Ciba Foundation Symposium 183

CIRCADIAN CLOCKS AND THEIR ADJUSTMENT

1995

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Introduction

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The amount of research into circadian rhythms has increased markedly in recent years. Important developments in our understanding of the mechanisms involved have taken place through the use of many different animal models and technological advances. As examples, consider the developments in the genetics of *Drosophila* and *Acetabularia*, the neurophysiology of the eye of *Bulla*, the neurophysiology, function and physiology of the rodent suprachiasmatic nuclei, and the adjustment of body clocks in humans by light and melatonin. We have learned about the pathways by which environmental stimuli can adjust the clock and have increasing knowledge of what such zeitgebers are. The ontogeny of the clock and the changing nature of the zeitgebers affecting it have become better defined. There has been a parallel increase in clinical and applied interests, as illustrated by work on shift-work, blindness and jet lag.

Such advances have been covered in scientific meetings and in the literature, of course, but in spite of an ever-increasing number of meetings dealing with chronobiological topics, two limitations remain to the process of integrating recent findings into the general body of knowledge. First, there is rarely enough time devoted to the discussion of new work. Second, the specialist nature of many meetings prevents the cross-fertilization of ideas and methodologies between the many disciplines with an interest in the subject. It is rare to have a meeting in which there is the necessary combination of authority and extended constructive and organized debate.

This symposium gives us an opportunity to discuss recent advances in the field unhampered by these limitations. I hope that the ensuing discussions will be lively and constructive, with continual interaction between experts from many different disciplines, so as to suggest possible approaches to current problems. We have chosen to start at one end of the scale, at the molecular level, and work up to the clinical end, but this does not mean that the fungal geneticists should be involved only at the beginning and the human chronobiologists only at the end; I hope all of you will ask questions over and over again. Even if we are working at markedly different levels of complexity, we are nevertheless dealing ultimately with the same thing, working towards some presumably common mechanisms. I also hope that some of the intellectual stimulation that

I know will be evident will be readily inferred by readers of the papers and recorded discussions.

Finally, I would like to pay tribute to my colleague Dr Peter Redfern, who played an integral part in making this symposium the success I am sure it will be, for his continual support and wisdom.

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

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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*.

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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**

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

[a]Period 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

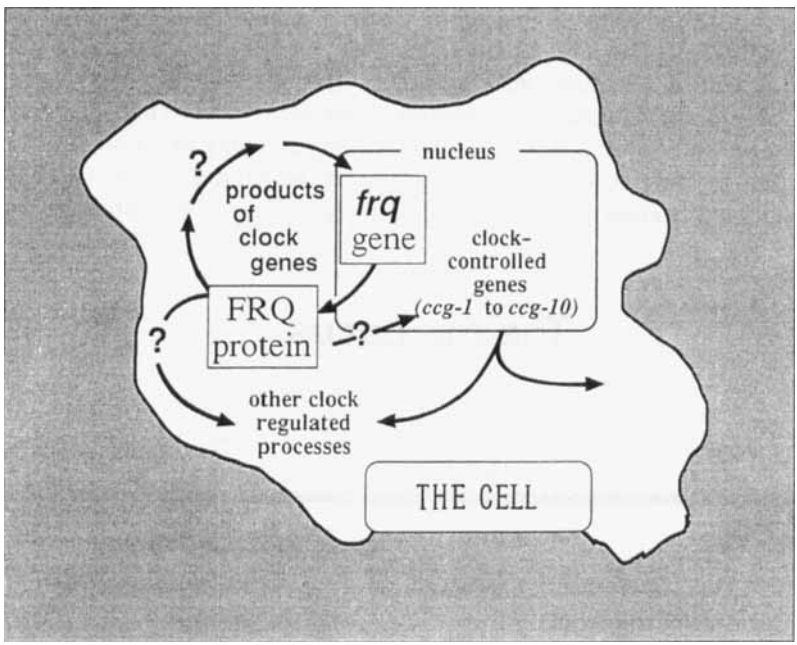

FIG. 1. The components of the circadian system in a cell. This figure implies more than we really know (for example, the location of the nuclear boundary), while leaving out some recognized effects such as the effects of light on the clock and on *frq* and the independent effects of light on the morning-specific clock-controlled genes, *ccg*s (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 *ccg*s) 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 *c*lock-*c*ontrolled *g*enes (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*,

