University of Groningen

The genetic basis of the circadian clock

Dunlap, Jay C.; Loros, Jennifer J.; Aronson, Benjamin D.; Merrow, Martha; Crosthwaite, Susan; Bell-Pedersen, Deborah; Johnson, Keith; Lindgren, Kristin; Garceau, Norman Y.

Published in:
Circadian Clocks and their Adjustment

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version
Publisher's PDF, also known as Version of record

Publication date:
1995

[Link to publication in University of Groningen/UMCG research database](#)

Citation for published version (APA):

Dunlap, J. C., Loros, J. J., Aronson, B. D., Merrow, M., Crosthwaite, S., Bell-Pedersen, D., Johnson, K., Lindgren, K., & Garceau, N. Y. (1995). The genetic basis of the circadian clock: identification of *frq* and *FRQ* as clock components in *Neurospora*. In *Circadian Clocks and their Adjustment* (183 ed., pp. 3-25). University of Groningen, Centre for Behaviour and Neurosciences.

Copyright

Other than for strictly personal use, it is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), unless the work is under an open content license (like Creative Commons).

The publication may also be distributed here under the terms of Article 25fa of the Dutch Copyright Act, indicated by the "Taverne" license. More information can be found on the University of Groningen website: <https://www.rug.nl/library/open-access/self-archiving-pure/taverne-amendment>.

Take-down policy

If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Downloaded from the University of Groningen/UMCG research database (Pure): <http://www.rug.nl/research/portal>. For technical reasons the number of authors shown on this cover page is limited to 10 maximum.

The genetic basis of the circadian clock: identification of *frq* and FRQ as clock components in *Neurospora*

Jay C. Dunlap, Jennifer J. Loros, Benjamin D. Aronson, Martha Merrow, Susan Crosthwaite, Deborah Bell-Pedersen, Keith Johnson, Kristin Lindgren and Norman Y. Garceau

Department of Biochemistry, Dartmouth Medical School, Hanover, NH 03755-3844, USA

Abstract. Genetic approaches to the identification of clock components have succeeded in two model systems, *Neurospora* and *Drosophila*. In each organism, genes identified through screens for clock-affecting mutations (*frq* in *Neurospora*, *per* in *Drosophila*) have subsequently been shown to have characteristics of central clock components: (1) mutations in each gene can affect period length and temperature compensation, two canonical characteristics of circadian systems; (2) each gene regulates the timing of its own transcription in a circadian manner; and (3) in the case of *frq*, constitutively elevated expression will set the phase of the clock on release into normal conditions. Despite clear genetic and molecular similarities, however, the two genes are neither molecular nor temporal homologues. The timing of peak expression is distinct in the two genes, *frq* expression peaking after dawn and *per* expression peaking near midnight. Also, although expression of *per* from a constitutive promoter can rescue rhythmicity in a fly lacking the gene, constitutive expression of *frq* will not rescue rhythmicity in *Neurospora frq*-null strains, and in fact causes arrhythmicity when expressed in a wild-type strain. These data suggest that *frq* is and/or encodes a state variable of the circadian oscillator. Recent molecular genetic analyses of *frq* have shed light on the origin of temperature compensation and strongly suggest that this property is built into the oscillatory feedback loop rather than appended to it. It seems plausible that clocks are adjusted and reset through adjustments in central clock components such as *frq*, and, by extension, *per*.

1995 Circadian clocks and their adjustment. Wiley, Chichester (Ciba Foundation Symposium 183) p 3–25

Living things generally have the capacity for endogenous temporal organization of cellular processes over the course of an approximately 24 h period. The cellular machinery that generates this ability is collectively known as the biological clock, and its output as a circadian rhythm. Investigators have gone to great lengths to prove that these circadian rhythms are in fact endogenous. In

the case of *Neurospora*, for example, such controls have included placing the organism on a rotating platform on the South Pole so that the position of the organism in cosmic space could be controlled (Hamner et al 1962) and even a trip into outer space (Sulzman et al 1984). Despite such machinations, the take-home message from work to date remains the same: that clocks consist of a biochemical–genetic feedback loop, the components of which are active at the intracellular level.

In order to understand, at the level of genetics and biochemistry, how cells keep time and regulate their metabolism, we have been concentrating our efforts on the analysis of the simplest rhythmic system in which both genetics and molecular biology can be used, *Neurospora crassa* (see Dunlap 1993 for review). In this system, 15 mutations associated with seven genetic loci have been identified as affecting the biological clock (Table 1). The genetic characteristics of the *Neurospora* clock suggest several things. There are a number of genes involved in or capable of affecting the operation of the clock, and it is likely

TABLE 1 *Neurospora crassa* mutations identified in screens for circadian clock genes

<i>Allele</i>	<i>Linkage</i>	<i>Period length (h)</i>	<i>Dominance/recessivity</i>	<i>Other clock properties affected</i>
<i>frq</i> ¹	VII R	16	Semi-dominant	
<i>frq</i> ²	VII R	19.3	Semi-dominant	
<i>frq</i> ³	VII R	24	Semi-dominant	Temperature compensation
<i>frq</i> ⁴	VII R	19.3	Semi-dominant	
<i>frq</i> ⁶	VII R	19.3	Semi-dominant	
<i>frq</i> ⁷	VII R	29	Semi-dominant	Temperature compensation, cycloheximide resetting
<i>frq</i> ⁸	VII R	29	Semi-dominant	Temperature compensation
<i>frq</i> ⁹	VII R	Uncompensated, conditionally arrhythmic	Recessive	Temperature compensation, nutritional compensation ^a , entrainment
<i>frq</i> ¹⁰	VII R	Uncompensated, conditionally arrhythmic	Recessive	Temperature compensation, nutritional compensation ^a , entrainment
<i>chr</i>	VI L	23.5	Semi-dominant	Temperature compensation
<i>prd-1</i>	III C	25.8	Recessive	Temperature compensation
<i>prd-2</i>	V R	25.5	Recessive	
<i>prd-3</i>	I C	25.1	Recessive	Temperature compensation
<i>prd-4</i>	I R	18	Dominant	Temperature compensation
<i>cla-1</i>	I R/VII R	27	Semi-dominant	Temperature compensation

^aPeriod length of the rhythm, when expressed, is known to be dependent on the growth medium in the null mutant *frq*⁹, and is inferred to be so dependent in the other null strain, *frq*¹⁰.

that many more clock-affecting loci have yet to be identified, because if one were approaching the point at which most of the clock genes had been found, one would expect most genes to have been identified more than once, yet few of the clock genes found so far have been identified more than once. Also, clearly, the *frequency* (*frq*) locus stands out as being important, because mutations at this single locus can result in both long and short period lengths and loss of temperature compensation.

