Isolation and Characterization of a Temperature-Sensitive Circadian Clock Mutant of *Neurospora crassa*

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ABSTRACT

A new circadian clock mutant has been isolated in *Neurospora crassa*. This new mutation, called *period-6* (*prd-6*), has two features novel to known clock mutations. First, the mutation is temperature sensitive. At restrictive temperatures (above 21°) the mutation shortens circadian period length from a wild-type value of 21.5 hr to 18 hr. At permissive temperatures (below 21°) the mutant has a 20.5-hr period length close to that of the wild-type strain. Second, the *prd-6* mutation is epistatic to the previously isolated clock mutation *period-2* (*prd-2*). This epistasis is unusual in that the *prd-2 prd-6* double mutant strain has an 18-hr period length at both the restrictive and permissive temperatures. That is, the temperature-sensitive aspect of the phenotype of the *prd-6* strain is lost in the *prd-2 prd-6* double mutant strain. This suggests that the gene products of the *prd-2* and *prd-6* loci may interact physically and that the presence of a normal *prd-2*$^+$ protein is required for low temperature to "rescue" the *prd-6* mutant phenotype. These results, combined with our recent finding that *prd-2* and some alleles of the *frq* gene show genetic synergy, suggest that it may be possible to establish a more comprehensive model of the Neurospora circadian clock.

GENETIC analyses of circadian clock mechanisms have resulted in the isolation of mutants in several organisms that exhibit alterations in one or more clock properties (reviewed in DUNLAP 1993). Of these, only the *frequency* (*frq*) gene of Neurospora and the *period* and *timeless* genes of Drosophila have been cloned and studied at a molecular level. Most previous work in Neurospora has focused on the *frq* gene for which five mutant allelic phenotypes are known. A molecular model for the Neurospora clock has been proposed, involving periodic transcription and translation of *frq*, that is controlled by negative regulation of *frq* transcription by FRQ protein (ARONSON *et al.* 1994; CROSTHWAITE *et al.* 1995). It seems likely, however, that a full understanding of the mechanism of the Neurospora clock and the role of each clock gene in the clock mechanism will require characterization and analysis of other clock components. In Drosophila, preliminary models of the clock involving the *period* gene were greatly enhanced with the discovery and characterization of *timeless* (SEHGAL *et al.* 1995; HUNTER-ENSOR *et al.* 1996).

In *N. crassa* there are seven known genes that, when mutated, alter the 21.5-hr period length of the circadian rhythm in asexual spore formation (DUNLAP 1993). Among these genes are both short period (*frq*^{1}, *frq*^{2}, *prd-4*) and long period (*frq*^{3}, *frq*^{7}, *prd-1, prd-2, prd-3*) mutants. Efforts to establish functional relationships between these genes have failed to reveal any synergistic interactions since all double and triple mutant combinations previously tested show additive (or multiplicative) behavior (FELDMAN *et al.* 1979; GARDNER and FELDMAN 1980). We report here the characterization of a new clock mutant strain in *N. crassa, period-6* (*prd-6*), which has a temperature-sensitive shortening of circadian period length and is thus the first known temperature-sensitive clock mutation. In addition, the *prd-6* mutation shows an unusual epistasis to the previously isolated clock mutation *period-2* (*prd-2*) that suggests a direct physical interaction between the products of the two genes.

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MATERIALS AND METHODS

Strains and culture conditions: All strains used in this study were isolated in this lab or were obtained from the Fungal Genetics Stock Center (FGSC). Unless otherwise indicated in the text, all strains contain the *band* (*bd*) mutation that allows clear expression of the circadian rhythm of conidiation without affecting the period length (SARGENT and WOODWARD 1969). The strains used in this study are shown in Table 1. Strains were handled according to standard procedures (DAVIS and DESERRES 1970) and maintained on minimal media with required supplements (see PERKINS *et al.* 1982).

Racetube assay of circadian rhythm: Racetube assays were performed on glucose-arginine media as described (SARGENT *et al.* 1966; SARGENT and KALTENBORN 1972) with supplements as required. Circadian period was calculated as previously described (FELDMAN and HOYLE 1973). Unless otherwise indicated all racetube assays were done at 25°.

