Genetic interactions between clock mutations in *Neurospora crassa*: can they help us to understand complexity?

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Recent work on circadian clocks in *Neurospora* has primarily focused on the *frequency* (*frq*) and *white-collar* (*wc*) loci. However, a number of other genes are known that affect either the period or temperature compensation of the rhythm. These include the *period* (no relationship to the *period* gene of *Drosophila*) genes and a number of genes that affect cellular metabolism. How these other loci fit into the circadian system is not known, and metabolic effects on the clock are typically not considered in single-oscillator models. Recent evidence has pointed to multiple oscillators in *Neurospora*, at least one of which is predicted to incorporate metabolic processes. Here, the *Neurospora* clock-affecting mutations will be reviewed and their genetic interactions discussed in the context of a more complex clock model involving two coupled oscillators: a FRQ/WC-based oscillator and a 'frq-less' oscillator that may involve metabolic components.

Keywords: *Neurospora*; circadian; oscillator; temperature compensation; *period*; *frequency*

1. INTRODUCTION

Genetic analyses of mutants have played major roles in dissecting complex biological processes, including biochemical pathways, the cell cycle and behavioural responses. Genetic approaches have also provided the foundation for our current understanding of the molecular aspects of the circadian clock system by identifying genes required for rhythmicity. Classical genetic analysis of the *Neurospora* clock identified a number of mutant strains defective in the circadian rhythm of asexual spore production (conidiation) (Feldman 1982, 1983). Currently, more than 20 loci are known that affect the 21.5 h period of the conidiation rhythm when mutated, ranging from slight effects on period to complete arrhythmicity. Some of these genes encode products of known biochemical functions, whereas others encode products whose functions are unknown (table 1). Of these genes, most studies have concentrated on the *frq* gene (reviewed in Dunlap 1999), for which multiple alleles have been isolated, and more recently on the *wc-1* and *wc-2* genes, which are required for both rhythmicity and normal light responses (Harding & Shropshire 1980; Crosthwaite *et al.* 1997). (For gene and abbreviated allele names see table 1.)

In this review, and consistent with Bell-Pedersen *et al.* (2001), we define a circadian oscillator as a self-sustained rhythm generator that produces rhythms with all of the properties of a circadian rhythm, and the clock as the entire timekeeping mechanism which includes one or more circadian oscillators and possibly non-circadian oscillators, input to the oscillators and rhythmic output

from the oscillators. A rhythm is defined as a regular oscillation in any observable variable.

There has been suspicion for years that the circadian clock is constituted through the coupling of more than one oscillator (e.g. Dunlap 1991, 1998; Mellow *et al.* 1999), but the molecular analysis of the existing mutants has provided insights into only one feedback loop. Molecular models of one of the circadian oscillators contributing to the clock in *Neurospora* have been proposed involving negative feedback (Dunlap 1999; Lee *et al.* 2000). In brief, it is proposed that WC-1 and WC-2 are transcription factors that activate *frq* expression since the levels of *frq* transcripts in strains with mutations in *wc-1* or *wc-2* are very low (Crosthwaite *et al.* 1997). FRQ protein, produced maximally at specific times of day from the accumulating *frq* transcript (Aronson *et al.* 1994b; Garceau *et al.* 1997), enters the nucleus (Luo *et al.* 1998) and is suggested to inhibit a complex of WC-1 and WC-2 (Lee *et al.* 2000); however, a direct inhibition has not been formally demonstrated. Turnover of FRQ, involving progressive phosphorylation (Liu *et al.* 2000), then according to the model would result in activation of *frq* transcription by WC-1 and WC-2. This simple feedback model has now been extended to include post-transcriptional regulation of WC-1 by FRQ because rhythmic accumulation of WC-1 protein, but not mRNA (which appears to be constitutively expressed), requires functional FRQ (Lee *et al.* 2000). Thus, while the actual biochemical function of FRQ is unknown, FRQ appears to have at least two roles in the clock system, one to indirectly repress its own synthesis through WC-1 and WC-2, and the other to positively affect WC-1 synthesis. One or more of these cycling proteins is predicted to directly regulate the output pathways from the oscillator(s) to

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Table 1. Rhythm mutants in *Neurospora crassa*.