Our current view of the *Neurospora* circadian programme is shown in Fig. 1, which makes several points worth noting. First, everything of interest at the most basic level of the oscillator is happening *within* the cell; cell-cell communication is not a factor in timekeeping at its simplest level. The problem of organismal rhythmicity is then broken up, at least metaphorically, into two separate issues—that of how the oscillator itself works, and that of how

FIG. 1. The components of the circadian system in a cell. This figure implies more than we really know (for example, the location of the nuclear boundary), while leaving out some recognized effects such as the effects of light on the clock and on *frq* and the independent effects of light on the morning-specific clock-controlled genes, *ccgs* (which are known to be direct rather than being simply transmitted to these genes through the clock). Question marks refer to pathways that are possible but not proven (e.g., FRQ directly affecting the *ccgs*) or to pathways which are known but which contain an unknown number of steps (e.g., the feedback loop connecting *frq* to itself via FRQ).

information about the time leaves the oscillator and effects modulation of the metabolism and behaviour of the cell and, eventually, the organism. Our view of this grows more and more complex as we fill in the arrows in the figure. Now, on the basis of evidence discussed here, we can firmly place *frq* and its protein product FRQ in the oscillatory loop. It appears likely that the capacity for temperature compensation also lies within the loop, not appended to it. It is still somewhat difficult to decide exactly where in this figure to draw the nuclear boundary, and whether to include FRQ in it sometimes or never. At the level of regulation of metabolism, some time ago we undertook the targeted isolation of a bank of genes specifically regulated on a daily basis by the clock, the *ccg* or clock-controlled genes (Loros et al 1989); it is possible that at least some of these clock-controlled genes are regulated directly by FRQ. I shall have little to say about these genes beyond what is in Fig. 1: they were all identified by either subtractive or differential hybridization and, somewhat surprisingly, are generally morning-specific genes.

Because on the basis of its genetics alone, *frq* might have been predicted to be a central component of the circadian clock, we have undertaken the analysis of its structure, function, and regulation. *frq* was cloned several years ago (McClung et al 1989) and is now known to encode several transcripts including at least the following: a short one going leftward, a long one going rightward, and another lying at least partially on top of the latter (Fig. 2). Through the isolation and analysis of cDNA clones corresponding to these products of *frq*,

The *frq* Locus

FIG. 2. A physical map of the region of DNA required for the rescue of recessive loss-of-function mutations in the *frq* gene; marks above the line represent restriction sites (a sizing bar gives an estimate of distance in kbp) (adapted from McClung et al 1989). Two *frq* transcripts (arrows) are drawn underneath a fragment capable of complementing the recessive *frq*⁹ allele, and the dotted line above the large rightward transcript represents a third transcript whose presence was inferred from the cDNAs. Parentheses indicate uncertainty as to the end points of transcripts. See text for details (Aronson et al 1994a).

we learned that the small transcript has no open reading frames (ORFs) running through it, suggesting that it does not encode a protein. It does, however, contain an intron that is spliced out. The large transcript region yields at least two transcripts, a long rightward-directed one being intron free and an overlapping one, which goes leftward, having an intron. Thus, of these transcripts, only one, the approximately 4.5 kb transcript transcribed to the right in the figure, has the potential to encode a protein.

Which of these transcripts are required for clock functioning? To answer this question we have used three separate approaches: physiological analysis of gene knock-outs, molecular analysis of the existing *frq* mutants and analysis of phylogenetic conservation. Knock-outs in either the large or the small transcript region, brought about by replacement or by methylation and disruption, can disrupt rhythmicity (Aronson et al 1994a). This result is consistent with the results of resection from either end (K. Johnson, unpublished work; McClung et al 1989), which has shown that all of the region encoding the large transcript, and most of that encoding the small transcript, is required for normal clock functioning. We (K. Johnson, unpublished work) have shown that knocking out the small transcript region alone or making deletions in the small transcript region disrupts rhythmicity. However, prevention of normal splicing of the intron (by destroying the 5' splice donor site) has no effect on rhythmicity. Finally, knocking out the large transcript region disrupts rhythmicity, as does a frame shift point mutation in the long ORF that results in the production of a truncated protein. These findings focus attention on the large transcript region, but leave open the question of whether one or both of the transcripts are essential.

To get at this question we have studied the existing *frq* mutants, because mutational analysis offers an unbiased way of finding out which parts of a region are important. When Ben Aronson looked to see where in the entire *frq* region the existing mutations mapped, and what aspects of *frq* they disrupted, he found that they all lie within the single long FRQ ORF; none of the mutations lies within *frq* regulatory regions, the small transcript, or the promoter region driving expression of the transcripts. Surprisingly, there is considerable redundancy within the allelic series. Alleles with identical phenotypes are, in fact, genetically identical; a meaningful distinction can no longer be made between *frq*², *frq*⁴ and *frq*⁶, or between *frq*⁷ and *frq*⁸. Finally, there is distinct clustering of the alleles around one region in the centre of FRQ. The three alleles with the most extreme differences in period length as compared with wild-type (*frq*¹, *frq*³ and *frq*⁷) map to an interval of just 12% (124 codons) of the protein, and the two most extreme alleles, *frq*¹ and *frq*⁷, lie within just 24 amino acids of each other.

The third approach we undertook in an effort to understand the function of *frq* was the study of its phylogenetic conservation. We (Morrow & Dunlap 1994) have now cloned and sequenced all or part of *frq* from five different

FIG. 3. Comparison of DNA sequences corresponding to the *frq* region from two different fungi, *Neurospora crassa* and *Sordaria fimicola*, reveals that the protein-coding region of *frq* is highly conserved. The *frq* gene, as defined by the region of DNA required to complement recessive loss-of-function mutations in *N. crassa*, is shown schematically as a horizontal line. Hallmarks of the different regions of *frq* are noted below the line. Insertions in the *Sordaria fimicola* genome as compared with the *N. crassa* genome are noted as bars above the horizontal line, and deletions as bars below the line. The open reading frame encoding the *frq* gene product (FRQ) is clearly the most highly conserved region (Morrow & Dunlap 1994). 5' UTR, untranslated region.

fungi representing three genera (*Neurospora crassa*, *N. discreta*, *N. intermedia*, *Gelasinospora cerealis* and *Sordaria fimicola*) and have found that there is considerable conservation within the long *frq* ORF, but not so much outside it (Fig. 3). Additionally, within the ORF there are preferentially conserved regions. Specifically, all of the sites identified in *N. crassa* as important to *frq* functioning are conserved, as are many of the hallmark features of FRQ, including the PEST regions (sequences highly enriched in the amino acids proline, aspartic acid, glutamic acid, serine and threonine, often found in proteins showing rapid turnover), the putative nuclear localization signal and the hyperacidic region. Among the parts of *frq* that are most highly conserved is the region surrounding the *frq*¹ and *frq*⁷ mutations in which even between the most highly diverged species (*Sordaria fimicola* and *Neurospora crassa*) there is just one conservative change over 75 codons. Despite about 15% overall divergence of sequence between the *frq* genes of these two fungi, there is also conservation of function. To examine this, we cloned the *Sordaria fimicola*

FRQ ORF into the context of the *N. crassa* gene so that the *N. crassa* regulatory sequences would control the expression of the *Sordaria* protein. This construct was then targeted back into *N. crassa* bearing loss-of-function mutations in *frq*. Under these conditions, the *S. fimicola* gene will rescue, i.e., restore, rhythmicity in the *N. crassa* mutant. This is particularly interesting in light of the fact that the rhythm being monitored in *Neurospora* was the typical rhythm in conidial production, but *Sordaria* does not produce conidia. Thus, what is conserved is not a 'developmental gene' in the classical sense, but rather a central control gene that is required for the operation of a circadian clock which can be used to initiate diverse developmental pathways.

