A RECESSIVE CIRCADIAN CLOCK MUTATION AT THE *FRQ* LOCUS OF *NEUROSPORA CRASSA*

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Manuscript received August 26, 1985
Revised copy accepted August 18, 1986

ABSTRACT

A circadian clock mutant of *Neurospora crassa*, the most distinctive characteristic of which is the complete loss of temperature compensation of its period length, maps to the *frq* locus where seven other clock mutants have previously been mapped. This mutant, designated *frq-9*, is recessive to the wild-type allele and to each of the other *frq* mutants; thus, it differs from the other mutants, which show incomplete dominance to wild type and to each other. Complementation analysis suggests either that the *frq* locus is a single gene or that *frq-9* is a deletion that overlaps adjacent genes. Preliminary efforts at fine structure mapping have indicated that recombination between certain pairs of *frq* mutations is less than 0.005%, a distance consistent with the locus being a single gene. The recessive nature of *frq-9*, coupled with complete loss of temperature compensation, suggests that this mutant may represent the null phenotype of the locus and that the *frq* gene is involved in the temperature compensation mechanism of the clock.—Genetic mapping studies have placed the *frq* locus on linkage group VIIR, midway between *oli* (oligomycin resistance) and *for* (formate auxotrophy), about 2 map units from each, and clearly indicate that *frq* and *oli* are separate genes.

A GENETIC approach to studying the mechanism of circadian rhythms has been used in several organisms, including Neurospora, Drosophila and Chlamydomonas (for review, see FELDMAN 1982). Mutants have been isolated in these organisms that show an altered period length of their circadian clock, a change in the response of the clock to light or temperature, or complete loss of rhythmicity.

In *Neurospora crassa*, 12 circadian clock mutants have been well characterized. Of these, seven map to a single locus called *frq* (frequency), located on the right arm of linkage group VII. Four of the *frq* mutants (*frq-1*, *frq-2*, *frq-4*, *frq-6*) have period lengths shorter than the wild-type value of 21.5 hr, whereas the other three (*frq-3*, *frq-7*, *frq-8*) have period lengths longer than wild type. The *frq* mutants are very tightly linked, since in pairwise crosses no

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wild-type recombinants have been isolated among more than 2000 progeny (FELDMAN and HOYLE 1973, 1976; GARDNER and FELDMAN 1980).

frq mutants have several characteristics that suggest that this locus plays an important role in the organization of the Neurospora clock (GARDNER and FELDMAN 1980). First, the mutations are highly specific. No phenotypic changes other than the alterations in circadian period length have been detected, including changes in growth rate, nutritional requirements or morphology. Second, in heterokaryons all of the *frq* mutants show incomplete dominance to the wild-type frq^+ allele and to each other. Furthermore, in the one case studied in detail ($frq\text{-}1/frq^+$), there was a gene dosage effect, such that the change in period length was proportional to the nuclear ratio (FELDMAN and HOYLE 1976).

An important and distinguishing characteristic of circadian rhythms in all organisms, including Neurospora, is that the period length of the rhythm is the same over a wide range of physiological temperatures; that is, the rate of the clock is temperature compensated ($Q_{10} = 1$). Although the clocks of the short period *frq* mutants show normal temperature compensation (FELDMAN and HOYLE 1976), those with long periods show a partial loss in temperature compensation—their period lengths increase slightly as the temperature is lowered (GARDNER and FELDMAN 1981).

In *Drosophila melanogaster*, four clock mutations map to a single locus on the *X* chromosome called *per*. As in Neurospora, there are both short period and long period *per* mutants, as well as two isolates that are arrhythmic, and all three mutant phenotypes show incomplete dominance to some extent (KONOPKA and BENZER 1971; SMITH and KONOPKA 1981). The *per* locus has been cloned (BARGIELLO and YOUNG 1984; ZEHRING *et al.* 1984), and a transcriptional map of the region has indicated the existence of a key 4.5-kb transcript (REDDY *et al.* 1984) that codes for a proteoglycan (SHIN *et al.* 1985; JACKSON *et al.* 1986; REDDY *et al.* 1986).

Recently, a new circadian clock mutant of Neurospora has been isolated, the most distinctive characteristic of which is the complete loss of temperature compensation of its period length (LOROS and FELDMAN 1986). The mutant also exhibits different period lengths on different media in contrast to the relatively constant period length of the wild-type strain. Neither of these characteristics has previously been found in any clock mutant, including those at the *frq* locus. In this paper we report the genetic characterization of this mutant and show that (1) it maps to the *frq* locus, (2) it is recessive to the wild-type frq^+ allele and to the other *frq* mutants, and (3) it does not show gene dosage effects on period length. We also report a detailed recombination analysis of the *frq* locus and its surrounding region. A previous report (DIECKMANN and BRODY 1980) has suggested that *frq* and a nearby locus coding for oligomycin resistance (oli^r) were allelic. Our genetic data clearly indicate that the two loci are separable, and this is consistent with more recent biochemical data from the same laboratory (BRODY, DIECKMANN and MIKOLAJCZYK 1985).