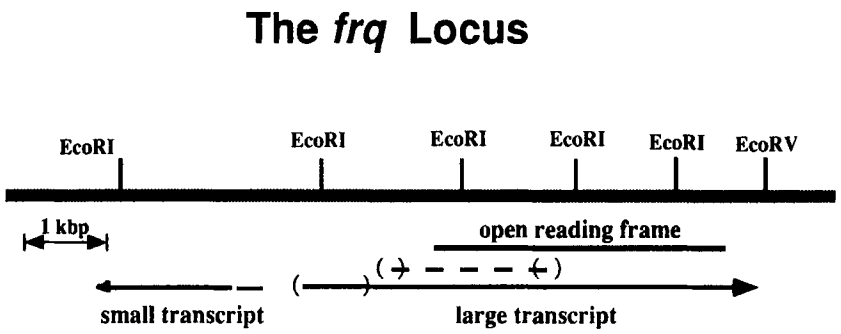

FIG. 2. A physical map of the region of DNA required for the rescue of recessive loss-of-function mutations in the *frq* gene; marks above the line represent restriction sites (a sizing bar gives an estimate of distance in kbp) (adapted from McClung et al 1989). Two *frq* transcripts (arrows) are drawn 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 (Merrow & Dunlap 1994) have now cloned and sequenced all or part of *frq* from five different

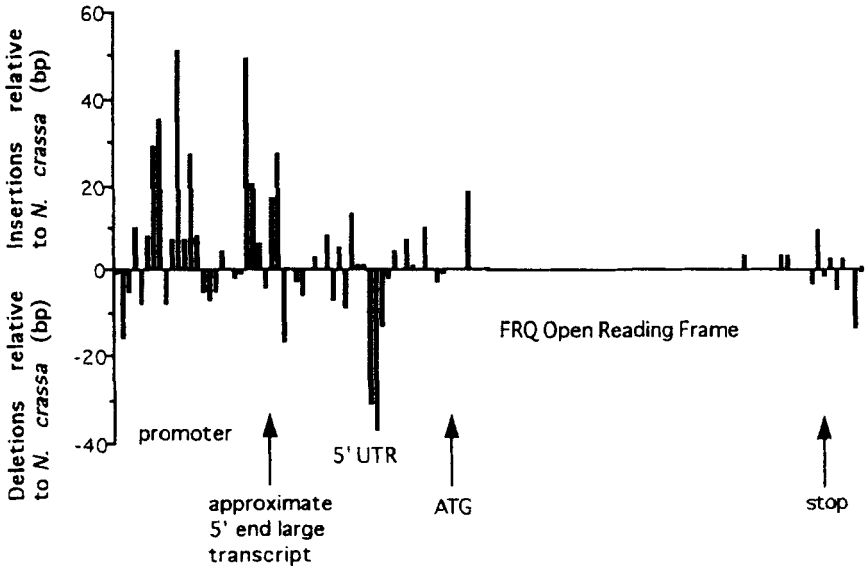

FIG. 3. Comparison of DNA sequences corresponding to the *frq* region from two different fungi, *Neurospora crassa* and *Sordaria fimicola*, reveals that the protein-coding region of *frq* is highly conserved. The *frq* gene, as defined by the region of DNA required to complement recessive loss-of-function 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 (Merrow & 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 fond 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 *ccg*. 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 *ccg*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 *ccg* 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 *ccg*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