(This table is adapted from Bell-Pedersen (2000).)

gene	allele name	period (h) at 25 °C	temperature compensation (+ or -)	dominance	growth rate (GR) (mm day ⁻¹) ^c and comments	references
wild-type	NA ^a	21.5	+	NA	GR 39.2	
<i>frequency</i>	<i>frq¹</i>	16.5	+	semi-dominant	GR 36.1	Feldman & Hoyle (1973)
	<i>frq²</i>	19.3	+	semi-dominant	GR 37.2	Feldman & Hoyle (1973), Gardner & Feldman (1980)
	<i>frq³</i>	24.0	—	semi-dominant	GR 36.7	Feldman & Hoyle (1973), Gardner & Feldman (1980)
	<i>frq⁷</i>	29.0	—	semi-dominant	GR 36.9	Feldman & Hoyle (1973), Gardner & Feldman (1980, 1981)
	<i>frq⁹</i>	variable	—	recessive	ND ^b	Loros & Feldman (1986), Loros <i>et al.</i> (1986)
	<i>frq¹⁰</i>	variable	—	recessive	gene disruption	Aronson <i>et al.</i> (1994a)
	<i>frq¹¹</i>	arrhythmic at 30 °C	—	ND	temperature sensitive > 30 °C	Nakashima & Onai (1996)
<i>period-1</i>	<i>prd-1</i>	25.8	—	recessive	GR 24.7	Feldman & Atkinson (1978), Feldman <i>et al.</i> (1979)
<i>period-2</i>	<i>prd-2</i>	25.5	+	recessive	GR 33.3	Feldman <i>et al.</i> (1979), Gardner & Feldman (1981)
<i>period-3</i>	<i>prd-3</i>	25.1	—	recessive	GR 31.2	Feldman <i>et al.</i> (1979), Gardner & Feldman (1981)
<i>period-4</i>	<i>prd-4</i>	18.0	—	semi-dominant	GR 38.3	Feldman <i>et al.</i> (1979), Gardner & Feldman (1981)
<i>period-6</i>	<i>prd-6</i>	18.0 at 22 °C	+	recessive	GR 27.7 temperature sensitive > 21 °C	Morgan & Feldman (1997)
<i>chrono</i>	<i>chr</i>	23.5	+	semi-dominant	GR 37.7	Feldman <i>et al.</i> (1979), Gardner & Feldman (1981)
<i>white collar-1</i>	<i>wc-1 ER53</i>	arrhythmic	ND	recessive	light insensitive	Harding & Shropshire (1980), Crosthwaite <i>et al.</i> (1997)
<i>white collar-2</i>	<i>wc-2 ER33</i>	arrhythmic	ND	recessive	light insensitive	Harding & Shropshire (1980), Crosthwaite <i>et al.</i> (1997)
	<i>wc-2 ER24</i>	29.7 at 25 °C	—	ND	temperature sensitive	Degli-Innocenti & Russo (1984), M. A. Collett and J. C. Dunlap (unpublished data)
<i>arginine-13 chain elongation</i>	<i>arg-13</i>	19 ^c	+	recessive	amino acid requirer	Lakin-Thomas <i>et al.</i> (1990)
	<i>cel</i>	variable ^d	—	recessive	fatty acid synthase deficient	Mattern & Brody (1979)
<i>choline-1</i>	<i>chol-1</i>	variable ^c	—	ND	phosphatidylcholine deficient	Lakin-Thomas (1998)
<i>cytochrome a-5</i>	<i>cya-5</i>	19	+	ND	cytochrome <i>aa</i> ₃ deficient	Brody <i>et al.</i> (1987)
<i>cytochrome b-2</i>	<i>cyb-2</i>	18	+	ND	cytochrome <i>b</i> deficient	Brody <i>et al.</i> (1987)
<i>cytochrome b-3</i>	<i>cyb-3</i>	20	+	ND	cytochrome <i>b</i> deficient	Lakin-Thomas <i>et al.</i> (1990)
<i>cytochrome-4</i>	<i>cyt-4</i>	20	+	ND	cytochrome <i>aa</i> ₃ and <i>b</i> deficient	Lakin-Thomas <i>et al.</i> (1990)
<i>cysteine-4</i>	<i>cys-4</i>	19 ^c	+	recessive	amino acid requirement	Feldman <i>et al.</i> (1979), Feldman & Widelitz (1977)
<i>cysteine-9</i>	<i>cys-9</i>	variable ^c	—	ND	thioredoxin reductase	Onai & Nakashima (1997)
<i>cysteine-12</i>	<i>cys-12</i>	19 ^c	+	recessive	amino acid requirement	Feldman <i>et al.</i> (1979), Feldman & Widelitz (1977)
<i>female fertility-1 ff-1 (glp-3)</i>		19	ND	ND	ND	Brody <i>et al.</i> (1987)

continued

Table 1. (Continued.)

