THE *frq* LOCUS IN *NEUROSPORA CRASSA*: A KEY ELEMENT IN CIRCADIAN CLOCK ORGANIZATION[1]

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ABSTRACT

Four new circadian clock mutants of *Neurospora crassa* have been isolated that alter the period length of the circadian conidiation rhythm. Three of these are at the *frq* locus on linkage group VIIR, where four other clock mutants are located. In contrast to wild type, which has a period length of 21.6 hr, *frq-6* has a period length of 19 hr, while *frq-7* and *frq-8* have period lengths of 29 hr and represent the largest effects of any single gene mutants on circadian periodicity. Thus, seven mutants have now been isolated that map to the *frq* locus, with period lengths ranging from 16.5 to 29 hr, and each mutant alters clock periodicity by an integral multiple of 2.5 hr. In addition, all *frq* mutants show incomplete dominance in heterokaryons. The large percentage of clock mutants that map to this locus, coupled with their unique properties, suggests that the *frq* locus plays an important role in clock organization.——The fourth mutant, designated chrono (*chr*), has a period length of 23.5 hr, shows incomplete dominance and is unlinked to either of the previously identified clock loci, *frq* or *prd* (formerly called *frq-5*). Double mutants between various combinations of clock mutants show additive effects and indicate no significant gene interaction among mutants at these three loci.

INTEREST in analyzing the cellular basis of circadian clocks has increased with the recent application of genetic techniques. In general, two complementary approaches have been used. On the one hand, mutants with altered circadian clock properties have been isolated in *Drosophila melanogaster* (KONOPKA and BENZER 1971), *Drosophila pseudoobscura* (PITTENDRIGH 1974), *Chlamydomonas reinhardi* (BRUCE 1972, 1974), and *Neurospora crassa* (FELDMAN and HOYLE 1973, 1976; FELDMAN and ATKINSON 1978). These mutations all affect nuclear genes and either alter the period length of the clock, or in one case, eliminate rhythmicity entirely.

In addition, a few Neurospora mutants with known biochemical lesions have been shown to have specific alterations in their circadian clock. For example, the mitochondrial mutant poky shows altered responses to visible light (BRAIN, WOODWARD and BRIGGS 1977), and cysteine auxotrophs alter their period length in response to exogenous sulfur concentration (FELDMAN and WIDELITZ 1977). In addition, the fatty acid auxotroph *cel* lengthens its period in response to un-

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saturated fatty acids (BRODY and MARTINS 1979), and oligomycin-resistant mutants have a shortened circadian period (DIECKMANN and BRODY 1980).

An interesting and unexpected observation has been the high percentage of clock mutants clustered at a single chromosomal region. In Neurospora, four of the five previously reported mutants are at the *frq* locus on linkage group VIIR. These *frq* mutants exhibit several interesting properties: (1) Each alters the period length of the clock by a specific number of hours, but the mutants differ from each other, with *frq-1* having a period length of 16.5 hr, *frq-2* and *frq-4*, 19 hr and *frq-3*, 24 hr, in contrast to frq^+, which has a period length of 21.6 hr. (2) All show incomplete dominance in heterocaryons with the frq^+ allele. In fact, in the case of *frq-1*, there is a gene-dosage effect in heterokaryons with different nuclear ratios (FELDMAN and HOYLE 1976). (3) The four *frq* mutants show normal growth and differentiation. The only phenotypic difference we have observed is the frequency of their circadian clock, observed as rhythmic conidial banding in cultures grown on race tubes (SARGENT, BRIGGS and WOODWARD 1966).

These properties show some similarities to the mutants obtained in Drosophila and Chlamydomonas. For example, in *D. melanogaster*, three of the five known mutants are clustered at the *per* locus on the *X* chromosome, and these three mutants have different phenotypes: short period, long period and arrhythmic. Furthermore, only arrhythmic is completely recessive (KONOPKA and BENZER 1971). In Chlamydomonas, although the four long-period mutants studied are unlinked to each other, only one of the three tested in diploids is recessive (BRUCE and BRUCE 1978).

The properties of the *frq* mutants indicate that this locus plays an important role in the organization and operation of the Neurospora clock and suggest that its further study may provide additional insight into clock mechanisms. This paper reports the isolation and characterization of four new clock mutants in Neurospora, three of which are at the *frq* locus, and summarizes our current knowledge of the unique properties of this genetic region.