MATERIALS AND METHODS

Strains: The following strains of *N. crassa* were obtained from the Fungal Genetics Stock Center, Arcata, California: *bd* (band), *pan-2* (pantothenate), *inl* (inositol), *for* (formate) and *un-10* (unknown metabolic defect, temperature-sensitive for growth). The oligomycin-resistant (*oli*^{r}) mutants (alleles *16-1*, *16-3* and *16-16*), were obtained from DAVID EDWARDS (Scripps Clinic and Research Foundation, La Jolla, California). The *frq* mutants were all previously isolated in this laboratory (FELDMAN and HOYLE 1973, 1976; GARDNER and FELDMAN 1980). Their period lengths at 25° are as follows: *frq-1*, 16.5 hr; *frq-2*, *frq-4*, *frq-6*, 19 hr; *frq-3*, 24 hr; *frq-7*, *frq-8*, 29 hr. All strains used in these studies contain the *bd* mutation, which allows clear expression of the circadian rhythm on race tubes (SARGENT, BRIGGS and WOODWARD 1966).

The mutant *frq-9* was isolated after ultraviolet light mutagenesis of the *bd* strain. Conidia were suspended in 10 ml of sterile distilled water in a standard Petri dish at a concentration of 1×10^5/ml and were irradiated to produce a killing rate of about 90%. The survivors were plated on sorbose complete medium (DAVIS and DESERRES 1970) and were screened for circadian rhythmicity of conidiation on race tubes (see below). The *frq-9* mutant was initially picked for further study because it did not show any rhythm on medium containing acetate as a carbon source. Its temperature-dependent period length was subsequently discovered on a glucose-containing medium (LOROS and FELDMAN 1986). The mutant has since been backcrossed to *bd*, *frq*$^+$ several times, with ordered tetrad analysis at each backcross, to eliminate several unlinked mutations causing slow growth and other morphological abnormalities. The studies presented here with *bd*, *frq-9* were carried out with either the isolate (621-313) or (820-38), both of which will be referred to simply as *frq-9*.

Culture conditions: Stock cultures were maintained on Horowitz complete medium at 25°, and crosses were carried out on Westergaard and Mitchell's crossing medium by standard procedures (DAVIS and DESERRES 1970). The circadian clock was assayed by measuring the period length of the circadian rhythm of conidiation for cultures grown on race tubes as previously described (DHARMANANDA and FELDMAN 1979). The medium contained Vogel's salts, 0.3% glucose, 0.5% arginine·HCl, 2% agar. Race tubes were 60-cm long, instead of the usual 20 cm, in order to collect data for up to 20 days. This was necessary because *frq-9* does not begin to express its conidial banding rhythm until 5–7 days after inoculation onto the race tube (LOROS and FELDMAN 1986). Determination of the phase and period length of the rhythm was also as previously described (DHARMANANDA and FELDMAN 1979), except that a digitizer (Summagraphics Bit Pad) interfaced to a Northstar Horizon microcomputer was used to collect and process the data. In addition to the altered period length expressed by *frq-9*, the mutant also fails to express any rhythm of conidiation when grown on a medium containing 1.2% anhydrous sodium acetate, 0.05% casamino acids and 2% agar (LOROS and FELDMAN 1986). The absence of conidial banding on this medium proved to be a simple and convenient way to assay the presence or absence of the *frq-9* allele and was used for scoring the marker in two-, three- and four-point crosses.

Scoring for auxotrophs was done on VOGEL's (1964) minimal medium in liquid culture. Concentrations of supplements to allow growth of auxotrophs were as follows: *for*$^-$, 250 mg/liter sodium formate plus 18.4 mg/liter adenine sulfate; *pan-2*$^-$, 10 mg/liter calcium pantothenate; *inl*$^-$, 100 mg/liter inositol. The *un-10* mutation, which results in the inability to grow on either minimal or complete medium at 34° but which grows like wild type at 25°, was scored simply by comparing growth at 25° with that at 34°. To score for *oli*^{r} (oligomycin-resistance), oligomycin stock solution (25 mg/ml in 95% ethanol) was added under sterile conditions after the autoclaved medium had cooled to 60° to yield a final concentration of 2 or 5 μg/liter (EDWARDS and UNGAR 1978).

Heterokaryon analysis was as previously described (DAVIS and DESERRES 1970; FELDMAN and HOYLE 1976) with the following exceptions: Strains used to construct heter-

okaryons were initially crossed to maximize matching of heterokaryon-incompatibility alleles. Heterokaryons were formed by overlaying drops of conidial suspensions of the "parental" strains onto 4% washed agar plates and incubating overnight. As a control, drops of the parental suspensions alone were inoculated. Hyphal tips were isolated directly from the growing heterokaryon cultures on the plates. Period length data for all heterokaryons were obtained on both the glucose-arginine and acetate-casamino acids media, and at 22° and 25°, to determine the temperature and media dependence of period length, since *frq-9* differs from both frq^+ and the other *frq* mutants in these respects (LOROS and FELDMAN 1986).