maternally inherited	<i>mi-2, mi-3, mi-5</i>	18–19	+	ND	cytochrome oxidase subunit 1 deficient	Brody <i>et al.</i> (1987)
oligomycin resistant	<i>oli^R</i>	18–19	+	semi-dominant	mitochondrial ATPase subunit 9	Dieckmann & Brody (1980)
<i>phenyl-alanine-1</i>	<i>phe-1</i>	19 ^c	+	ND	ergosterol synthesis deficient	Lakin-Thomas <i>et al.</i> (1990)
<i>rhythm-1</i>	<i>rhy-1</i>	arrhythmic at 30 °C	+	ND	temperature sensitive > 30 °C	Chang & Nakashima (1998)
<i>unknown-18</i>	<i>un-18</i>	24.5 at 22 °C	ND	ND	temperature sensitive above 22 °C, RNA polymerase subunit	Onai <i>et al.</i> (1998)

^a NA indicates not applicable.

^b ND indicates no data.

^c Period length is reduced by increasing starvation for the required supplement.

^d The period length of these strains can be altered by changing the supplementation of the medium.

^e The growth rate was measured at 25 °C on standard race tube media containing 1 × Vogel's salts, 0.3% glucose, 0.5% arginine.

control the rhythmic processes of the cell, including the developmental rhythm. For a more detailed account of this proposed feedback model see Lee *et al.* (2000) and Bell-Pedersen *et al.* (2001).

A model for the clock as a single oscillator is inherently pleasing since it could theoretically account for the mechanism by which an oscillator that is linked to the environment and downstream rhythmic processes functions to generate self-sustained and metabolically compensated rhythms (Goldbeter 1996; Gonze *et al.* 2000). Such single oscillator mathematical models have historically played an important role in the development of the field; however, a simple model cannot explain all the available data in *Neurospora* regarding the circadian clock, including the well-documented rhythms that remain in *frq*-null strains (Loros & Feldman 1986; Aronson *et al.* 1994b; Merrow *et al.* 1999; Bell-Pedersen *et al.* 2001). To explain this residual rhythmicity, the presence of additional oscillators in the *Neurospora* cell seems likely; however, to date, molecular analyses of the existing mutations have contributed only to an understanding of the FRQ–WC loop, and therefore the nature of the putative additional oscillator(s) has not been established.

Because null mutants of *frq* can be entrained by temperature cycles, it has been suggested that the entrainment has allowed a cryptic, temperature-entrainable oscillator to be uncovered in the absence of the *frq*-based feedback loop (Merrow *et al.* 1999; Iwasaki & Dunlap 2000). Because *frq*-null strains have lost temperature compensation (Loros & Feldman 1986; Aronson *et al.* 1994a), but may still be entrained by temperature cycles, we shall treat these two temperature effects as separate mechanisms. Attempts to entrain the *frq⁰* strain by various light–dark cycles were unsuccessful (Merrow *et al.* 1999; Lakin-Thomas & Brody 2000; Chang & Nakashima 1997). These negative results provided the basis for a model in which FRQ is part of a light-input pathway to a temperature-entrainable oscillator (Merrow *et al.* 1999). However, it should be noted that previous experiments demonstrated that the *frq⁰* strain could be entrained by 5 min light pulses given once every 24 h (Loros & Feldman 1986). This discrepancy has not yet been

resolved. In any case, further suggestion of the participation of FRQ in a light-input pathway comes from studies of double-mutant strains carrying either of the lipid-deficient, rhythm-affecting, *cel* or *chol-1* mutations plus a null allele at the *frq* or *wc* locus. The double-mutant strains are rhythmic with periods similar to the *cel* or *chol-1* single-mutant strains (Lakin-Thomas & Brody 2000). These strains also are less sensitive to light and cannot be entrained to light–dark cycles.