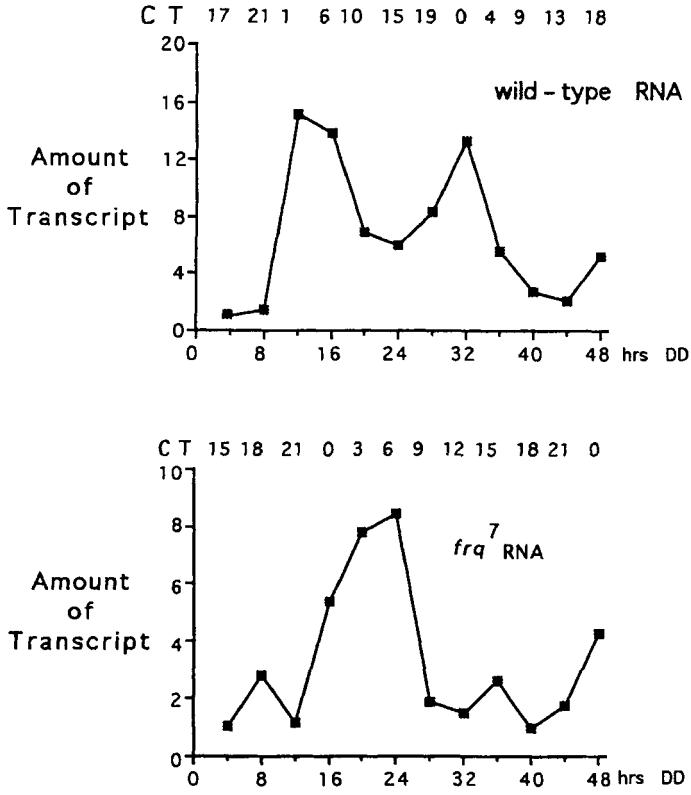

FIG. 4. *frq* is a morning-specific gene which regulates the timing of its own synthesis. Relative amounts of the large (approximately 4500 nucleotide) *frq* transcript found in *N. crassa* mycelia isolated at different times are plotted. Data were collected in two genetic backgrounds, frq^{+} (*top*) 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 $qa\text{-}1^{F}$ gene, whose activation is normally blocked by complexation with the product of the $qa\text{-}1^{S}$ 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-2*FRQ 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 CT 9–11) following the release from quinic acid induction. This predictability of phase thus deserves further discussion.

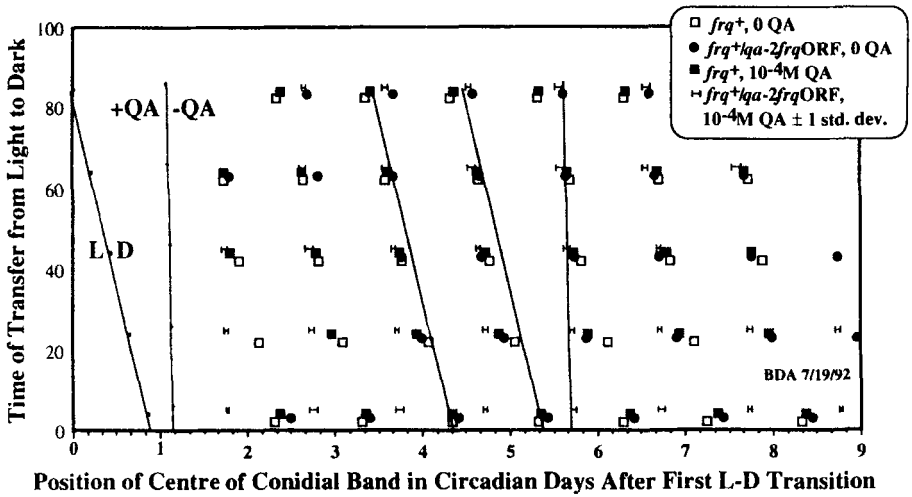

FIG. 5. Steps from high to low expression levels of an FRQ-encoding transcript set the clock to a unique phase. Cultures (*bd; frq*$^+$ and *bd; frq*$^+$ [pBA40 transformant]) were grown in liquid medium in constant light in the presence or absence of 10^{-4} M inducer (quinic acid, QA). Starting at 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-2frq*ORF 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-2frq*ORF 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

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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 connnecting 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.

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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.