Fine structure analysis of the *oli-frq* region: Since no selection method is available to isolate rare recombinants based on circadian clock periodicity, fine structure analysis was attempted using selectable outside markers on either side of the *frq* locus. Initially, *un-10* (about 10 map units proximal) and *for* (about 2 map units distal) were used as the selectable markers and were crossed into the appropriate *oli* and *frq* strains. Since a crossover between *oli* and *frq* would also produce a crossover between *un-10* and *for*, crosses were set up using the following general scheme:

$$\frac{un\text{-}10^-,\ oli^r,\ frq\text{-}X,\ for^+}{un\text{-}10^+,\ oli^s,\ frq\text{-}Y,\ for^-}$$

(The diagram shows the zygote produced by crossing the two haploid strains, the genotypes of which are given above and below the line. "*frq-X*" and "*frq-Y*" are used to designate two different *frq* alleles.) In such a cross, if one selects simultaneously for $un\text{-}10^+$ and for^+, all selected progeny have a crossover between these two outside markers, and these progeny can then be screened for oligomycin resistance and clock periodicity.

Once it was clearly established that *oli* and *frq* were easily separable (see RESULTS), three-point unselected crosses were carried out using *oli*, *frq* and *for* to confirm that the method of selecting crossover progeny using outside markers did not bias the results in some unexpected manner. These results showing that *oli* was proximal to *frq* allowed us to set up a similar scheme using *oli* as the outside proximal marker to look for recombinants between different *frq* alleles as follows:

$$\frac{oli^r,\ frq\text{-}X^+,\ frq\text{-}Y^-,\ for^-}{oli^s,\ frq\text{-}X^-,\ frq\text{-}Y^+,\ for^+}$$

In this case, selection for oligomycin resistance and prototrophy (*i.e.*, for^+) resulted in progeny with a crossover between *oli* and *for*, which were then screened for clock periodicity. In the example above, if *frq-X* were truly to the left of *frq-Y*, recombinants between the two *frq* alleles should yield the wild-type frq^+. If the order were reversed (*i.e.*, *frq-Y* to the left of *frq-X*), the recombinants would be the double mutant. Note that in this cross the $frq\text{-}X^+$ allele is in coupling with oli^r.

Since the phenotype of the double mutant could resemble either of the single mutants and therefore be undetectable in this scheme, a second cross was carried out in which the coupling between the *frq* alleles and *oli* was reversed, as follows:

$$\frac{oli^r,\ frq\text{-}Y^+,\ frq\text{-}X^-,\ for^-}{oli^s,\ frq\text{-}Y^-,\ frq\text{-}X^+,\ for^+}$$

In this cross, where $frq\text{-}Y^+$ is in coupling with oli^r, if *frq-Y* is to the left of *frq-X*, a crossover between them will produce wild-type frq^+. By carrying out both of these crosses for each pair of *frq* alleles studied and by screening for progeny with either wild-type or a unique periodicity, we could eliminate the possibility that the phenotype of the double mutant progeny would prevent detection of the crossover event.

The actual selection of progeny was carried out by suspending ascospores in 5 ml of top agar containing Westergaard and Mitchell's salts, biotin (5 μg/liter), 0.1% glucose, 6% sorbose, 0.8% agar. The ascospores were heat shocked in the top agar for 40 min

TABLE 1

Mapping *frq-9* by a three-point unselected cross

Zygote genotype and percent recombination	Parentals	Single crossovers		Double crossovers
		R1	R2	R1 and 2
oli^s + *for*$^-$	108	2	4	0
oli^r *frq-9* + (4.0 2.2)	101	7	1	0

at 60° and were plated on a bottom agar that was the same composition except that it contained 3% agar. The agar was washed three times with distilled water before use. When using oligomycin resistance as a selectable marker, oligomycin (in 95% ethanol) was added to both the top and bottom agar after the autoclaved medium had cooled to 60°, to a final concentration of 2 or 5 μg/ml. Plates were incubated at 34° for 3–5 days (growth was intentionally very slow at this high sorbose concentration), and colonies were examined under a dissecting microscope to ensure that each colony arose from only a single ascospore.

RESULTS

A new clock mutant maps to the *frq* locus: A new circadian clock mutant of Neurospora has been isolated that has a temperature-dependent period length (LOROS and FELDMAN 1986). This mutant segregates as a single nuclear gene and fails to recombine with either *frq-1* or *frq-7*. In a two-point cross with *frq-1*, no wild-type recombinants were recovered among 198 progeny. In a similar cross with *frq-7*, no wild-type recombinants were recovered among 139 progeny. These results suggested that the mutation mapped in or very near the *frq* locus; therefore, the mutant was designated *frq-9*.