Together, these data provide support for the 'zeitnehmer' (meaning time-taker) model of Roenneberg & Merrow (1998) in which the FRQ–WC-based feedback loop is proposed to be part of a light-input pathway to a separate oscillator (see also Roenneberg *et al.* 1998). This oscillator, in turn, imparts rhythmicity on the FRQ–WC input loop (Roenneberg & Merrow 1998; Merrow *et al.* 1999). The input zeitnehmer loop, involving FRQ and WC proteins, was not considered to be part of a central master circadian oscillator. In this model, the central oscillator is a metabolic feedback loop, similar to that observed in the unicellular dinoflagellate *Gonyaulax* (Roenneberg & Merrow 1999). In *Gonyaulax*, metabolism is not only an output of the clock, but also contributes information towards the generation of circadian rhythms (Johnson & Hastings 1989; Roenneberg 1994). Mathematical modelling of the zeitnehmer clock by Roenneberg & Merrow (1999) demonstrated that a rhythmic zeitnehmer input loop could allow a damped metabolic oscillator to become self-sustained and compensate for metabolic variations in the cell.

The compensation property, a hallmark of circadian rhythms, is based on the observation that the period of circadian rhythms is maintained when organisms are grown at different temperatures or on different growth media. It is predicted that at least some of the individual components of the circadian oscillators are temperature-dependent, but that these components interact in such a way that allows the final output to be temperature compensated. The zeitnehmer model posits that the temperature-compensation mechanism of the clock may be partly responsible for shielding the clock against metabolic variations since the main effect of temperature is to alter the rates of metabolic reactions (Roenneberg &

Merrow 1999). This is not a new hypothesis—the relationship between temperature compensation and metabolic compensation received considerable attention very early on in the field (for example see Feldman & Stevens 1973; Pittendrigh *et al.* 1973). One prediction of the *zeitnehmer* model is that some mutant strains defective in temperature compensation might also be defective in metabolic compensation. Indeed, several mutants that affect temperature compensation also affect metabolic functions (table 1). The phenotypes of the *frq⁹*, *cel* and *chol-1* mutant strains, which have lost both temperature compensation and nutritional compensation (Loros & Feldman 1986; Lakin-Thomas *et al.* 1997), support a connection between temperature and metabolic compensation. The *frq⁹* strain is sensitive to both the type and concentration of the carbon source in the growth medium. More recently it was shown that the *prd-6* mutation suppresses temperature-compensation defects (see § 3). In addition, *prd-6* mutant strains appear resistant to the slight period lengthening caused by addition of histidine to media or use of maltose or galactose as the carbon source (Sargent & Kaltenborn 1972; L. Morgan, unpublished data). These data support the *zeitnehmer* model prediction that a connection exists between compensation for temperature and metabolism.

A second model to account for the residual rhythmicity is reminiscent of a coupled two-oscillator system, first proposed in by Pittendrigh (1960) and used to explain the responses of pupal eclosion in *Drosophila* in response to light and temperature. In his model, a central A oscillator is light-entrainable and drives a temperature-entrainable B oscillator. The B oscillator is considered to be peripheral—it is coupled to, and phased by, the A oscillator. The temperature compensation is derived from information encoded by the A oscillator. In the case of *Neurospora*, the A oscillator might be predicted to be the light-responsive FRQ–WC-based oscillator and the B oscillator would represent a temperature-entrainable FRQ-less oscillator (FLO) (Iwasaki & Dunlap 2000). However, unlike Pittendrigh's original model in which circadian properties are derived from the A oscillator, there is no tendency now to place either the FLO or the FRQ–WC loops in a peripheral role; rather both would contribute to the clock. In essence, the *zeitnehmer* and the A&B oscillator models are not very different. By analogy, the FLO in the A&B oscillator system could represent the metabolic oscillator in the *zeitnehmer* model. In both models, the FLO derives its phase control from at least two sources; directly from temperature inputs, and indirectly from light inputs through linkage with the light-responsive FRQ–WC oscillator.