Genetic mapping: Genetic crossing and linkage analyses were conducted using random ascospores as described (DAVIS and DESERRES 1970). Auxotrophic markers were scored for growth on Vogel's minimal media with and without supple-

TABLE 1

Strains used in this study

Strain	Genotype	Source
808-8	*bd a*	Laboratory stock
1313-3	*bd a*	This study (reisolate)
1290-1	*bd prd-6 A*	This study
1373-1	*bd prd-6 A*	This study (reisolate)
1373-25	*bd prd-6 a*	This study
613-43	*bd prd-2 a*	Laboratory stock
P1257	*bd prd-2 (prd-6) A*	This study
1257-(81, 154)	*bd prd-2 prd-6 A* (two separate isolates)	This study
1388-(46, 34)	*bd prd-2 prd-6 cyh-2^R a* (two separate isolates)	This study
1447-42	*bd prd-6 ilv-2* (39709)	This study
1447-6	*bd inl* (37401)	Laboratory stock
1318-901	*bd prd-2 cyh-2^R A*	This study
1360-8	*bd prd-6 lys-2* (37101) *a*	This study
1360-3	*bd prd-6 lys-2* (37101) *A*	This study
3434	*alcoy csp-2 a*	FGSC
1228-992	*bd am* (32213) *inl* (37401) *A*	This study
1318-349	*bd cyh-2^R his-1* (C84) *a*	This study
1330-16	*bd inl* (37401) *ilv-2* (39709) *A*	This study
1464-79	*bd prd-6 inv a*	This study
1466-4	*bd pab-2 A*	This study
1471-6	*bd asn* (C123) *A*	This study

Numbers in parentheses indicate specific alleles used. Since the P1257 strain has a questionable genotype, the *prd-6* mutation is given in parentheses. FGSC, Fungal Genetics Shock Center.

ments (VOGEL 1957; PERKINS *et al.* 1982). To allow for scoring of circadian period, the *bd* mutation was first crossed into the strains used for mapping.

Linear growth measurements: The linear growth rate (mm/day) was calculated as the average of at least 5 days growth on racetubes containing glucose-arginine.

Heterokaryon analysis: Heterokaryons were constructed and analyzed essentially as previously described (DAVIS and DESERRES 1970; FELDMAN and HOYLE 1976). Period length was calculated as an average of six replicate racetubes assayed at 25°. Nuclear ratios were determined by plating on unsupplemented or single and double supplemented plates. Nuclear ratios were calculated assuming an average of 2.5 nuclei per conidium (DAVIS and DESERRES 1970).

RESULTS

Initial isolation of *prd-6*: Strains carrying the *prd-6* mutation were isolated as an unusual segregant class from a backcross of a *prd-2* clock mutant strain (P1257) (25.5-hr period length) to a wild-type *prd-2*$^+$ strain (808-8) (21.5-hr period length). Strains carrying the *prd-2* mutation had been previously isolated following UV mutagenesis of the wild-type strain (FELDMAN *et al.* 1979) and maintained in the laboratory. The period length of the unusual progeny was 17.7 ± 0.4 hr at 25°. In another cross involving the same *prd-2* parental strain (P1257) crossed to a *prd-2*$^+$ *am inl* strain (1228-992), some progeny with similar 18-hr period lengths were also observed. These results suggested that the mutation causing the 18-hr period length arose spontaneously in this *prd-2* isolate (P1257). One of the 18-hr progeny (1290-1) was then backcrossed to the wild-type strain (808-8) several times. The 18-hr period length phenotype was shown to segregate as a single gene mutation (Figure 1) that cosegregated with a slow growth rate (Figure 2). This mutation was called *period-6* (*prd-6*).