Several conclusions can be drawn from our studies of functional and mutational analysis and phylogenetic conservation. FRQ is a large protein, of about 1000 amino acids, whose general structure and role in the operation of the clock are conserved. Single amino acid substitutions within it are sufficient to affect both period length and temperature compensation, and loss-of-function mutations result in conditional arrhythmicity and complete loss of clock compensation.

One can also speculate about *frq*. It seems likely that it is tightly regulated; a very long 5' untranslated region preceding the ORF may play an important role in regulating *frq* functioning post-transcriptionally. The small *frq* transcript may have no explicit role in running the clock, but the region encoding it apparently must be present, perhaps to regulate the transcription of the large ORF-containing region. The role of the third transcript remains a mystery also; it is of very low abundance and may be irrelevant, or it may play a role in regulating the level of the large ORF-containing transcript. However, it seems clear that there is a single ORF encoding a protein, FRQ, that is the *trans*-acting product of the locus. Although we can only guess at the real biochemical role FRQ plays in the oscillator, it is noteworthy that the FRQ ORF contains a strong hyperacidic region in its C-terminal part that is reminiscent of known transcriptional activators from several systems (Leuther et al 1993), and that we (M. Merrow) have recently shown that FRQ, when tethered 5' to a yeast promoter by fusion to the DNA-binding domain of GAL4, is capable of activating transcription in yeast.

The regulation of *frq* suggests that FRQ is a component of the clock

Although the results presented thus far are consistent with a role of FRQ in the oscillator, they can also be explained in other ways. An understanding of the regulation of this gene is therefore essential. It has long been appreciated that if FRQ is a component of the clock, and if the clock is a feedback loop, then there is a real possibility that the synthesis of FRQ is regulated by the clock; i.e., if there is a loop defining the clock, in it FRQ could control the clock and the clock control *frq*. We tried for several years to find evidence

for control of *frq* expression by the clock under the conditions we commonly use for biochemical analysis of clock-related processes, namely, starvation conditions in the dark. We chose these conditions because under them the clock runs normally but differentiation is drastically curtailed. Thus, we had reasoned, we should be able to focus on a normal clock without being distracted by developmental regulation. Although under these conditions, *frq* transcript(s) are extremely inabundant, we (J. Loros) recently succeeded in adapting conditions using very highly labelled riboprobes and showing that the synthesis of *frq* is regulated by the clock (Fig. 4). In this regard then, *frq* is a morning-specific *cgc*. In separate experiments, we (K. Johnson) have shown that the level of *frq* transcript in the loss-of-function strain *frq*⁹ remains high at all times of day, consistent with *frq* and FRQ regulating their own expression and synthesis through negative feedback (Aronson et al 1994).

These findings clearly put *frq* in the feedback loop, because mutations in the gene affect the timing of its own expression. Interestingly, *frq* expression peaks in the morning and bottoms out in the early evening, generally around dusk. In this regard, *frq* is clearly different from *per*, the transcription of which peaks in the early night. This is an important observation: the first two clock genes to be cloned and analysed at the molecular level both show regulation of the levels of their transcripts (and can therefore be placed with some, if not complete, certainty within the feedback loop), but they are out of phase with one another. Finally, as an aside, it should be noted that this observation provides a ready explanation for the phase-resetting effects of inhibitors of translation (Dunlap & Feldman 1988, Khalsa & Block 1992) and transcription (Dunlap et al 1994, Raju et al 1991): *frq* must be transcribed and the transcript translated on a daily basis for the clock to run, and the time of maximum sensitivity to inhibitors of both transcription and translation is in the subjective morning, about the time of the maximum *frq* transcript level.

The other transcription that is going on in the subjective morning, of course, is the transcription of the various *cgc*s. More than 10 of these genes have now been identified and nearly all are morning specific; this is a surprise, because we had expected the times of maximum *cgc* transcript concentration to be scattered throughout the day rather than being heavily clustered within just one part of the day. This finding provides the basis for several speculations. First, because we have shown that FRQ is capable of activating transcription from a heterologous promoter, it is possible that the protein might be synthesized rapidly and then act immediately to turn on the morning-specific genes. In this manner, it could be viewed as an activator of conidiation, a role that fits several of the phenotypes associated with loss-of-function mutations at *frq*. Alternatively, because *frq* expression is being turned on in the morning, it is possible that whatever is turning on the *cgc*s might also be turning on *frq*.

Several testable predictions can be made to verify the hypothesis that *frq* and FRQ are central components of the feedback-based oscillator: (1) just

FIG. 4. *frq* is a morning-specific gene which regulates the timing of its own synthesis. Relative amounts of the large (approximately 4500 nucleotide) *frq* transcript found in *N. crassa* mycelia isolated at different times are plotted. Data were collected in two genetic backgrounds, *frq*⁺ (top) and the long period mutant *frq*⁷ (bottom). In each case, the *frq* message is seen to oscillate with the period length of the clock appropriate to the strain. The RNAs peak at different times in darkness but at the same subjective clock times, in the early subjective morning (Aronson et al 1994b). CT, circadian time.

as loss-of-function mutations eliminate rhythmicity, constitutive elevated expression of FRQ should result in arrhythmicity; and (2) reducing FRQ concentrations in a step-wise fashion from these elevated levels to normal concentrations should reset the clock to a unique phase.

To test these predictions, we (Aronson et al 1994b) developed an inducible promoter system for *Neurospora*, using as a basis the *qa-2* gene and regulatory system (Giles et al 1985). The *qa-2* gene is regulated in a positive manner by the *qa-1^F* gene, whose activation is normally blocked by complexation with the product of the *qa-1^S* gene. In the presence of inducer (a non-essential metabolite, quinate), the repressor dissociates from the activator, which then

turns on the structural genes, including *qa-2*. With this system, a 300-fold induction can be achieved within about six hours (Chaleff 1974). We generated a construct (pBA40) in which the FRQ ORF was cloned immediately following the initiating ATG of the *qa-2* gene, so that in the presence of inducer *frq* message (but without the long 5' untranslated leader) would be synthesized and FRQ made. RNA analysis confirmed that FRQ-encoding RNA was made in the presence but not in the absence of inducer.