Neither model provides a biochemical explanation for the temperature-compensation mechanism of the clock. However, both models imply that this feature is embedded within the FRQ–WC feedback loop. Mathematical models have suggested that turnover of oscillator components has a critical role in the regulation of the oscillator's period (Ruoff *et al.* 1999) and this has been experimentally confirmed. In *Neurospora*, mutation of one of the FRQ phosphorylation sites (Ser513) results in a reduction in the rate of FRQ degradation and the mutant strain has a long period of about 30 h (Liu *et al.* 2000). In addition, *frq* mRNA levels in the long-period *frq⁷* mutant

(which also has a defective temperature-compensation mechanism), are significantly higher than in wild-type strains (Aronson *et al.* 1994b), suggesting less efficient turnover. These data may indicate that the long period in these mutants can be attributed to a slower degradation rate of a component of the feedback loop. By extension, in order for the period to remain compensated at varying temperatures, one might expect that the turnover rate of FRQ should also be compensated. Thus, mutants that affect temperature compensation might be expected to perturb the stability or activity of oscillator components.

With these ideas in mind, it is apparent that a full understanding of the *Neurospora* clock, along with the ability to distinguish between models that incorporate multiple oscillators, will be aided by the identification and characterization of additional clock components.

Most of the rhythm-affecting loci in *Neurospora* have secondary effects, including alterations in growth rate, or they have known biochemical defects (table 1). As a result, the genes specified by the mutations have not usually been considered to be major components of a central circadian oscillator required for a functional feedback loop (Lakin-Thomas 2000). Despite this, we need to bear in mind the possibility that the circadian clock mechanism might incorporate metabolic feedback loops, as well as housekeeping functions (Lakin-Thomas 2000; Roenneberg & Merrow 2001). Thus, the characterization of these additional clock-affecting loci will probably provide important information regarding the complexity of the *Neurospora* circadian system, and may provide a route to the components of the putative additional oscillators. In addition, because a number of the mutant alleles affect the ability of the clock to maintain a constant period at temperatures within the physiological range of *Neurospora* (between 18 °C and 30 °C) these mutant loci may provide clues to the mechanism behind the process of temperature compensation.

This review, written to commemorate David Saunders' retirement, focuses on genetic interactions that exist between mutations known to affect rhythmicity in *Neurospora* and discusses how the known genetic and physiological data may fit a two-oscillator model of the circadian clock.

2. GENETIC INTERACTIONS AMONG THE CLOCK-AFFECTING MUTANT LOCI

To determine if genetic interactions occur between loci that are involved in circadian timekeeping, a number of double-mutant strains were constructed and analysed. Because *Neurospora* is a haploid organism, the dominance relationship at each locus can be ignored, whereas the effects of possible interactions between loci that affect the same phenotype can be characterized. The possible classifications include the following two (Lakin-Thomas & Brody 1985).

First, the two loci act independently such that the effects are additive whereby each mutation alters the phenotype by a fixed amount, or the effects are multiplicative whereby the mutations alter the phenotype by a fixed percentage. For example, if one mutant allele lengthens a 21.5 h endogenous period by 2 h and the second allele increases the period by 3 h, the double-mutant strain

would have a period that is increased by 5 h if the effects are additive and would have a period of 26.8 h ($23.5/21.5 \times 24.5/21.5 \times 21.5$) if the effects are multiplicative.

Second, the two loci are dependent on each other. Here, the result could be either (i) epistasis, whereby one mutation masks the effects of mutation at the second locus, (ii) intergenic suppression, whereby a mutation in one gene seemingly reverses the action of a mutation in a second gene such that a wild-type phenotype is observed, or (iii) synergy, whereby the two loci affect the phenotype in an interactive manner that is not predicted by any of the other classes.

With a few exceptions (table 2), in strains carrying two clock mutations the effects of the mutations are cumulative, being represented either by additive or multiplicative effects (Feldman *et al.* 1979; Lakin-Thomas & Brody 1985). In these cases, two long-period mutants produced a strain with a period that was longer than either of the single-mutant strains; two short-period mutants produced a strain that had a period that was shorter than either single-mutant strain; and strains with both a long- and short-period allele yield a strain with an intermediate period. These data suggest that the loci have independent effects on the period of the conidiation rhythm.