Further confirmation of the map location of *frq-9* came from crosses between *frq-9* and the flanking markers *for* and *oli* (see below). In two-point crosses, *frq-9* showed a 1.9% recombination frequency with *for* (12 recombinants among 618 progeny), a value quite similar to that found with the other *frq* mutants. In the crosses *oli*^{s} *frq-9* × *oli*^{r}, *frq-7* and *oli*^{s}, *frq-9* × *oli*^{r}, *frq-1*, recombination between *frq-9* and *oli* was 3.8% (seven recombinants among 184 progeny) and 3.7% (eight recombinants among 212 progeny), respectively. Although slightly higher than the recombination frequency between *oli* and the other *frq* mutants, it is still within the normal variation seen in crosses between two loci using different alleles (PERKINS *et al.* 1982). Furthermore, no wild-type recombinants were recovered between *frq-9* and either *frq-7* or *frq-1* in these crosses, bringing to 733 the number of progeny scored in crosses between *frq-9* and other *frq* alleles without finding any wild-type recombinants.

Finally, a three-point cross of *oli*^{r}, *frq-9*, *for*$^+$ × *oli*^{s}, *frq*$^+$, *for*$^-$ was carried out to confirm that *frq-9* maps between *oli* and *for*, as do the other *frq* mutants (Table 1). Map distances in this cross were similar to those obtained in the previous crosses.

Mapping the *oli-frq-for* region of linkage group VIIR: It had previously

TABLE 2

Mapping *frq* and *oli* by a four-point unselected cross

Zygote genotype and percent recombination	Parentals	Single crossovers[a]		
		R1	R2	R3
+ *oli*^{r} + *for*$^-$	71	9	1	2
un-10 *oli*^{s} *frq-1* + (5.9 2.4 2.4)	80	1	3	2

[a] No double crossovers were recovered from this cross.

TABLE 3

Mapping *frq* and *oli* by four-point selected crosses

Zygote genotype	Total *un-10*$^+$, *for*$^+$ recombinants	*oli*^{s}, *frq*$^-$	*oli*^{r}, *frq*$^+$	*oli*^{r}, *frq*$^-$ (double mutants)	*oli*^{s}, *frq*$^+$ (wild type)
un-10$^-$ *oli*^{s} *frq-7* + / + *oli*^{r} (*16-1*) + *for*$^-$	341	233	60	48	0
un-10$^-$ *oli*^{s} *frq-1* + / + *oli*^{r} (*16-1*) + *for*$^-$	266	135	81	50	0
un-10$^-$ *oli*^{s} *frq-7* + / + *oli*^{r} (*16-16*) + *for*$^-$	104	53	26	25	0

been reported that *oli* and *frq* were very tighly linked, and the suggestion was made that *oli* and *frq* mutants were allelic (DIECKMANN and BRODY 1980). Therefore, we carried out four-point unselected crosses involving two *oli* and two *frq* alleles, as well as two "outside" markers—*un-10* (proximal) and *for* (distal)—to determine the map order and distance of these markers. A total of 787 progeny from four pairwise combinations of *frq* and *oli* alleles were examined. The results of one such cross are shown in Table 2, giving the gene order *un-10-oli-frq-for* and a recombination frequency between *oli* and *frq* of 2.4%. The other three crosses gave similar results, with an average map distance between *frq* and *oli* of 1.9 map units.

Since this recombination frequency is not consistent with the suggestion of allelism between *oli* and *frq*, we carried out a second set of crosses using the same markers, but initially selecting for recombinants between *un-10* and *for* (see MATERIALS AND METHODS). Since these outside markers are about 12 map units apart, scoring only among the recombinant progeny for *oli* and *frq* would increase the resolution of the mapping by a factor of about 8. Table 3 shows the results of three such crosses, in which a total of 711 *un-10*$^+$, *for*$^+$ selected recombinants were screened for *oli* and *frq*. This is equivalent to about 5700 unselected progeny. Once again, the results confirm the order of the genes

TABLE 4

Period lengths of *oli,frq* double mutants

Strain	Period length (hr)	Change in period length from wild type (hr)
frq$^+$,*oli*^{s} (wild type)	21.5	
frq$^+$,*oli*^{r} *16-1*	20.2	−1.3
frq$^+$,*oli*^{r} *16-16*	20.1	−1.4
frq-7,oli^s	29.0	+7.5
frq-7,oli^r *16-1*	26.6	+5.1
frq-7,oli^r *16-16*	26.3	+4.8
frq-1,oli^s	16.6	−4.9
frq-1,oli^r *16-1*	15.4	−6.1
frq-1,oli^r *16-16*	15.8	−5.7

and yield an average recombination frequency between *oli* and *frq* of about 2.1%, in good agreement with the results of the unselected crosses.

These crosses also allowed us to isolate a large number of *oli, frq* double mutants to determine whether there is any interaction between mutants at the two loci with respect to their effects on clock periodicity. The data summarized in Table 4 show that (1) the two *oli*^{r} mutations themselves (*i.e.*, with *frq*$^+$) shorten the period length by about 1–1.5 hr, as previously reported (DIECKMANN and BRODY 1980); and (2) the two *oli*^{r} mutations also shorten the period length of *frq-1* and *frq-7* by about the same amount and do not offer any indication of synergism between mutations at the two loci. Although there is a slightly greater shortening effect of *oli* in *frq-7* than in *frq-1*, this is within the range of variability seen among different isolates of a cross with varying genetic background (SARGENT and WOODWARD 1969; FELDMAN and HOYLE 1973).

