277:14048–14052

Wang GK, Ousley A, Darlington TK et al 2001 Regulation of the cycling of timeless (tim) RNA. J Neurobiol 47:161–175

Yang Z, Sehgal A 2001 Role of molecular oscillations in generating behavioral rhythms in Drosophila. Neuron 29:453–467

Yu Q, Jacquier AC, Citri Y, Hamblen M, Hall JC, Rosbash M 1987 Molecular mapping of point mutations in the period gene that stop or speed up biological clocks in *Drosophila melanogaster*. Proc Natl Acad Sci USA 84:784–788

Zehring WA, Wheeler DA, Reddy P et al 1984 P-element transformation with *period* locus DNA restores rhythmicity to mutant, arrhythmic *Drosophila melanogaster*. Cell 39:369–376

Zeng H, Hardin PE, Rosbash M 1994 Constitutive overexpression of the *Drosophila period* protein inhibits *period* mRNA cycling. EMBO J 13:3590–3598

DISCUSSION

Weitz: I have a question regarding the ectopic clocks. Is PDF driven in those cells, or are there any other PDF-like transcripts?

Rosbash: I mentioned in passing that when you double stain for PDF, it is restricted to its original homes. There is no PDF expression in any of these ectopic locations. Therefore we don't know what connects from these locations to behavioural outputs.

Kyriacou: The implications of that experiment with *dClock* are that in the more primitive insects, where the oscillators are located more out in the optic lobes, *dClock* may have had a direct effect on placing those cells there, in the same way that homeotic master control genes can put *Drosophila* eyes anywhere.

Rosbash: That is the implication. I think a prediction and a line of experimentation is called for that is simply to look at clock expression in development in different animals.

Sehgal: What you find for *Clock* rescue by *pdf* — that it is really weak — is also true for *per* and *tim*.

Rosbash: That is correct. The point here is that expression in the lateral neurons (by other people's experiments) is insufficient. So there is nothing weird about that *Clk* result. What hadn't been done before is the *cry* rescue, which is much better. My guess would be that if we use *per* or *tim*, we would get the same result.

Sehgal: What you call 'ectopic *Clk*' expression is not necessarily ectopic *Clk*. We don't know what the expression pattern of *Clk* is. It is ectopic on the basis of the fact that it is not in the subsets of cells that you think are circadian-relevant.

Rosbash: To put your question another way, is the rest of the brain really negative for *Clk* expression?

Sehgal: Or even for *per* and *tim* expression.

Rosbash: In the 10 years that this has been described, no one has seen PER or TIM protein or mRNA expression outside of these five centres. When you use *pergal4* or *timgal4* drivers, it is indeed present elsewhere.

Sehgal: It is everywhere.

Rosbash: It is not everywhere. It is in a lot of other places. These places don't correspond to these five centres. I'm sceptical that these are locations of *bona fide* *Clk* gene expression.

Sehgal: According to some people, the *per* and *timgal4* drivers are markers for all the neurons, or maybe even all the cells in the adult. I agree that they are not found everywhere, but the expression is widespread. One explanation is that the drivers are promiscuous. The other is that there are low levels of expression in these places that we don't detect any other way. I would think that this is kind of supported by some of the *doubletime* data, where, in a *doubletime* mutant we get *per* expression in ectopic locations. This would argue that *per* is synthesized in a lot of locations where you normally don't detect it because it is destabilized by *doubletime*.

Young: This could be an amplifier.

Sehgal: That is what I am thinking.

Rosbash: This is part and parcel of the question as to whether there is something special about the cells which now appear in this experiment to have properties which allow them to show cycling clock gene expression, and even connect up with behaviour. This is entirely possible. One of these properties could be some low level of clock gene expression which is there normally.

Stanewsky: In addition to these five groups of cells there are all the glial cells in the brain.

Rosbash: Definitely; I was just referring to neurons.

Stanewsky: Do you know what type of cells these are? John Ewer showed that mosaics which only have glial expression can rescue behaviour. It could be that using this promoter you get up-regulation in glial cell expression.