In contrast, both *prd-2 prd-6* and *prd-3 prd-6* double-mutant strains have a period of 18 h at 25 °C, identical to the period of the *prd-6* mutant strain (table 2) (Morgan & Feldman 1997, 2001). These data demonstrate that the short-period *prd-6* mutation is epistatic to both the *prd-2* and *prd-3* long-period mutations; the nature of the epistasis suggests possible physical interactions between their encoded proteins (Morgan & Feldman 1997). The period of a *prd-2 prd-3* strain is longer than values predicted by mutations acting independently, suggesting that they affect the same process (Feldman & Atkinson 1978; Lakin-Thomas & Brody 1985). Mutant *prd-1 prd-2* strains have a period that is close to each of the single mutants, suggesting that their products act within the same pathway. However, the *prd-1* mutation shows an additive effect on period in combination with the *prd-3* or *prd-6* mutations, indicating that *prd-1* is not in the same epistasis group as *prd-3* and *prd-6* (Morgan & Feldman 2001).

Each of the *prd-2*, *prd-3* and *prd-6* mutations has an allele-specific interaction with the long-period *frq⁷* mutation. The *prd-2 frq⁷* and *prd-3 frq⁷* strains have periods at 25 °C that are significantly longer than would be predicted by additive or multiplicative calculations (table 2). Mutant *prd-6 frq⁷* strains have a period of 20.5 h, significantly shorter than predicted. Together, the genetic interactions with *frq⁷* implicate the protein products of these loci having either a direct or indirect role in the FRQ–WC feedback loop.

The particular *frq* alleles show different behaviour in combination with the *prd* alleles. Only the long-period *frq⁷* and *frq³* alleles have been found to genetically interact with the *prd* loci (table 2). These data suggest that the mutations of *frq* occur within distinct functional domains of the protein, and that these can affect the clock mechanism in distinct ways. Both the *frq⁷* and *frq³* mutations border a conserved glycine-rich region of the protein (Aronson *et al.* 1994a) believed to have a role in protein–protein interactions (Lewis & Feldman 1995; Lewis *et al.* 1997). Thus, it is possible that these mutations

Table 2. Circadian period of double-mutant strains.

(These data are adapted from Morgan & Feldman (2001). Periods were measured at 25 °C. The predicted periods were calculated based on the periods of the single-mutant strains in table 1. Only those double mutants that show a measured period value that differs by more than 2 h from the predicted values are shown. For a complete summary of all combinations of mutants, see Morgan & Feldman (2001), Lakin-Thomas & Brody (1985, 2000) and Lakin-Thomas *et al.* (1990).)

genotype	period (h)	predicted periods (h)	
		additive	multiplicative
<i>prd-1 prd-2</i>	25.6 ± 1.4	29.8	30.6
<i>prd-2 prd-3</i>	32.7 ± 0.9	29.1	29.8
<i>prd-2 prd-6</i>	18.1 ± 0.4	22.0	21.3
<i>prd-2 frq⁷</i>	37.7 ± 0.8	33.0	34.4
<i>prd-3 frq⁷</i>	41.2 ± 1.5	32.6	33.9
<i>prd-3 prd-6</i>	18.0 ± 0.6	21.6	21.0
<i>prd-3 frq³</i>	30.4 ± 0.8	27.6	28.0
<i>prd-6 frq⁷</i>	20.7 ± 0.5	25.5	24.3

affect the ability of FRQ to interact in a complex with the products of at least one or more of the *prd-2*, *prd-3* or *prd-6* gene products and that alteration of this interaction results in a longer period. It is interesting that only the long-period *frq⁷* and *frq³* alleles, which are the only alleles to show a genetic interaction with the *prd* genes, affect temperature compensation, whereas the short period alleles do not. This suggests that when the rate of the clock is slowed by an alteration of the interaction between FRQ and the PRD proteins, temperature compensation is also affected.

Other genetic interactions are known. Both the *prd-1* and *oli^R* mutations show epistasis to the *cel* mutation on media supplemented with linoleic acid. Double-mutant strains containing either *cel* or *chol-1* and various *frq* alleles demonstrated that the long periods of *cel* and *chol-1* were shortened in strains containing short-period *frq* alleles. Double mutants of *chol-1* or *cel* and *frq* or *wc* nulls are arrhythmic with full supplementation, but with a long-period rhythm on media where the period lengthening effects of the *cel* or *chol-1* mutation are observed (Lakin-Thomas & Brody 1985, 2000; Lakin-Thomas 1998). Some of these interactions will be discussed in more detail below (§ 4).