Attempts at fine structure mapping of the *frq* locus: In an effort to construct a fine structure map of the *frq* locus, including *frq-9*, several four-point selected crosses were carried out using either *un-10* or *oli* as the proximal selectable marker and *for* as the distal marker. Since *un-10* and *for* are about 12 map units apart, scoring for clock periodicity only those progeny which were produced by a crossover between these two markers should increase the resolution of the analysis by a factor of 8. On the other hand, since *oli* and *for* are only 4 map units apart, scoring selected progeny recombinant for these two markers should increase the resolution by a factor of about 25. These results are summarized in Table 5. In crosses using *un-10*, no progeny with wild-type circadian period lengths were found among 529 selected progeny. This is equivalent to screening more than 4000 unselected progeny, and it suggests that recombination frequencies between the *frq* alleles used in these crosses are less than 0.025%. Although the phenotype of the double mutant is not predictable, no individual was found with a period length different from either of the parental types.

To further increase the resolution of this analysis, additional crosses were

TABLE 5

Recombination between *frq* alleles—four-point selected crosses

frq alleles in cross[a]	Total no. of selected progeny	No. of wild-type recombinants
A. Crosses using *un-10* and *for* as selected markers		
frq-7 × *frq-9*	319	0
frq-1 × *frq-3*	210	0
Total	529	0
B. Crosses using *oli* and *for* as selected markers		
frq-1 × *frq-2*	253	1
frq-1 × *frq-3*	384	0
frq-1 × *frq-7*	422	0
frq-2 × *frq-7*	74	0
frq-3 × *frq-7*	691	1
frq-3 × *frq-9*	247	0
frq-7 × *frq-9*	297	0
Total	2368	2

[a] For details of crosses and selection of progeny, see MATERIALS AND METHODS.

carried out using *oli* as the proximal outside marker. Two different schemes were used in these crosses. In one set the oli^s phenotype was selected by microscopic examination of germinating ascospores, since oli^s ascospores germinate more quickly than do those containing oli^r. In the second set, oli^r was selected by germinating the ascospores on medium containing oligomycin. In the first set, no wild-type or unique period lengths were found among 297 selected progeny (equivalent to more than 7000 unselected progeny). In the second set, two progeny with wild-type period lengths were isolated out of a total of 2368 selected progeny. Since this represents the equivalent of about 60,000 unselected progeny, one can calculate an average recombination frequency between *frq* alleles of about 0.003%. However, since several crosses yielded no recombinants and since only one recombinant was isolated in each of two crosses, it is not meaningful to calculate a map for the *frq* alleles. A preliminary report suggesting higher frequencies of recombination than those found here was based on the misidentification of heterokaryons as recombinants (FELDMAN and DUNLAP 1983).

Dominance relations of *frq-9*: The new *frq* mutant is recessive in forced heterokaryons with frq^+. Figure 1 shows data for heterokaryons tested on acetate-casamino acids medium at 25°, where *frq-9* does not exhibit conidial banding. It can be seen that, even in cases where *frq-9* was present in more than 90% of the nuclei, the heterokaryons expressed a clear rhythm with a period length equal to that of frq^+. These heterokaryons were also tested on glucose-arginine medium at 25°, where frq^+ has a period length of about 21.6 hr, and *frq-9* has a period length of about 18.2 hr. Figure 1 shows that once again *frq-9* is recessive to frq^+, with the wild-type period length expressed in

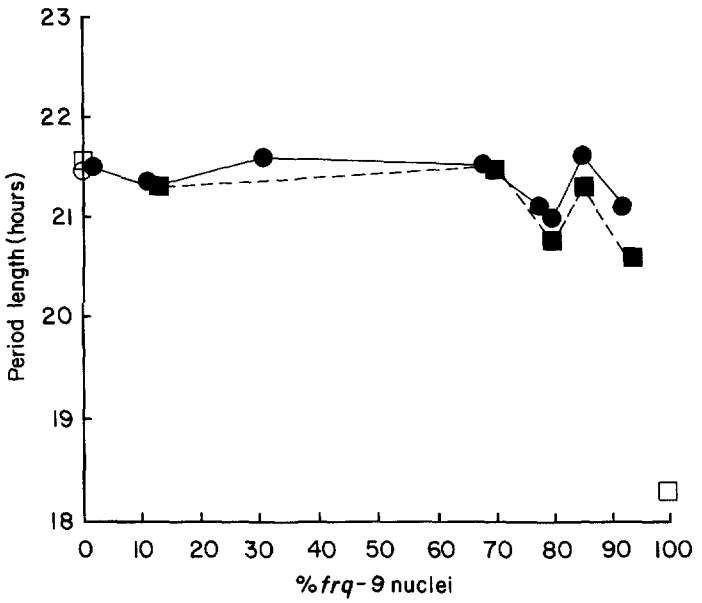

FIGURE 1.—Relationship between period length and nuclear ratio in *frq-9*/*frq*$^+$ heterokaryons. Open symbols represent the period lengths of the parent strains, and solid symbols represent the period lengths of the heterokaryons. ■ and □, glucose-arginine medium; ● and ○, acetate-casamino acids medium (Note: *frq-9* does not band on acetate-casamino acids medium).