Rosbash: To put it more generally, if you do a misexpression experiment and get a behavioural consequence with this vector, it is impossible to distinguish whether it is because of up-regulation in the usual locations or because of ectopic expression in the new locations. This is due to the fact that the construct also expresses more heavily in the traditional clock cells. Therefore it could be the up-regulation in the traditional clock cells that leads to the behavioural consequences.

Stanewsky: Did you do a double stain against ELAV (this is a protein expressed in all neurons), for example?

Rosbash: No, but you can see that many of these cells are gigantic, i.e. almost certainly neurons.

Hardin: Have you used either a *per* or *timgal4* driver to do the rescue with *Clk*?

Rosbash: This was the first thing we tried and they are both lethal. And we don't get any aberrant behaviour with *pdfgal4* or *crygal4*, which both over-express in those lateral neurons.

Young: Your interpretation of the *cry* versus *pdf* promoter is that it is a cell-type pattern difference. But CRY is going to oscillate with the same phase as CLK in wild-type flies. Is this necessarily a cell-type difference, or could it be the fact that you are supplying a cycling promoter?

Rosbash: We assume that when we use GAL4, because of its stability, there is little or no cycling left because that protein is so stable. Without an unstable protein it doesn't matter what happens at the RNA level. Secondly, I should have made the point that swapping the promoters on *Clk* has only very modest behavioural consequences. The general sense and feel is that fooling around with promoters doesn't do a great deal, at least from the behavioural point of view. The cell-type issue is legitimate, and although it is very hard to draw a relationship between the cell types and behaviour, I would be shocked if the promoters were doing much here.

Young: We have used the same argument about GAL4. You can make sure by using another route, but that is probably it.

Sassone-Corsi: I was interested in the
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Rosbash: Who knows. No one ha
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Sassone-Corsi: I was interested in the *Clk* recessive allele. I was wondering about the expression profile. You mentioned some alternative splicing.

Rosbash: One splice doesn't occur, but there is probably some re-initiation of something to provide a little bit of transcript that gives rise to either a bit of normal protein or almost normal protein. There are two reasons for drawing this conclusion. One is that by PCR analysis of the transcripts there is still some stuff there that could give rise to normal protein. Second, there is low but not super-low *per* and *tim* expression, and there is still weak amplitude RNA cycling. In other words, these proteins are made in greatly increased amounts compared with the dominant *Jrk* mutation. We infer that the amplitude of cycling is so low relative to wild-type that the flies are behaviourally arrhythmic. The period is only marginally affected. By and large this is an amplitude mutant and not a period mutant.

Sassone-Corsi: Is the alternative splicing present in the same cells?

Rosbash: Who knows. No one has got good *Clk in situ* hybridization so far.

Van Gelder: I'd like to switch to discussing the microarray experiments. There is a question about the relatively low concordance between the different groups who have done microarrays in what is a very similar experimental paradigm. 10% of these genes show up on multiple lists but that majority show up on only a single list. There are several possible reasons for this: statistics, how one decides whether something is oscillating or not, environmental conditions and day 1 DD versus day 3 DD. But this is a really important question because at a genomic level, what does the clock do? Is transcription the output or is it not?

Rosbash: You have touched on two different issues. I would leave aside the question of whether transcription is the output. Let's talk about why the numbers are so different. We have thought a lot about this. One big factor is indeed the method of analysis. What we have done is taken our method of analysis. We had access to Mike Young's raw data and we carried out our method of analysis on both data sets. We also got Straume's method of analysis. The different methods of analysis gave completely different results on the same data set. Then we tried one method of analysis on two data sets, but we still got different results. In other words, we still don't know why the conclusions are so different, but it is at least due to two differences between laboratories.

Van Gelder: I have a problem with the data you showed. There were genes with 30% peak to trough amplitude by RT-PCR. There is no way that you can distinguish less than a twofold change with any reliability on RT-PCR.