Most of the *prd* mutations are recessive, implying loss of function, yet they still maintain circadian rhythms albeit with altered period. Furthermore, many of the *prd* mutations have slow growth and some exhibit reduced conidiation (table 1). Whether these pleiotropic effects are a consequence of altered output or a more direct effect of the mutation on growth and development is not known. In support of a more direct role for these genes in oscillator function, the epistasis between period defects caused by the *prd* mutations does not extend to the pleiotropic effects of the mutations. For example, in a *prd-3 prd-6* strain, the growth rate is slower than each of the parental strains, despite the epistasis in their clock phenotypes. These data suggest that the clock defects in the mutant *prd* alleles are separate from the growth rate or conidiation defects (Morgan & Feldman 2001). Genetic interactions between the *prd* mutations and *frq* alleles also

suggest that the *prd* mutations have a more direct effect on the clock.

3. EFFECTS OF THE *prd* ALLELES ON TEMPERATURE COMPENSATION

Some of the *prd* mutations cause alterations in the temperature-compensation mechanism of the circadian rhythm (Gardner & Feldman 1981) (table 1). For example, *prd-3* strains show overcompensation at temperatures above 25 °C. This effect is significantly amplified in the presence of the *prd-2* mutation, which alone has a normal temperature-compensation mechanism. The period of *prd-2 prd-3* strains gets longer with increasing temperature (the opposite of temperature-dependent reactions) suggesting that a temperature-dependent component has lost activity and that the normal activity of the compensatory mechanism of the clock results in overcompensation (Morgan & Feldman 2001).

Temperature compensation may result from interaction between the two oscillators such that a defect in one of the oscillators results in over- or undercompensation (Hastings & Sweeney 1957). The *prd-6* mutation is epistatic to the overcompensation phenotype caused by the combination of the *prd-2* and *prd-3* mutations, and suppresses the loss of temperature compensation caused by the *frq⁷* mutation (Morgan & Feldman 2001). This suggests that the interaction of the two oscillators may require a wild-type *prd-6* allele and that PRD-6 may provide a link between the proposed temperature-dependent oscillator and the FRQ-WC-based oscillator.

4. THE EFFECTS OF METABOLIC MUTATIONS ON THE CLOCK

While the majority of *Neurospora* mutations tested to date show no effect on the circadian rhythm (Lakin-Thomas *et al.* 1990), some mutations which disrupt normal metabolism can alter the period (and, in some cases, temperature compensation) of the rhythm (table 1). These mutations include the *cel* and *chol-1* mutations that are deficient in lipid synthesis, the *cys-9*, *arg-13* and *phe-1* mutations that have altered amino acid metabolism, and a number of mutations that affect mitochondrial energy metabolism (reviewed in Lakin-Thomas *et al.* 1990; Dunlap 1993). Historically, these mutations have not been considered to be central components of a circadian oscillator; however, the phenotypes of the mutations clearly demonstrate that metabolic defects can alter the properties of the overt circadian rhythm. With appropriate supplementation, the *cel* and *chol-1* mutations can cause a robust long-period conidiation rhythm (albeit outside the circadian range) in *frq*-null (or *wc*-null) strains with the same period as the *cel* and *chol-1* single mutants. These data might suggest the existence of a second oscillator being linked to cellular metabolism (Lakin-Thomas & Brody 2000). The two oscillators are likely to be coupled since the period of the system is affected by the *frq* allele. For example, the short period *frq¹* allele shortens the long period observed in the *chol-1* or *cel* backgrounds (Lakin-Thomas 1998). However, when FRQ is absent, the rhythms lose some circadian characteristics, including light entrainment and compensation for changes in

temperature and metabolic state. One caveat that we need to keep in mind is that the *cel* and *chol-1* mutations alter membrane composition, which may influence a number of signalling pathways; thus, the indirect versus direct effects of these mutations on the clock are still debatable.

Mutations that alter mitochondrial energy metabolism (e.g. *oli^R*, *mi-2*), and the *prd-1* mutation, show epistasis to the *cel* mutation (Lakin-Thomas & Brody 1985). These data imply that a genetic link exists between the two oscillators: the *prd-1* mutation is epistatic to the *cel* mutation, the *prd-1* and *prd-2* mutations exhibit epistasis, the *prd-6* mutation is epistatic to the *prd-2* mutation, and the *prd-2* and *prd-6* mutations show an interaction with the *frq⁷* mutation. Together these data support the existence of a genetic pathway between metabolism and *frq* (figure 1).