heterokaryons containing more than 60% *frq-9* nuclei. Even in cases where 90% of the nuclei carry *frq-9*, only a slight shortening of the period was seen. Furthermore, when tested at 22°, no evidence of the temperature-dependent phenotype of *frq-9* was seen; that is, period lengths were the same as those at 25° (data not shown).

frq-9 is also recessive to four other *frq* alleles (Table 6). All of these have previously been shown to exhibit incomplete dominance to *frq*$^+$ and to each other (FELDMAN and HOYLE 1976; GARDNER and FELDMAN 1980). As in previous experiments, neither the *pan-2* nor the *inl* markers used to force the heterokaryons affected period length, since changing the coupling of these markers with the *frq* alleles had no effect on the period length of the heterokaryons (Tables 6 and 7).

Gene dosage effects of *frq-1* and *frq-7*: It had previously been shown that, in heterokaryons involving *frq-1* and *frq*$^+$, there was a gene dosage effect between the percentage of *frq-1* nuclei and the change in period length from the wild-type value (FELDMAN and HOYLE 1976). In that case the difference in period length between the two parental strains was about 5 hr. To emphasize the difference between *frq-9* and the other *frq* alleles and to obtain additional data concerning the gene dosage effects of the *frq* mutants which show incomplete dominance (*i.e.*, all except *frq-9*), heterokaryons were constructed between *frq-7* and *frq*$^+$, on the one hand, and *frq-7* and *frq-1*, on the other. The data in Table 7 confirm the incomplete dominance of *frq-7* previously reported

TABLE 6

Heterokaryons of *frq-9*

Genotype of heterokaryon[a]	Nuclear ratio	Temperature (°C)	Period length (hr)[b]		
			Parents		
			frq-9	*frq-X*[c]	Heterokaryon
(*frq-9,inl*) (*frq-1,pan*)	48/52	25	18.3	16.8	16.8
		22	22.0	16.1	16.9
(*frq-9,pan*) (*frq-1,inl*)	68/32	25	18.3	16.8	16.2
(*frq-9,inl*) (*frq-4,pan*)	76/24	25	18.3	19.1	18.7
		22	22.0	19.2	18.8
(*frq-9,pan*) (*frq-4,inl*)	62/38	25	18.3	19.1	18.9
		22	22.0	19.2	19.6
(*frq-9,inl*) (*frq-3,pan*)	64/36	25	18.3	24.1	23.9
		22	22.0	23.9	23.7
(*frq-9,pan*) (*frq-3,inl*)	52/48	25	18.3	24.1	23.8
		22	22.0	23.9	23.9
(*frq-9,inl*) (*frq-7,pan*)	73/27	25	18.3	28.4	28.3
		22	22.0	31.6	30.6
(*frq-9,pan*) (*frq-7,inl*)	56/44	25	18.3	28.4	28.1
		22	22.0	31.6	30.5

[a] *inl* = inositol-requiring; *pan* (pan-2) = pantothenate-requiring. Parentheses indicates markers within a single nucleus.

[b] Period length was measured on glucose-arginine medium.

[c] *frq-X* refers to the *frq* allele other than *frq-9*.

TABLE 7

Heterokaryons between *frq-7* and *frq*$^+$

Genotype of heterokaryon[a]	Nuclear ratio	Temperature (°C)	Observed[b] period length (hr)	Predicted[c] period length (hr)
(*frq*$^+$,*inl*) (*frq-7,pan*)	40/60	25	24.7	25.7
		22	25.4	27.5
(*frq*$^+$,*inl*) (*frq-7,pan*)	54/46	25	22.6	24.7
		22	24.5	26.1
(*frq*$^+$,*pan*) (*frq-7,inl*)	51/49	25	23.6	24.9
		22	24.2	26.4

[a] Symbols as in Table 6.

[b] Period length was measured on glucose-arginine medium.

[c] Predicted period length was calculated assuming a change in period length proportional to the nuclear ratio. Period length of *frq*$^+$ was 21.5 hr at both 22° and 25°. The period length of *frq-7* was 28.4 hr at 25° and 31.6 hr at 22°.