Prediction 1: constitutively elevated expression of frq will result in arrhythmicity

Strains of *N. crassa* wild-type with respect to the clock were transformed with the pBA40 construct and grown in the presence of increasing concentrations of inducer. In this experiment, then, the experimental strain (*frq*⁺ transformed with pBA40) is its own control. At zero or at low concentrations of inducer (up to about 10⁻⁶ M), conidial banding and the clock appear normal. [The operation of the *Neurospora* circadian clock is expressed as a daily clock-regulated change in developmental potential and hyphal morphology. Each subjective morning, the mycelia at the growing front of a culture are endowed with the capacity to differentiate from vegetative surface mycelia into aerial hyphae that can eventually give rise to asexual spores (conidia). Mycelia that are laid down several hours later, near noon and throughout the rest of the circadian day, however, do not have this strong potential to differentiate. Thus, following a week of growth across an agar surface, the periods of subjective morning are represented as yellow/orange zones or 'bands' of conidia and differentiated or differentiating mycelia, interspersed with interbands of predominantly vegetative surface mycelia which were laid down at other times of day.] At higher concentrations of quinate, banding appears to broaden and become obscure after several days, and at the highest concentrations (greater than 10⁻⁵ M) the banding rhythm is completely lost after the first day. The first prediction, that increased constitutive expression of *frq* should cause arrhythmicity, is thus confirmed.

There are several points worth making about this experiment. This is a conditional phenotype resulting from the manipulation of a known clock component. Rhythmicity is not lost instantaneously, but rather gradually, and more rapidly at higher concentrations of inducer, and therefore FRQ. Also, unexpectedly, there are no major effects on period length, although there could have been small effects, of up to a few hours, that were obscured by the broadening and coarsening of the rhythm. This may reflect tight compensation of the rhythm, which is held steady at higher and higher concentrations of FRQ until compensation fails and arrhythmicity ensues. Finally, it should be noted that we have been unable to rescue normal rhythmicity in an *frq*-null strain through constitutive expression of FRQ at any level. This suggests that FRQ must

not only be present, but also that it must be regulated in the correct manner, i.e., because we have exquisite control of FRQ synthesis, we predict that the missing aspect of expression is not quantitative but rather temporal control.

These findings also suggested to us that *frq* and FRQ might have two roles in the cell, one as a component of the clock and the other simply as a transcriptional activator of the developmental process resulting eventually in conidiation. Such a role for FRQ in regulating conidiation would be consistent with the known phenotype of *frq*-null strains which produce fewer conidia than *frq*⁺ strains. It is possible that FRQ's developmental effect might be exerted at lower concentrations than that on the clock, with the result that at concentrations of FRQ at which we would see effects on period, the observation of the rhythm is already obscured.

Prediction 2: in a wild-type strain constitutively expressing frq under the influence of an inducer, step-wise decreases in the concentration of the inducer should completely reset the phase of the clock

The transformed strains described above were grown in liquid medium in constant light in the presence of 10⁻⁴ M inducer, the minimal concentration necessary for the full phenotypic effect. Controls, either untransformed strains with and without inducer, or transformed strains without inducer, were grown under the same light regimen. Every five hours over a period of 20 h, a full circadian cycle, groups were transferred from light to dark to set the clock to phases covering the cycle. Three hours after the final transfer into the dark, all of the cultures, experimental and control, were transferred out of liquid medium, washed, and transferred to race tubes. The first conidial bands (signifying expression of a functional clock) appeared one or more days later, and the phase of the clock at the time of transfer onto the race tubes was inferred from the phase of these bands (Fig. 5).

There are several points to note about this experiment. First, the transition from light to dark clearly sets the phase of all cultures except those bearing the inducible construct in the presence of inducer. Second, in the experimental strain, a stepped decrease in *frq* expression clearly sets the phase of the rhythm absolutely; prediction 2 is thus confirmed. Third, all experimental cultures (i.e. all cultures of *frq*⁺/*qa*-2FRQ ORF + 10⁻⁴ M quinic acid, irrespective of their light-to-dark transfer time) are set (by the shift out of inducer) to the same unique phase point corresponding to around circadian time (CT) 9–11 (approximately the same phase reached following strong resetting by light). This third point represents an unexpected bonus in this experiment, because only the resettability of the clock by the high FRQ to low FRQ shift was required for the model to pass the test, and not the ability to predict the phase (in this case to CT9–11) following the release from quinic acid induction. This predictability of phase thus deserves further discussion.

FIG. 5. Steps from high to low expression levels of an FRQ-encoding transcript set the clock to a unique phase. Cultures (*bd*; *frq*⁺ and *bd*; *frq*⁺ [pBA40 transformant]) were grown in liquid medium in constant light in the presence or absence of 10⁻⁴ M inducer (quinic acid, QA). Starting at time 0, at staggered five-hour intervals, groups of cultures (six individual for each treatment and transfer time) were transferred from constant light to darkness to set the clocks out-of-sync to phases scanning the circadian cycle. Five hours after the final light-to-dark (L-D) transfer, all cultures were washed free of old medium and inducer, and placed onto race tubes where they could express their rhythm in conidiation. The first bands of conidiation appeared one or two days later, and, given the knowledge that the centre of a conidial band corresponds to approximately CT 0, the phase of the clock at the time of transfer onto the race tubes (and therefore out of inducing conditions) was inferred from the phase of these bands. A more detailed explanation of the experiment follows. The figure can be viewed as a schematic representation of a series of five sets of race tubes running from left to right, where the position of each symbol (□, ●, ■ and +) corresponds to the position of the conidial band (the point representing CT 0) for each tube on that day. The x-axis has units of time, but biological time rather than sidereal time is used; one circadian day (approximately 23 sidereal hours for the strains in this experiment) contains 24 circadian hours, so each division represents four circadian hours. Each symbol is plotted at the average time of day of the centre of a conidial band on a set of six replicate race tubes on one circadian day, such that the same symbol recurs at intervals of one circadian day. The positioning of the symbols, however, differs among the sets because the clocks in each tube were set to different times of day by the different pretreatments of light/dark and ± QA. Standard deviation is shown only for the experimental samples; in all other cases, the error bars were contained within the width of the symbol used. In the three control strains—*frq*⁺ without QA (uninducible strain without inducer), *frq*⁺ with QA (uninducible strain with inducer), and *frq*⁺/*qa-2frqORF* without QA (inducible strain without inducer)—the clocks should be set solely by the time of the L-D transition. The experimental strain was *frq*⁺/*qa-2frqORF* with QA in the liquid culture medium (inducible strain with inducer); the third control above verifies that the clock in this strain can respond to a light-to-dark transfer, but the clock in this strain also should be