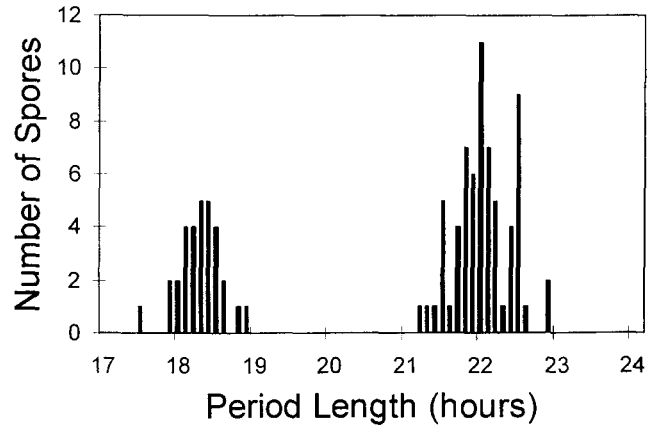

FIGURE 1.—Distribution of period lengths among random spore progeny of a cross of a *prd-6* strain (1373-1) to a *prd-6*$^+$ strain (1313-3). Period length was scored at 25°. The average period lengths of the two groups were 18.3 ± 0.3 hr (n = 31) and 22.0 ± 0.4 hr (n = 66).

Genetic mapping: In an initial cross of the *prd-6* strain (1373-1) to the *alcoy csp-2* strain (3434), linkage was observed between *cot-1* and *prd-6* indicating that *prd-6* was on LG IV or LG V. A series of three- and four-point crosses with markers on linkage group VR was performed and the results are summarized in Table 2.

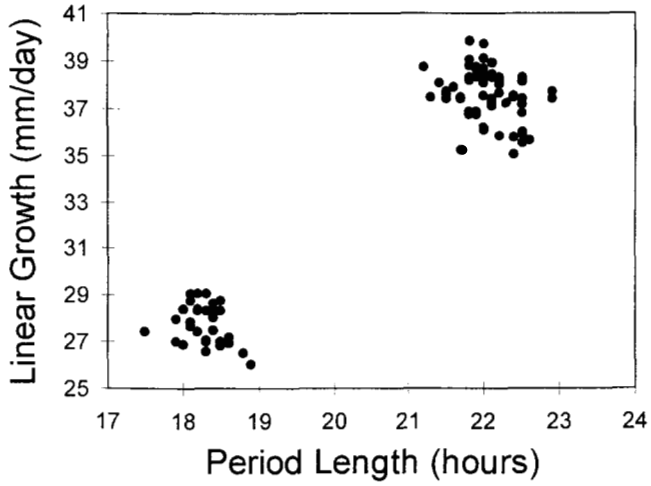

FIGURE 2.—Cosegregation of short period length and slow growth rate among random spore progeny of a cross of a *prd-6* strain to a *prd-6*$^+$ strain. Both period length and linear growth rate were assayed at 25°. The period lengths are the same as those shown in Figure 1. The average growth rates of the two groups were 27.8 ± 0.8 mm/day (n = 31) and 37.6 ± 1.0 mm/day (n = 66).

In a cross between strain 1360-3 (*prd-6 lys-2*) and strain 1318-349 (*cyh-2*^{R} *his-1*), the *prd-6* gene mapped ~20 map units distal to *his-1*. In a cross of strain 1464-79 (*prd-6 inv*) to strain 1466-4 (*pab-2*), *prd-6* mapped ~2.3 map units proximal to *pab-2*.

Analysis of dominance: To determine whether the *prd-6* mutation is dominant or recessive, a forced heterokaryon was constructed between a *prd-6 ilv-2* (isoleucine- and valine-requiring) strain (1477-42) and a *prd-6*$^+$ *inl* (inositol-requiring) strain (1477-6). Conidia from the two strains were mixed at ratios of 1000:1, 1:1, and 1:1000, and grown on minimal media. The period length of the heterokaryons was assayed on race tubes at 25° and nuclear ratios were determined by plating on appropriate media. At nuclear ratios of ~1:1 (*prd-6/prd-6*$^+$) the period length of the heterokaryon strain was clearly wild type (Table 3). This indicates that *prd-6* is recessive to *prd-6*$^+$.

TABLE 3

Analysis of *prd-6/prd-6+* heterokaryons

Strain	Nuclear ratio *prd-6/ prd-6+*	Period length (hr)
1313-3	—	21.9 ± 0.2
1373-1	—	18.4 ± 0.2
Heterokaryon 1	33:67	20.7 ± 0.7
Heterokaryon 2	42:58	21.5 ± 0.1
Heterokaryon 3	45:55	21.3 ± 0.1

Period length was calculated as the average of six replicate racetubes.