Rosbash: I disagree. You can pick out any one graph or gene and we can have a lengthy discussion, but I don't agree with that characterization. Most of the curves

are very similar with the two methods. In other words, with real-time analysis and internal standard curves that is just incorrect. But if 70% of the data depend on 30% amplitudes, then things are indeed dicey. In our paper we set a minimal criterion of 50% amplitude change. The majority of the things we have tested with RT-PCR are greater than this.

Van Gelder: Mike Young's group validated their targets by Northern blot or RNAse protection. Yet those still don't show up in your list.

Rosbash: Our methods were too stringent.

Weitz: They had different methods for selecting waveform; it is not just a matter of stringency.

Rosbash: I would have posed the question a slightly different way at the outset. Everyone has different numbers and the overlap is small. You could break down the question even further: do we have large numbers of false negatives, or large numbers of false positives?

Van Gelder: We know that we don't have that many false negatives because we all found the canonical cycling genes. If the specificity had been very low we wouldn't have recovered those genes.

Rosbash: That is a little facile. We found them all too. In fact, in attempting to get the methods to do this we changed the parameters so that we could find them all. You in fact didn't find them all.

Van Gelder: Actually, we did find them all with the exception of *Takeout.*

Rosbash: Nor did Mike Young find them all.

Young: The list we gave had a particular cut-off, and they were recorded further down the list.

Rosbash: This gets very complicated. How far down the list do you go? This is a work in progress and most people are continuing to do other kinds of experiments.

Young: You can ask not only whether you have oscillation, but also whether the phase fits what you see in your microarray. And you can use the mutants to gain insight into the problem. You can ask what the response of the mutants is. As you gather additional pieces of information, your case gets either stronger or weaker.

Van Gelder: The reason I bring this up gets back to this question of output. How much cycling gene expression is facultative, and how much is mandatory for the organism? Our feeling from doing the analysis and doing the overlap analysis from the other groups' data is that there is only a core set of 25–30 genes that show up repeatedly as oscillating. These appear to be necessarily oscillating: the remainder are either false positives or facultatively oscillating genes that in one particular set-up will show oscillation and in another will disappear.

Rosbash: I think that will be a minor part of the explanation.

Van Gelder: I have a second point. The one thing we have all found, which is stunning and sometimes gets lost in the discussion of the oscillating genes, is how many genes there are whose constitutive level of expression is markedly affected by

the clock genes. We don't have an ex
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strains; and the statistical significance

Rosbash: That has been brought up
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Rosbash: Liver gene expression is

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the clock genes. We don't have an explanation for this. We found over 400 genes showing markedly different basal levels of expression between wild-type and per^0 strains; and the statistical significance of these effects is phenomenal.

Rosbash: That has been brought up at this meeting several times. But there are simple explanations: these could be RNAs with long half-lives.

Van Gelder: If you are asking from both an evolutionary and genomic perspective what the clock genes do, and we took the naïve approach that we didn't really understand that clock genes oscillated, we would do chip experiments and conclude that clock genes are master regulators of static gene expression levels of a huge array of functions. We need to challenge the output model that the sole or major function of these genes is to drive transcriptional rhythmicity, and to also consider the possibility that one of the things that they are doing is setting static levels of gene expression and varying them with environmental conditions.

Menaker: You are focused on methods of analysis here. But it seems to me that if you are going to try to get concordance among experiments from different laboratories, you really have to worry about the conditions of the experiments. Michael Rosbash showed us nice data indicating that damped oscillations can grow in constant darkness in some of the cells. If you take this seriously, and you are trying to compare data sets that were generated after 1 d DD with those generated after 3 d DD, there is a problem.

Rosbash: I don't think of that initial response as damping. I think those cells freak out because of the lack of light cues at the appropriate time. There are enough data from the different groups for us to take comparable data and for this no longer to be a confounding feature.

Kyriacou: Would you then apply these 30 core genes to the mammalian work, where the correspondence is actually much greater among the various studies?

Rosbash: Liver gene expression is a much simpler situation than the fly head.