If the feedback loop constituting the clock is made up of a number of state variables (i.e., more than just *frq* and FRQ) that oscillate with respect to one another, and whose phases with respect to one another yield the final 'overt' phase, then it might have been supposed that the phase to which the clock would be reset following the high to low FRQ shift would, *a priori*, not be predictable, since this final phase would depend not only on the level of FRQ and *frq* but also on the values of other state variables at the time the oscillation was re-initiated. None the less, the data show that the clock is reset to the low point of the *frq* transcript cycle, the same point as would be predicted if the oscillator were made up of only *frq* and FRQ. Does this mean there are no additional components awaiting discovery? This is possible, but seems highly unlikely given the complex properties attributable to the oscillator, including the limits of entrainment and temperature and nutritional compensation. Thus, we favour the alternative, that there are several state variables yet to be identified, and suggest the following interpretation for our data: the extremely strong clock-resetting stimulus represented by the high concentration of FRQ may artificially drive not only *frq* and FRQ, but also the other state variables, to the unique phase points they assume when *frq* is at its low point in the normal cycle. The analogy could easily be made to resetting by light. A short pulse of light, of minutes in duration, will reset (advance or delay) the clock to the daytime part of the cycle no matter where in the cycle the clock is when it sees the light. However, the clock can end up anywhere in the day—a span of some 10 h—depending on where it was before the pulse. A long

influenced by the presence of a high level of FRQ (brought about by the presence of the inducer QA) and *may* be reset by the step decrease in QA when the culture is transferred out of QA-containing medium (this is the experiment). The *y*-axis plots the time of transfer from light to dark, and a line drawn through the L-D transfer times (marked L-D) for all five sets of cultures runs, approximately 30° from vertical, obliquely across the first day. The line connecting the times at which all of the cultures were washed and plated onto race tubes (five hours after the final L-D transfer) is nearly vertical and is marked +QA - QA. After several days of growth, a line connecting all of the band centres for all the controls would run obliquely across the figure (at an angle of about 30°), nearly parallel to the line marking the time of the L-D transfer; this is because the light-to-dark transfer has started the clocks of those cultures at five-hour intervals. To illustrate this, lines actually paralleling the L-D transfer line are plotted four and five days into the experiment; the data points fit closely to these lines. However, if the +QA to -QA shift has reset the clocks in the experimental strains, then a line connecting the centres of these experimental cultures should be close to vertical, paralleling the +QA to -QA line; clearly this is the case, and to illustrate this, a line actually parallel to the +QA - QA line has been drawn on Day 5. This line runs through all of the experimental points (H), verifying that the +QA to -QA shift, not the L-D shift, was the predominant determinant of the phase in these samples. Thus, the shift from high levels of FRQ-encoding transcript to the normal feedback-regulated level has completely reset the clock. See text for more details (Aronson et al 1994b).

pulse of light (over 12 h), however, will drive the clock to the unique phase point corresponding to subjective dusk—a span of about one hour—regardless of where the clock was when the long pulse of light began. In our case, the ‘pulse’ of FRQ was both quite strong (in comparison with the normal level of *frq* transcript and FRQ in the cell) and more than 12 h long, so it may be that this high level of FRQ is driving all of the state variables, rather than just *frq*. If so, then our results are the results expected and are easily interpreted: elevated FRQ represses its own synthesis and eliminates feedback. Release from the effects of high FRQ concentrations resets the clock to the low point in the oscillation, a point corresponding very generally to the region surrounding dusk, the point noted above to be the nadir in the daily *frq* cycle.

Conclusion: *frq* and FRQ have characteristics of components, very probably state variables, in the circadian oscillator

We have reported data showing that: (1) point mutations in the *frq* gene affect both period length and temperature compensation; (2) loss-of-function mutations result in loss of stable rhythmicity and loss of compensation; (3) the protein-coding portion of the *frq* gene is highly conserved and the function of the gene in the operation of the clock is also conserved: *frq* is a clock gene, not just a conidiation gene; (4) *frq* is within the feedback loop: it regulates the timing of its own expression by negatively feeding back, at least indirectly, to repress its own expression; (5) constitutive expression of *frq* and FRQ appears to stop the clock (resulting in arrhythmicity); (6) step reductions in the concentration of FRQ set the clock to a unique phase, CT 9–11, around late day to dusk.

By now concentrating on the identification of the factors governing the expression of *frq*, we hope to complete the roster of clock genes in this circadian system.

Acknowledgements

This work was supported by NIH grants to J.C.D. (GMS34985, MH44651), an NSF grant to J.J.L. (MCB-9307299) and the Norris Cotton Cancer Center Core grant. B.D.A. was supported by NIH Postdoctoral Fellowship GM14465.

References

- Aronson BD, Johnson KA, Dunlap JC 1994a Circadian clock locus *frequency*: protein encoded by a single open reading frame defines period length and temperature compensation. Proc Natl Acad Sci USA 91:7683–7687
- Aronson BD, Johnson KA, Loros JJ, Dunlap JC 1994b Negative feedback defining a circadian clock: autoregulation of the clock gene *frequency*. Science 263:1578–1584

- Chaleff J 1974 Induction of quinate metabolizing enzymes in *Neurospora crassa*. J Gen Microbiol 81:357–372
- Dunlap JC 1993 Genetic analysis of circadian clocks. Annu Rev Physiol 55:683–728
- Dunlap JC, Feldman JF 1988 On the role of protein synthesis in the circadian clock of *Neurospora crassa*. Proc Natl Acad Sci USA 85:1096–1100
- Dunlap JCL, Crosthwaite S, Feldman JF, Loros JJ 1994 An inhibitor of transcription resets the phase and lengthens the period of the *Neurospora* clock. J Biol Rhythms, under revision
- Giles NH, Case ME, Baum J et al 1985 Gene organization and regulation in the *qa* (quinic acid) gene cluster of *Neurospora crassa*. Microbiol Rev 49:338–358
- Hamner K, Finn J, Sirohi G, Hoshizaki T, Carpenter B 1962 A biological clock on the South Pole. Nature 195:476–480
- Khalsa SBS, Block GD 1992 Stopping the biological clock with inhibitors of protein synthesis. Proc Natl Acad Sci USA 89:10862–10866
- Leuther KK, Salmeron JM, Johnston SA 1993 Genetic evidence that an activation domain of GAL4 does not require acidity and may form a β -sheet. Cell 72:575–585
- Loros JJ, Denome S, Dunlap JC 1989 Molecular cloning of genes under the control of the circadian clock in *Neurospora*. Science 243:385–388
- McClung CR, Fox BA, Dunlap JC 1989 The *Neurospora* clock gene *frequency* shares a sequence element with the *Drosophila* clock gene *period*. Nature 339:558–562
- Marrow MW, Dunlap JC 1994 Intergeneric complementation of a circadian rhythmicity defect: phylogenetic conservation of structure and function of the clock gene *frequency*. EMBO (Eur Mol Biol Organ) J 13:2257–2266
- Raju U, Koumenis C, Nunez-Reguero M, Eskin A 1991 Alteration of the phase and period of a circadian oscillator by a reversible transcription inhibitor. Science 253:673–675
- Sulzman F, Ellman D, Fuller C, Moore-Ede M, Wassmer G 1984 *Neurospora* rhythms in space: a reexamination of the endogenous–exogenous question. Science 225:232–234