Temperature sensitivity of *prd-6*: The period length of the *prd-6* strain (1373-1) was determined at temperatures between 17° and 34° (Figure 3). The initial rationale for this experiment was to determine whether the temperature compensation property of the *prd-6* strain

TABLE 2

Mapping of *prd-6* by three- and four-point crosses

Parental genotypes and percent recombination	No. of progeny						
		Single crossovers			Double crossovers		
	Parentals	R1	R2	R3	R1 + 2	R1 + 3	R2 + 3
13.5 12.5 19.8							
lys-2 + + *prd-6*	24	3	2	3	0	2	3
+ *cyh-2* *his-l* +	36	7	4	9	1	0	2
21.7 3.3 30.0							
lys-2 + + *prd-6*	17	4	0	5	0	2	0
+ *am* *inl* +	15	5	1	8	0	2	1
25.9 9.3							
ilv-2 *inl* +	15	10	1	—	0	—	—
+ + *prd-6*	21	3	3	—	1	—	—
<1.1 7.4							
prd-6 *inv* +	40	0	2	—	0	—	—
+ + *asn*	47	0	5	—	0	—	—
2.3 2.3							
prd-6 + *inv*	151	4	3	—	0	—	—
+ *pab-2* +	155	2	3	—	1	—	—

The top number of each pair of complementary classes represents progeny carrying the *lys-2* (first two crosses), *ilv-2*, or *prd-6* (last two crosses) mutations. Regions (R) are numbered left to right.

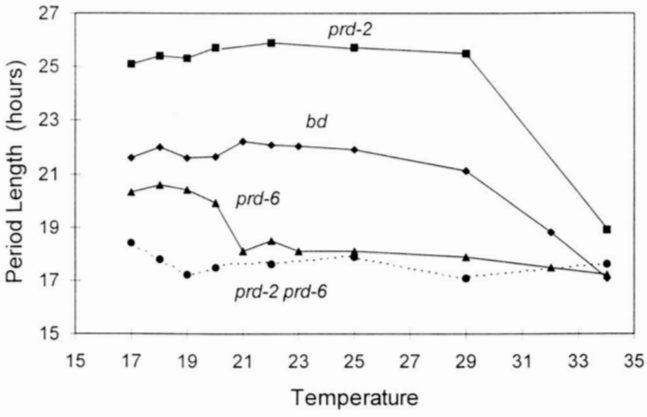

FIGURE 3.—Period lengths of wild-type, *prd-6*, *prd-2*, and *prd-2 prd-6* strains at different temperatures. The average SD for each strain was as follows: wild-type, 0.5 h; *prd-6*, 0.6 h; *prd-2*, 0.4 h; *prd-2 prd-6*, 0.5 h.