(GARDNER and FELDMAN 1980) and are consistent with a gene dosage effect on period length similar to that seen with *frq-1* (FELDMAN and HOYLE 1976), although heterokaryons with nuclear ratios that differed significantly from 50:50 were not generated. The gene dosage effects are illustrated even more

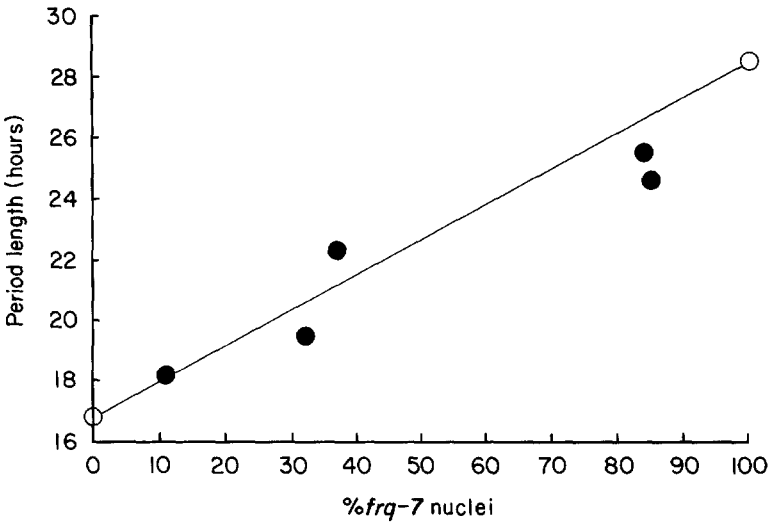

FIGURE 2.—Relationship between period length and nuclear ratio in *frq-7/frq-1* heterokaryons. Period lengths were measured on glucose-arginine medium at 25°. O, the period lengths of the parent strains; ●, the period lengths of the heterokaryons.

dramatically in the *frq-1/frq-7* heterokaryons, the period lengths of which are shown in Figure 2. Once again, as in the case of the *frq-1/frq*$^+$ heterokaryons, the change in period length from either parent is proportional to the percent of nuclei from the other parent.

DISCUSSION

A new circadian clock mutant of *N. crassa*, the most distinctive feature of which is the complete loss of temperature compensation of its period length, has been mapped to the *frq* locus on linkage group VIIR. The mutant has been designated *frq-9*.

Although seven other clock mutants previously isolated have been mapped to this locus (FELDMAN and HOYLE 1973, 1976; GARDNER and FELDMAN 1980), *frq-9* is unique in several respects. First, it is the only mutant to show complete loss of temperature compensation of its period length. *frq* mutants with period lengths that are shorter than wild type (*frq-1, frq-2, frq-4, frq-6*) exhibit normal temperature compensation, whereas *frq* mutants with period lengths that are longer than wild type (*frq-3, frq-7, frq-8*) exhibit only a partial loss of temperature compensation (GARDNER and FELDMAN 1981). Among the long period *frq* mutants, *frq-7* and *frq-8* have period lengths longer than *frq-3* and also show a greater loss of temperature compensation. It is possible, then, that a gradation in phenotype exists among long period mutants and that *frq-9* represents a more extreme mutation of this type than has previously been isolated.

Second, and consistent with the above suggestion, is the observation that *frq-9* is recessive to *frq*$^+$ and to each of the other *frq* mutants in heterokaryons. [It is important to note that the four mutants tested include two short-period

mutants and two long-period mutants and that they represent all four mutant phenotypes identified at the *frq* locus (the other three *frq* mutants duplicate the phenotypes of *frq-4* or *frq-7*).] This, too, is in contrast with the behavior of the other *frq* mutants, which show incomplete dominance with frq^+ and with each other. Since *frq-9* is recessive, it could represent a null mutation at this locus. The other long period *frq* mutations, with partial loss of temperature compensation, might represent mutations in which partial function of the gene was retained. In this regard, it is worth noting that *frq-9* was isolated after UV light mutagenesis, whereas each of the other *frq* mutations was induced with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine.

A previous question about the *frq* locus that we had raised, but not answered, was whether the *frq* locus contained one or several complementation groups (FELDMAN and HOYLE 1976). The question was difficult to approach because all of the mutants showed incomplete dominance, and results of complementation tests were therefore equivocal. With the availability of a recessive mutant, this question can once again be posed.

frq-9 is recessive to each of four other *frq* mutations. A "classical" interpretation of this result suggests two possibilities. It could be, of course, that all of the mutations fall within a single complementation group. Alternatively, the *frq* locus could include more than one complementation group, and *frq-9* could be a deletion that overlaps all of them. Although clustering of related genes is not as common in eukaryotes as in bacteria, there do exist gene clusters of related function in fungi, such as the *qa* cluster in Neurospora (CHALEFF 1974; CASE and GILES 1976), *gal* in *Saccharomyces cerevisiae* (DOUGLAS and HAWTHORNE 1964; BASSEL and MORTIMER 1971; OSHIMA 1982, for review) and the conidiation genes of *Aspergillus nidulans* (ZIMMERMAN *et al.* 1980; ORR and TIMBERLAKE 1982). Testing whether *frq-9* can revert to wild type would, theoretically, be one way to determine whether it is a deletion; however, this has not been feasible due to the absence of a selection method for wild-type revertants based on clock phenotype.