DISCUSSION

Menaker: Would you accept a correction to Fig. 1, since it's recently been clearly demonstrated that prokaryotes have circadian rhythms (Kondo et al 1993)? Figure 1 seems to me to be too complex to represent the minimal unit; a much simpler organization can sustain circadian rhythmicity. That's absolutely fascinating and has some interesting possible consequences. It means that we have pushed back the origin of circadian rhythmicity a whole kingdom's worth, and perhaps to one and a half billion years ago when we have the earliest fossil record of blue-green algae. That has two potential further consequences. One is that it is possible that the clocks of eukaryotic cells were introduced by prokaryotic symbiosis—that is, that the clock mechanism itself comes from prokaryotes. The second potential consequence, which is more relevant to this discussion, is that if in fact clocks evolved as long ago as this finding suggests, and if there has been a common line of descent—which is of course a big if—they have had to adjust over that one and a half billion years to a remarkable slowing down of the earth's rotation. The mechanism which evolved under those

early conditions has been modified evolutionarily over the last one and a half billion years into what we think of as a circadian rhythm. Perhaps the easiest way to have done that would have been by adjusting the delay region of a system comprising feedback with delay. If that's what happened, we would expect to see mutations causing quantal changes in period, which might act through insertion of successive delay elements.

Dunlap: I would agree that Fig. 1 could be modified; let's call it the clock in a eukaryotic cell rather than just the cell. The results of Kondo et al (1993) are right and support Beatrice Sweeney's last major contribution before her death, which was to show that there were circadian period lengths and temperature compensation in the cell division rhythms of *Synechococcus*, the same prokaryotic system (Sweeney & Borgese 1989). These findings really do push the origin of clocks further back.

The issue of quantal elements is more dicey. Many of the arguments about such quantal elements in the clock were originally made on the basis of Feldman's mutants in *Neurospora* (Feldman 1982). He had reported independently isolated strains with mutations altering the normal 21.5 h period length of the *Neurospora* clock. One had a period of 16 h, and three strains with ostensibly different mutations had a period of 19 h, suggesting that a quantal element of about three hours could be added or subtracted to change the period length. Similarly, going from wild-type with a period of 21 h to a long period mutant of 24 h and two nominally different mutations altering the period to 29 h suggested there were quantal elements adding, here, 2.5 and three times 2.5 (7.5) h to the period length. However, one of the primary assumptions was wrong: Feldman's mutations were almost certainly not independent after all. All three supposedly independently isolated 19 h period alleles are genotypically identical; they have the same single base pair alteration giving rise to the same amino acid change in FRQ, a change from alanine to threonine at position 895. Similarly, the two ostensibly different 29 h period alleles have the same base change resulting in a glycine to aspartic acid substitution at position 459 of FRQ. The odds of this happening by chance are exceedingly low, suggesting that the phenotypically identical isolates were simply reisolates of the same, single, original mutant strains.

Given this, the whole 'quantal element' picture has fallen apart. There is one mutant at 16 h and one at 19 h. There could also be short period *frq* mutants with period lengths of 17 and 18 h that we haven't found yet, but there is now no reason to assume that they cannot be found. Similarly, with just one mutant with a period of 24 h and one with a period of 19 h, we can't be sure that there are no potential alleles giving period lengths between these two. That doesn't mean that your argument about adding delays is wrong, but rather that there is no genetic support for the idea that period lengths can be changed by adding or subtracting quantal elements.

Loros: It's important to point out that the mutants with long periods, 24 and 29 h, are also altered in temperature compensation. You have to remember the period length is not 24 and 29 h invariably, but only at 25 °C. *Neurospora* has a physiological range from 18–20 °C up to 30 °C.

Edmunds: Some years ago Klevecz (1976) proposed that there might be a quantal element in the length of the cell division cycle. He based this idea on his analysis of the variance of the published generation times of mammalian cell lines in culture, as determined by time-lapse cinematography of individual cells. He discovered what appeared to be a quantal element (G_q) of about 3.75 h. Although in retrospect the evidence may have been a bit shaky, the idea of building up longer period lengths (that is, generation times) up to about 26 h by adding on successive integer multiples of this basic quantal period (which he took to reflect a fundamental limit cycle oscillator underlying the cell division cycle) remains interesting and has its analogue in the possibility that the circadian oscillator derives its period from a multiplication (frequency demultiplication) of some basic ultradian period (see Edmunds 1988, p 111–118). This notion came long before we knew anything much about the supposed quantized array of *Neurospora* circadian τ s. Now that an entirely new area in cell cycle regulation has opened up, replete with restriction and execution points that mediate the transit of a cell across the division cycle and help to explain the variability in the length of the G1 (and other) phase(s)—with a little help from the cyclin oscillator and the *cdc2* or *cdc28* gene products—perhaps the concept of quantal elements should be revisited.

Takahashi: Because Jay Dunlap was too modest to say this, I would like to point out that the experiment shown in Fig. 5 is the most definitive to show that any gene is actually part of a feedback loop. Dr Dunlap may have said that, but I'm not sure that everyone appreciated the significance of this experiment. For none of the clock genes that have been studied, *per* in particular, has such a definitive experiment yet been done. The inducible regulation of *frq* and its resetting of the phase of the conidiation rhythm is remarkable. A similar approach has been taken in *Drosophila* using a heat-shock promoter construct to drive *per* expression (Edery et al 1994). The complication with that experiment, however, is that the heat pulse resets the rhythm in control animals, which is not the case with the inducer, quinic acid, in Dr Dunlap's *Neurospora* system.

In *Neurospora*, constitutive expression of *frq* stops the clock in a wild-type background and also does not rescue the *frq*-null mutant. That is not the case in *Drosophila*. What are your thoughts on that?

Dunlap: The results in *Drosophila* to which you are referring, which supposedly show that constitutive expression of *per* from a heat-shock promoter is capable of rescuing rhythmicity in a *per* loss-of-function fly (Ewer et al 1988)—if taken as correct and at face value—are not consistent with feedback-regulated transcription from *per* being a part of the clock. Either oscillatory

per transcription isn't necessary for the clock, or the supposedly constitutive expression isn't constitutive after all. In contrast, our experiments suggest just what we would have predicted, that the clock gene (*frq*) transcript and clock protein (FRQ) must not only be present, but must also be present at the right time of day—having them there all the time isn't sufficient for a functional clock. There is a caveat to our experiments, though. As we turned on *frq* expression constitutively the rhythm got worse and worse over about two days until it finally degraded into arrhythmicity. *frq*, acting through FRQ, appears to regulate its own transcription, but we (M. Merrow & J. Dunlap, unpublished work) also have some results suggesting that FRQ can activate transcription; as well as being a component of the feedback loop, FRQ may actually turn on conidiation genes. If it turns on conidiation genes at concentrations lower than those required for its operation in the clock, we may be masking the effect on the clock; that is, the clock could, conceivably, have been running at intermediate levels of induction with an altered period length. By the time we increase the concentration of *frq* mRNA enough to see arrhythmicity in conidiation, we haven't yet got long period lengths. However, if we could look at the operation of the clock at even higher concentrations of FRQ, we might see long periods. It was to disprove this possibility that we carried out the FRQ-induced clock resetting experiments shown in Fig. 5.