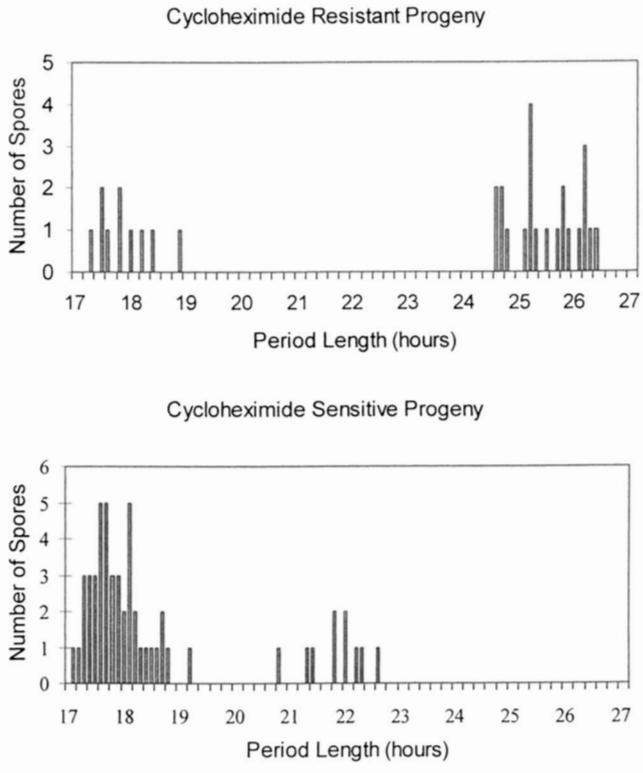

FIGURE 4.—Distribution of period lengths from random spore progeny of a cross of a *prd-2 cyh-2*^{R} strain to a *prd-6 lys-2* strain. The average period lengths of each group are as follows: cycloheximide resistant, 17.8 ± 0.5 hr (n = 10) and 25.4 ± 0.6 hr (n = 22); cycloheximide sensitive, 17.8 ± 0.5 hr (n = 44) and 21.8 ± 0.5 hr (n = 10).

was altered by the mutation, since strains carrying other clock mutations (*e.g.*, *frq*^{7}, *frq*^{9}, *prd-4*) show partial or complete loss of temperature compensation (GARDNER and FELDMAN 1981; LOROS and FELDMAN 1986). Although the temperature compensation feature of the *prd-6* strain (1373-1) did not appear to be altered, we found that the *prd-6* mutation itself is a temperature-sensitive mutation. At temperatures above 21° the *prd-6* strain (1373-1) showed a short period length of 18 hr, while below 21° the *prd-6* strain (1373-1) had a nearly wild-type period length of ~20.5 hr. Within each of these two temperature ranges (17–21° or 22–34°) however, the period length did not change [*i.e.*, within each temperature range, the period length of the *prd-6* strain (1373-1) was temperature compensated]. Surprisingly the period length remained well compensated above 30°, the point at which temperature compensation is partially lost in the wild-type strain (SARGENT *et al.* 1966; GARDNER and FELDMAN 1980; see Figure 3).

***prd-6* is epistatic to *prd-2*:** The possibility of an interaction between *prd-6* and *prd-2* was first suggested when two of the original isolates that showed an 18-hr period length (1257-81, 1257-154) were backcrossed to the wild-type strain (808-8). In these crosses (as opposed to backcrosses using the 1290-1 or 1373-1 isolates) the *prd-2* (25.5-hr period) phenotype reappeared among a small number of the progeny (6/73 and 10/102, respectively). Since *prd-2* and *prd-6* are 20–30 map units apart on LG VR (*prd-6* distal to *prd-2*), this result suggested that these two 18-hr isolates were in fact *prd-2 prd-6* double mutant strains with *prd-6* epistatic to *prd-2*. A test of this hypothesis was carried out by reconstructing a *prd-2 prd-6* double mutant strain from each of the two single mutants. This was done by crossing a *prd-2 cyh-2*^{R} strain (1318-901) with a *prd-6 lys-2* (1360-8) strain.

The *prd-2 cyh-2*^{R} (1318-901) strain was a rare double mutant we had previously isolated from crosses demonstrating tight linkage between *prd-2* and *cyh-2*^{R} (<0.1% recombination) (LEWIS 1995). The *prd-6 lys-2* double mutant strain (1360-8) was derived from a *prd-6* single mutant strain (1373-1) that had been backcrossed to the wild-type strain (808–8) and had yielded no *prd-2* (25.5-hr period length) segregants. *lys-2* had previously been shown to be ~1–2 map units proximal to both *prd-2* and *cyh-2*^{R} (LEWIS 1995). Due to this close linkage it was assumed that any progeny carrying *cyh-2*^{R} would likely be carrying the *prd-2* mutation as well. Cycloheximide-resistant progeny from this cross were scored for lysine auxotrophy and period length at 25°. Among 32 cycloheximide-resistant progeny, 22 showed a 25.5-hr period length and 10 showed an 18-hr period length (Figure 4). The 18-hr progeny that were *lys-2*$^+$ were assumed to be the most likely candidates for carrying both the *prd-2* and *prd-6* mutations. Therefore, two of the 18-hr, *lys-2*$^+$, *cyh-2*^{R} progeny (1388-34, 1388-46) were backcrossed to a wild-type strain (1313-3) and in each cross both 18- and 25.5-hr mutant period lengths were observed among the progeny. This confirmed that each of these 18-hr strains (1388-34, 1388-46) was carrying the *prd-2* mutation and, therefore, that *prd-6* is epistatic to *prd-2*.