Another approach to analyzing the genetic organization of the *frq* locus was to try to establish a fine structure map. Again, the absence of a selection for rare recombinations based on clock phenotype means that one must screen the progeny directly. Previous experiments had failed to uncover any wild-type recombinants in pairwise crosses of *frq* mutants among more than 2000 progeny. Therefore, we used a method to increase the resolution of the crosses by selecting for recombinants between closely linked outside markers on either side of *frq* and then screening only those progeny which contained a crossover between those markers. In one set of crosses we used *un-10* (about 10 map units proximal to *frq*) and *for* (2 map units distal), and in the other set we used *oli* as the proximal marker, because it is only 2 map units from *frq*. Although we screened more than 2500 selected progeny, equivalent to more than 65,000 unselected progeny, we isolated only two wild-type recombinants and no obvious double mutants. Although such small numbers were insufficient to construct a fine structure map, they did clearly indicate the tight linkage between

the *frq* mutants and offer a preliminary estimate of the recombinational size of the locus. Using the two recombinants obtained here, we find that the *frq* mutants are within 0.003 map units of each other, a value certainly consistent with the notion that *frq* is a single gene. For example, for *ad-3A* and *ad-3B*, DESERRES (1969) estimated 0.06 map units per gene, whereas CASE and GILES (1958) found recombination frequencies between alleles of *pan-2* of 0.01–0.1 map units. Recent fine structure analysis of more than 50 alleles of the *am* locus (RAMBOSEK and KINSEY 1983) using both flanking marker and deletion mapping techniques showed that recombination frequencies between mutants mapping at opposite ends of the gene were about 0.04–0.07 map units.

The *frq* locus of Neurospora appears similar to a clock locus in *D. melanogaster* called *per*, where short-period (per^s), long-period (per^l), and arrhythmic (per^0) mutants have been isolated. As in Neurospora, most of the mutations at the *per* locus show incomplete dominance. Since all of these mutants were induced with ethylmethane sulfonate, it is likely that they represent point mutations and by classical analysis fall in the same complementation group. However, the recent cloning of DNA in the *per* region has allowed a molecular analysis of this region, and complementation analysis by *P* element-mediated transformation has revealed a degree of complexity not anticipated from the genetic studies (ZEHRING *et al.* 1984; HAMBLEN *et al.* 1986) and has also indicated a rather complex transcriptional pattern (BARGIELLO and YOUNG 1984; REDDY *et al.* 1984), the interpretation of which awaits further detailed analysis. It seems likely, then, that the final answers concerning the organization of the *frq* locus in Neurospora will also require a detailed molecular analysis of this region.

It has been suggested (DIECKMANN and BRODY 1980) that oli^r (oligomycin-resistant) mutations are allelic to the *frq* mutants, since some oli^r strains have period lengths 1–2 hr shorter than wild type and since *oli* and *frq* are closely linked. Since the *oli* gene codes for a subunit of mitochondrial ATPase (SEBALD, MACHLEIDT and WACHTER 1980), there was a further suggestion that *frq* also codes for this polypeptide or another subunit of the ATPase. However, our data show clearly that the two loci are separable and are about 2 map units apart. PERKINS and BARRY (1977) have estimated that there are about 25–40 kb of DNA per map unit in Neurospora. On that basis, *oli* and *frq* would be separated by about 50–80 kb of DNA, enough to code for 20–30 genes. Thus, it is unlikely that *oli* and *frq* code for the same polypeptide. Furthermore, since there is no evidence of interaction or synergism between *oli* and *frq* in double mutants, it also seems unlikely that they code for different subunits of the same protein. More recently BRODY, DIECKMANN and MIKOLAJCZYK (1985) have reached a similar conclusion about the distinction between *frq* and *oli* by showing that the *frq-1* and *frq-7* mutations do not affect the structure of the protein encoded by *oli*.

The loss of temperature compensation in *frq-9* supports our previous suggestions (GARDNER and FELDMAN 1981; DUNLAP and FELDMAN 1985), based on results with the other *frq* mutants, that this locus is intimately involved in

the mechanism of temperature compensation of the Neurospora clock. A specific role for the *frq* gene product can be incorporated into various models for temperature compensation which have been proposed. For example, in the theoretical "second-reaction inhibitor" model of HASTINGS and SWEENEY (1957), *frq* could code either for a fundamental clock protein that has lost sensitivity to the compensating inhibitor or for a protein that controls the compensating reaction itself. In the membrane model of NJUS, SULZMAN and HASTINGS (1974), in which membrane fluidity is maintained by varying the degree of saturation of the membrane lipids, *frq* might code for a protein that affects lipid synthesis. In this regard it has recently been found that *frq-9* is deficient in a b-type cytochrome in the endoplasmic reticulum that is involved in the desaturation of fatty acids (BORGESON 1985).

The availability of *frq-9* will be of value in determining the function of the *frq* locus, not only in direct biochemical studies, but also in the molecular isolation and analysis of the *frq* gene itself.

We thank R. METZENBERG and D. PERKINS for helpful discussions and advice, RICHARD DENISON for help in the original isolation of the mutant, and KAREN HUTCHINSON and JANICE WILLIAMS for constructing many of the strains used in these experiments. This research was supported by grant PCM-8117869 from the National Science Foundation.

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Communicating editor: R. L. METZENBERG