The results in *Drosophila* are somewhat difficult to understand. On the one hand, elegant experiments by Hardin et al (1990) have shown that *per* is transcriptionally regulated and so is similar to *frq* except that the time of day of the *per* RNA peak is different—*per* is an evening gene whereas *frq* is a morning gene. On the other hand, supposed constitutive expression of *per* under the control of a heat-shock promoter is sufficient to rescue the rhythm. That suggests that the transcriptional rhythm, which we now know to be a fact in two out of two regulated systems (*Neurospora* and *Drosophila*), isn't critical for the clock in *Drosophila*. It is essential to determine whether transcription from the heat-shock-driven *per* promoter is actually constitutive or whether it's rhythmic. If the transcription is rhythmic, then there must be a second loop, one loop built around the clock gene—*frq* or *per*—feeding back on itself, and the other built around the clock protein feeding back on itself, and either of those two loops should be sufficient for rhythmicity.

Takahashi: A third possibility is that there is an intronic regulatory element in the *per* gene.

Dunlap: If that is the case, heat-shock-driven *per* expression is not really constitutive. I have a hard time understanding why there should be rhythmic transcription if it is not necessary; I believe it is, and that the heat-shock-driven *per* expression is not, after all, completely constitutive.

Block: I'm not convinced that Dr Dunlap's elegant experiment shows definitively that *frq* expression is an intrinsic part of the timing loop. Consider, for example, an oscillator that we know something about, such as membrane

potential oscillations in cardiac cells. In this case we know that calcium influx controls the potassium channels which are responsible for membrane potential oscillations. We can now do a thought experiment: if you dramatically increase a membrane conductance such as chloride conductance and, for the sake of argument, let's say that chloride conductance does not normally vary rhythmically (and therefore cannot be a state variable in the oscillator), the increase in chloride conductance will clamp the membrane potential and thus stop rhythmic changes in membrane potential. If you allow chloride conductance to return to its normal state, the oscillation will almost certainly start from some fixed point in the cycle. In other words, you would get many of the effects that Dr Dunlap observed, but the difference is that we know the chloride conductance is not a rhythmic element in the feedback loop. However, the issue is more complicated: chloride conductance might well be rhythmic, because of voltage sensitivity of the chloride channels, and yet still not be responsible for generating the rhythmic membrane potential changes.

My feeling is that the experiment Dr Dunlap described is not a definitive experiment, but part of an argument, one of a set of observations that can be taken together along with the fact that *frq* expression is rhythmic to argue that rhythmic *frq* expression is part of the timing loop. However, for this argument to be convincing, it will have to be shown that the oscillation stops if *frq* expression is held constant at any level, not just at a high level of expression.

Takahashi: This experiment by itself is not sufficient, but the aggregate of all the experiments that Jay Dunlap has done together with the constitutive expression of *frq* are quite convincing.

Dunlap: The *frq* gene encodes a product which regulates its own transcription and therefore is involved in feedback. The loss-of-function mutations at *frq* result in high-level expression of *frq* mRNA, which implies negative feedback. Finally, artificially driving constitutive high levels of FRQ-encoding transcript, via the *qa-2* promoter in an *frq*⁺ background, results in arrhythmicity, and a stepped reduction from these high levels both allows rhythmicity to resume and sets the phase of the subsequent rhythm, starting it from the unique phase point defined by the low point in the *frq* transcript oscillation. Those four things suggest that *frq* must be a clock component. Additionally, we have done just the experiment that you suggested, holding the level of *frq* transcript constant, in an *frq* loss-of-function background, at many intermediate levels; in no case could we rescue rhythmicity in this *frq*-null strain. Thus, *frq* cannot simply be supplying a support function as modelled in Dr Block's thought experiment. *per* could be doing this, but not *frq*. There may be ways of interpreting all these results that don't put *frq* in the feedback loop but I haven't come across them yet.

Mrosovsky: I wonder whether it is really true that temperature compensation is intrinsic to the rhythmic mechanism itself. There are some tropical plants, discussed in Bünning's book (1973, p 76), which apparently don't have temperature compensation, but have circadian rhythms.

Dunlap: It would be interesting to find out if they really have a circadian rhythm, which depends somewhat on what we define as a circadian rhythm. I have a slide, which I often use, outlining the characteristics of clocks—a period length close to 24 h which is reset by stepped changes in light and temperature, and temperature compensation. Among the fungi, there are some ‘rhythmic’ strains which are completely uncompensated. In the physiological temperature range they have period lengths of around a day, but when you push them up to 30 °C or to below 15 °C you can get period lengths that run from six to 40 h. These are typically viewed as non-circadian oscillatory systems.

Loros: If these plants anticipated a change from light to dark, or dark to light, this would help you to define their rhythms as real circadian rhythms that may have lost the capacity for temperature compensation because of the tropical conditions.

Edmunds: This discussion could be extended to ultradian rhythms and their relation to circadian rhythmicity. Jay Dunlap asked whether these plants’ rhythms are truly circadian rhythms if they lack a temperature-compensated free-running period. Could they be ‘half-way houses’ in the evolutionary development of a fully formed circadian clock? This has been suggested for the circadian rhythm of change between resting and actively moving phases in the marine plasmodial rhizopod *Thalassomyxa australis*, which shows a pronounced temperature dependence (Q_{10} of 2.7 between 12 and 24 °C) (Silyn-Roberts et al 1986). On the other hand, some ultradian rhythms have been reported to have temperature-compensated periods (see Lloyd & Rossi 1992, p 1–117). One’s hypothesis might be, then, that there has been a gradual evolution of temperature-compensated and entrainable circadian rhythms from temperature-compensated ultradian periodicities (see Edmunds 1988).

Waterhouse: Dr Dunlap, you suggested that in your *Neurospora* mutants there is a correlation between the loss of rhythmicity and the loss of temperature compensation. How strong is this correlation? Does one part of the genome have multiple functions, or can they be split?

Dunlap: Out of the series of seven single gene mutations affecting the *Neurospora* clock, well over half result in alterations in both period length and temperature compensation of the oscillator. There are no cases in which temperature compensation alone is affected, although there are cases where period length has been altered without an effect on temperature compensation.

Waterhouse: Can you say anything more quantitative about this, about the degree or percentage to which they have been changed?