Because of the identification of *prd-6* as a temperature-sensitive mutation, we also measured the period length of the *prd-2 prd-6* double mutant strain (1388-46) in the temperature range of 17–34°. Our initial

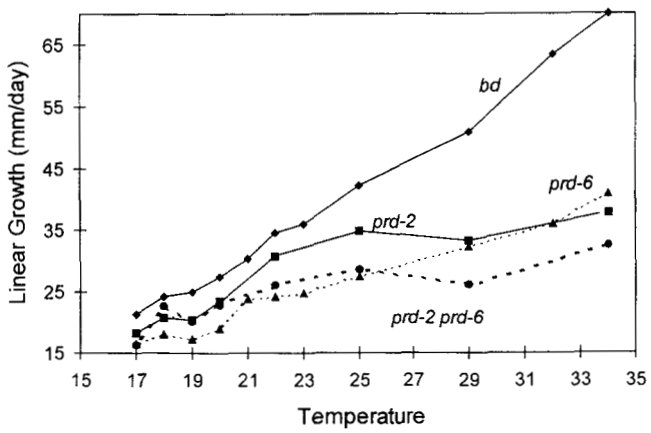

FIGURE 5.—Linear growth rates of wild-type, *prd-6*, *prd-2*, and *prd-2 prd-6* strains at different temperatures. The average SD for each strain was as follows: wild type, 0.9 mm; *prd-6*, 0.9 mm; *prd-2*, 0.7 mm; *prd-2 prd-6*, 0.7 mm.

expectation was that at low (permissive) temperatures, where *prd-6* is functionally wild type, the *prd-2* mutation would be "uncovered" and the period length of the double mutant strain would show the *prd-2* (temperature compensated) period length of 25.5 hr. However, the period length of the double mutant strain (1388-46) was ~18 hours, even at the low permissive temperatures (Figure 3). Thus, it appears that the presence of a wild-type *prd-2*$^+$ allele is required for low temperature to "rescue" strains carrying the *prd-6* mutation. In the absence of a functional *prd-2*$^+$ product, *prd-6* loses the temperature-sensitive nature of its altered clock phenotype.

Temperature effects on growth: While the period length alteration caused by *prd-6* seems to be "rescued" at low temperatures, the slow growth phenotype does not show any apparent temperature sensitivity (Figure 5). The growth rate of the *prd-6* strain (1373-1), like the *prd-6*$^+$ strain (1313-3), increased linearly with increasing temperature. The *prd-2 prd-6* double mutant strain (1388-46) also shows the slow growth rate of the *prd-6* single mutant strain (1373-1). In addition, at temperatures above 25° the *prd-2 prd-6* double mutant strain (1388-46), like the *prd-2* single mutant strain (613-43) (GARDNER and FELDMAN 1981; see Figure 5), shows little change in growth rate. These results suggest that the phenotypic effects of the *prd-6* mutation on growth rate may be a secondary consequence of the primary defect and only indirectly related to its effect on the clock.

DISCUSSION

We have isolated a new circadian clock mutant of *N. crassa* that exhibits a shortening of the period length of its circadian rhythm in conidiation from the wild-type value of 21.5 to 18 hr. This mutant strain, called *prd-6*, has two properties unlike other known clock mutations.