5. OH GOOD, ANOTHER CLOCK MODEL!

Because epistasis can occur either upstream or downstream, accurate ordering of the *prd-1*, *prd-2*, *prd-3*, *prd-6* and *frq* genes in a pathway is ambiguous. Despite this complication, a model based on the known genetic interactions is presented in figure 1. The epistatic relationship between *prd-1* and *prd-2*, along with their similar phenotype, suggests that they act within the same pathway. Because *prd-1* shows additive behaviour with *prd-3*, *prd-6* and *frq⁷*, it probably resides in a different pathway than that of the *prd-6* epistasis group. The genetic interaction data also suggest that *prd-2*, *prd-3* and *prd-6* act in a pathway that regulates FRQ activity to affect period and temperature compensation. Strains with the *prd-2* or *prd-3* mutation have a long period, and this may reflect reduced turnover of FRQ in the mutants. Furthermore, while each of the *prd-2* and *prd-3* mutations alone has only small effects on temperature compensation, in combination, the mutations cause extreme overcompensation. Mutation of *prd-6* results in a shorter period, suggesting that a possible function of *prd-6* is to negatively regulate the activity of FRQ; when *prd-6* is defective the clock runs faster as a result of more active FRQ. Taken together, the model predicts that in the *prd-2* and *prd-3* mutant strains, the long period is the net result of overactivity of *prd-6*. In addition, *prd-6* may provide a link to the putative temperature-dependent metabolic oscillator since mutations in *prd-6* have an increased range of temperature compensation, suppresses the temperature-compensation defects in *frq⁷* or *prd-2 prd-3* strains, and are resistant to some media conditions previously shown to affect period (Morgan & Feldman 1997).

Currently, we cannot distinguish between the *zeitnehmer* model and the A&B oscillator models. In any case, the model presented here makes certain testable predictions. Most notably, the *prd* alleles would be expected to have an effect on *frq* levels and/or FRQ activity and turnover. In addition, the *prd-6* mutation might be expected to show epistasis to the clock-affecting metabolic mutations. Control of the eventual output driving the conidiation rhythm may be independent of the FRQ-WC oscillator since *frq*-null mutants can entrain to temperature cycles, and the *cel* or *chol-1* mutant strains display rhythmic behaviour in a *frq*-null background. As of yet, there are no known mutations that result in an inability to entrain to a temperature cycle; however, such a mutant might be

Figure 1. Model for the genetic interactions between the PRD alleles and FRQ. Dotted lines indicate that these interactions are speculative. See § 5 for details of the model.

expected to have a normal FRQ–WC oscillator, but an altered FLO.

6. SUMMARY AND FUTURE PROSPECTS

Although the other *Neurospora* clock-affecting mutations have been largely ignored for the past several years, recent characterization of genetic interactions between the *prd* mutations and the recent cloning of the *prd-2* and *prd-6* genes (J. F. Feldman, unpublished data) have revived interest in these genes.

The composition of the circadian clock is still unresolved; however, it is clear that the circadian clock system does not exist in isolation from the environment and requires a mechanism to be synchronized to environmental cycles and at the same time be shielded from metabolic fluctuations. Furthermore, the experimental data forces us to face the conclusion that the circadian system comprises a population of oscillatory systems. Single-oscillator models will remain of heuristic value in providing the foundation for our current understanding of the feedback loops that interact to make a clock, yet they cannot account for the complexity of the available data. A multiple-oscillator model can be imposed on the physiological and genetic data, but the lack of molecular data still keeps the connection between the FRQ–WC oscillator and the rest of the cell a mystery. In particular, all of what we know about the independent role of the FLO–metabolic oscillator is derived from the ability, through mutations and genetically engineered strains, to manipulate or to eliminate altogether the FRQ–WC feedback loop. Until the FLO–metabolic oscillator can be similarly manipulated, we are constrained to modelling and phenomenology. Ultimately, molecular and biochemical explanations for the observed genetic interactions and altered physiology will be required to understand more fully the nature of the circadian oscillators and the role of the *prd* genes in the circadian system.

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