Dunlap: The number of different alleles affecting both periodicity and compensation is too small. *frq*³ has a period length of 24 h, *frq* wild-type one of 21 h, and *frq*⁷ and *frq*⁸ 29 h. 24 divided by 21 is about 1.15, which is the change in Q_{10} in *frq*³. So, the data fit quite well with the prediction that period length and temperature compensations are altered to the same extent, but the sample size is so small that this calculation is almost meaningless.

Turek: You say that no one has found a *Neurospora* mutant with an unchanged period that does not show temperature compensation. Has there been intensive screening for mutants with altered temperature compensation as there has been for period mutants?

Dunlap: No.

Turek: So the fact that you haven't found any may not be significant.

Dunlap: That's correct. Absence of evidence is not evidence of absence.

Kronauer: If the period is shortened, does the Q_{10} become less than one?

Dunlap: No. In fact, long period length is not required for a change in Q_{10} , and there can be a long period length without a change in Q_{10} . One of the striking things about the mutant strains with short periods is that they do not have altered Q_{10} values.

Menaker: Could one look at this question more directly by asking whether *frq* or FRQ are involved in the oscillations in other fungi that do not show temperature compensation? In an old paper, Jerebzooff (1965) describes a whole series of uncompensated oscillations in fungi. Why not investigate whether *frq* is involved in any of these?

Dunlap: That is certainly possible and would be interesting.

Resning: You raised the question of whether temperature compensation is a built-in component of the loop or is appended to the loop. What do you consider would demonstrate that a component was built in, for example, that the rates of synthesis and degradation of the mRNA are not dependent on temperature? Could you measure the amount of *frq* mRNA and determine the half-life at different temperatures?

Dunlap: We could do that. It's too soon, at least in this system and probably also in *Drosophila*, to try to produce a quantitative model. We know what some of the components are and we are beginning to describe how they act on one another, but we can't do that quantitatively yet. All I can say at the moment is that there's a lot more mRNA for *frq* in a mutant stain with a long period than there is in one with a short period. We looked for four and a half years for an oscillation of *frq* transcript under starvation conditions, and couldn't find one because there was very little mRNA for *frq* under those growth conditions. We then found that if you add glucose you suddenly see a large amount of *frq* mRNA, yet the clock runs along perfectly normally in either high or low glucose, with lots of *frq* mRNA or with very little. It stunned me that you can add glucose to a starving culture and not reset the clock although in the presence of glucose there's a lot more *frq* mRNA. That says that the level of *frq* transcript itself contains no information about timing. It is apparently the level of *frq* mRNA as compared with something else that's important for keeping time.

Hastings: Can I ask a question about the phase response curves and the nature of the altered periods? Is it possible to talk about the short period as being the result of deletion of part of the temporal programme and the long period the

result of an insertion, or do you view the short period as an equal acceleration across all phases and the long period as an equal slowing down of the oscillator across all phases? Does information from phase response curves allow you to differentiate between those two possibilities?

Dunlap: We don't have any good data speaking to that one way or the other. Studies in *Drosophila* (Konopka 1979) suggest that part of the cycle, the dead zone, is shortened in *per* mutants with shortened periods and lengthened in those with lengthened periods. Also, Nakashima (1985) has data in *Neurospora* that he has interpreted similarly to say that there is a zone of the *Neurospora* clock that is specifically lengthened or shortened. The data are good in *Drosophila*, but the error bars in the *Neurospora* phase response curves are larger and the distinction between parametric lengthening and specific lengthening of a particular zone is difficult to make.

Ralph: You said that there are no identified mutations in the promoter regions. The feedback control of the *frq* transcript implicates the promoter or regulatory regions of the long transcript as a logical place to look for both new mutations and entrainment pathways into the underlying biochemical loop generating the rhythm. You could go so far as to say that the promoter region is also a possible site of connection with the temperature compensation mechanism. What is known about this promoter region?

Dunlap: Almost nothing is known about it, and all your points are well taken. It's interesting that none of the mutations has turned up there.

Takahashi: Is it fair to say that the short transcript covers the promoter of the long open reading frame and that your deletion of the short transcript region is a promoter deletion?

Dunlap: That's how we are looking at it now, but to say that is tantamount to saying that the short transcript, which contains an intron and is polyadenylated, is superfluous to clock functioning. To establish this as the most likely alternative took a great deal of work, but I think we have done this now. Our interpretation is that FRQ is encoded by the long open reading frame within the large transcript and that the necessary *frq* regulatory regions extend across the region encoding the small transcript.

References

- Bünning E 1973 The physiological clock, 3rd edn. Springer-Verlag, New York
 Edmunds LN Jr (1988) Cellular and molecular bases of biological clocks. Springer-Verlag, New York
 Edery I, Rutila JE, Rosbash M 1994 Phase shifting of the circadian clock by induction of the *Drosophila period* protein. *Science* 263:237–240
 Ewer J, Rosbash M, Hall J 1988 An inducible promoter fused to the *period* gene of *Drosophila* conditionally rescues adult *per*-mutant rhythmicity. *Nature* 333:82–84
 Feldman J 1982 Genetic approaches to circadian clocks. *Annu Rev Plant Physiol* 33:563–608

- Hardin P, Hall J, Rosbash M 1990 Feedback of the *Drosophila period* gene on cycling of its mRNA. *Nature* 343:536–540
- Jerebzoﬀ S 1965 Manipulation of some oscillating systems in fungi by chemicals. In: Aschoﬀ J (ed) *Circadian clocks*. North-Holland, Amsterdam (Proc Feldaﬀing Summer Sch) p 183–194
- Klevecz RR 1976 Quantized generation time in mammalian cells as an expression of the cellular clock. *Proc Natl Acad Sci USA* 73:4012–4016
- Kondo T, Strayer CA, Kulkarni RD et al 1993 Circadian rhythms in prokaryotes: luciferase as a reporter of circadian gene expression in cyanobacteria. *Proc Natl Acad Sci USA* 90:5672–5676
- Konopka R 1979 Genetic dissection of the *Drosophila* circadian system. *Fed Proc* 38:2602–2605
- Lloyd D, Ross EL 1992 Ultradian rhythms in life processes. Springer-Verlag, London
- Nakoshima H 1985 Biochemical and genetic aspects of the conidiation rhythm in *Neurospora crassa*: phase shifting by metabolic inhibitors. In: Hiroshige T, Honma K (eds) *Circadian clocks and zeitgebers*. Hokkaido University Press, Sapporo, p 35–44
- Sweeney BM, Borgese MB 1989 A circadian rhythm in cell division in a prokaryote, the cyanobacterium *Synechococcus* WH7803. *J Phycol* 25:183–186
- Silyn-Roberts H, Engelmann W, Grell KG 1986 *Thalassomyxa australis* rhythmicity. I. Temperature dependence. *J Interdiscip Cycle Res* 17:181–187