First, the period length of the *prd-6* (1373-1) mutant is temperature sensitive. At temperatures above 21° the *prd-6* strain (1373-1) has a period length of 18 hr. At temperatures below 21° the *prd-6* strain (1373-1) has a nearly wild-type period length of 20.5 hr. Thus, *prd-6* represents the first example of a temperature-sensitive clock mutant. Although clock mutants with changes in temperature responses have been isolated previously, they were altered in the temperature compensation feature of the clock. (GARDNER and FELDMAN 1981; LOROS and FELDMAN 1986). Temperature compensation is a property of all circadian rhythms whereby the period length of the clock does not change with different ambient temperatures ($Q_{10} = 1$). Strains carrying the *frq*^{3} or *frq*^{7} mutations show partial loss of temperature compensation in which there is a gradual shortening of period length with increasing temperature (*i.e.*, the clock runs slightly faster at higher temperatures) expressed as a Q_{10} of 1.2 and 1.4, respectively. Strains carrying the *frq*^{9} mutation show complete loss of temperature compensation with a Q_{10} of 2.0 (*i.e.*, the clock runs twice as fast at 30° than at 20°). In contrast, the *prd-6* mutant strain (1373-1) exhibits a compensated period length within each of the two temperature ranges (the permissive temperature below 21° and the restrictive temperature above 21°). The *prd-6* strain (1373-1) also shows an additional difference in temperature response from wild-type strains. In wild-type strains, the temperature compensation of the clock is partially lost above 30° and the period length shortens as temperature is increased. In the *prd-6* strain (1373-1), however, temperature compensation remains intact even at temperatures up to 34°. It is not known how (or whether) this change is related to the temperature-sensitive phenotype.

A second novel feature of *prd-6* is that it is epistatic to the previously known clock mutation *prd-2*, which has a long 25.5-hr period length. At 25°, the *prd-2 prd-6* double mutant strain (1388-46) has a period length of 18 hr, identical to that of the *prd-6* single mutant strain (1373-1). This type of gene interaction has not previously been observed among Neurospora clock mutations (strains carrying multiple clock mutations have exhibited period lengths predicted simply by combining the effects of each mutation). The only other reported case of gene interaction involving genes affecting the Neurospora clock involves the period lengthening effects of the *cel* mutation, which is deficient in fatty-acid synthesis (LAKIN-THOMAS and BRODY 1985).

Of particular interest is the behavior of the *prd-2 prd-6* double mutant strain (1388-46) at the low (permissive) temperature. Because the *prd-6* single mutant strain (1373-1) is functionally wild-type at low temperature we had expected low temperature to "uncover" the *prd-2* mutation and for the 1388-46 double mutant strain to express the 25.5-hr period length of the *prd-2* strain. However, at the permissive low temperature the *prd-2 prd-6* double mutant strain (1388-46) shows the same

short 18-hr period length that it does at the restrictive high temperature. In other words, low temperature "rescues" the temperature-sensitive *prd-6* allele as a single mutant strain (where there is a functional *prd-2* gene product) but does not "rescue" *prd-6* in a double mutant strain where the *prd-2* gene product is either nonfunctional or absent. Traditionally epistatic interactions suggest that gene products affect different steps of a common pathway. The unusual nature of the interaction between *prd-2* and *prd-6*, however, suggests an alternative interpretation. Low temperature rescue of temperature-sensitive alleles usually suggests that the temperature-sensitive gene product can only fold correctly into a functional three-dimensional configuration at the low temperature. Since the *prd-6* phenotype is rescued only in the presence of a functional *prd-2* gene product, this might suggest a direct physical interaction between the *prd-2* and *prd-6* gene products.

The interaction between *prd-6* and *prd-2* takes on additional significance in light of our recent finding of a strong synergistic interaction between *prd-2* and long period alleles of the *frequency* (*frq*) locus (MORGAN *et al.* 1996), since *frq* has been shown to play a central role in the organization of the Neurospora clock (GARDNER and FELDMAN 1980; ARONSON *et al.* 1994). Although recent molecular models of circadian clocks in Neurospora focus on *frq* as the central element of the clock, these interactions suggest that a full understanding of the clock in general, and the specific role of *frq* in the clock, will require identification and analysis of other clock components encoded by other clock genes just as the analysis of the Drosophila *timeless* mutation has provided new insights into the Drosophila clock and the role of the *period* gene in clock mechanisms. To this end we have mapped *prd-6* close to both *pab-2* and *ad-7* on LG VR, and *prd-2* between *lys-2* and *cyh-2* also on LG VR. We have identified a cosmid that contains *prd-2* (LEWIS 1995), and we should be able to clone *prd-6* by a chromosome walk from the cloned *pab-2* gene (ROBB *et al.* 1995). These clones should offer new opportunities to identify the molecular nature of *prd-2* and *prd-6* and the nature of their interaction with *frq*. Such experiments are currently in progress.

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