MATERIALS AND METHODS

Strains: The following strains of *Neurospora crassa* were obtained from the Fungal Genetics Stock Center, Arcata, California: *bd, alcoy, chol-2, inl* (allele 37401) and *pan-2* (allele Y153M66). The mutants *bd frq-1, bd frq-2, bd frq-3* and *bd frq-4* were previously isolated in this laboratory (FELDMAN and HOYLE 1973, 1976). The mutant formerly designated *bd, frq-5* (FELDMAN and ATKINSON 1978) has been renamed *bd prd* (FELDMAN, GARDNER and DENISON 1979), since it is unlinked to the other *frq* mutants and is on linkage group IIIC. All strains used in these studies carry the *bd* mutation, which allows clear expression of the circadian conidial banding on race tubes (SARGENT, BRIGGS and WOODWARD 1966). The *bd* gene affects only the overt expression of the rhythm, not the underlying clock mechanism (SARGENT and WOODWARD 1969; FELDMAN and HOYLE 1973).

General procedures: Methods for maintaining stock cultures, carrying out crosses, mutagenesis with N-methyl-N′-nitro-N-nitrosoguanidine and mutant screening were all as previously described (FELDMAN and HOYLE 1973). Scoring for conidial banding on race tubes was also as previously described (FELDMAN and HOYLE 1973) except that data collection and processing were carried out using a digitizer (Bit Pad, Summagraphics Corp., Fairfield, Conn.), interfaced with

a Northstar Horizon Microcomputer and computer programs written in Basic. Construction and analysis of heterocaryons were done by standard procedures (DAVIS and DESERRES 1970) as adapted for circadian rhythm studies (FELDMAN and HOYLE 1976).

RESULTS

Mutants at the frq *locus:* Three new mutants have been isolated that map to the *frq* locus on linkage group VIIR. Each of these new mutants alters the "free-running" period length of the circadian conidiation rhythm from the wild-type value of 21.6 hr. These mutants have been designated *frq-6* (period length = 19.1 hr), *frq-7* (29 hr) and *frq-8* (29 hr). All three mutants segregate as single nuclear genes when backcrossed to frq^+. Figure 1 shows the distribution of progeny from a cross of *frq-7* × frq^+ and illustrates the 1:1 segregation among random spores. Single gene segregation was consistent with the 4:4 segregation observed in one ordered tetrad. Similar results were observed in crosses of *frq-6* × frq^+ (116 random spores, three ordered tetrads) and *frq-8* × frq^+ (82 random spores, three ordered tetrads).

As in the past, no wild-type recombinants were recovered in crosses between pairs of *frq* mutants. These crosses included *frq-6* × *frq-1* (30 random spores), *frq-6* × *frq-2* (31 random spores), *frq-6* × *frq-4* (24 random spores), *frq-7* × *frq-3* (84 random spores) and *frq-7* × *frq-8* (93 random spores). Including our previous studies (FELDMAN and HOYLE 1973, 1976), we have now examined more than 1,000 progeny of pairwise crosses between *frq* mutants without isolating any wild-type recombinants.

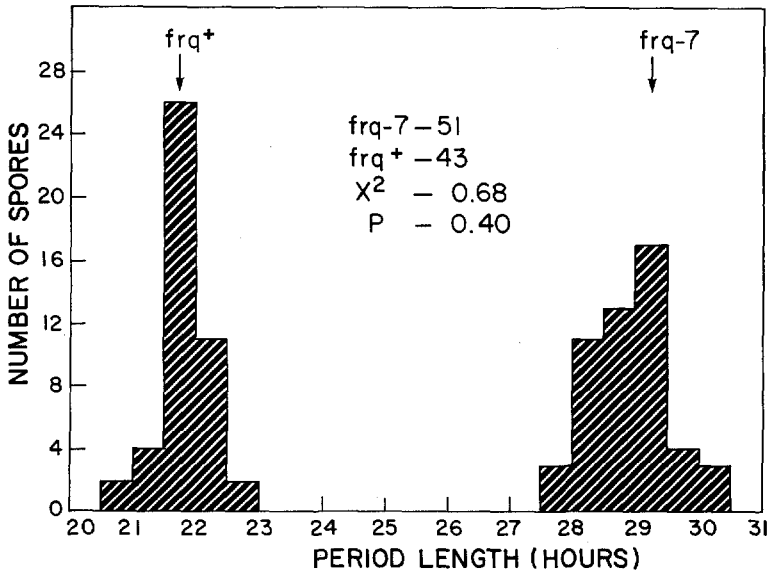

FIGURE 1.—Distribution of period lengths among progeny of a cross between *frq-7* and frq^+. Position of arrows indicates period lengths of parents. Mean period lengths of progeny were 21.6 ± 0.3 hr for frq^+, 28.6 ± 0.6 hr for *frq-7*.

Although *frq-7* and *frq-8* each have period lengths of about 29 hr, they were isolated in separate mutagenesis experiments and represent different isolates. *frq-6* is phenotypically identical with both *frq-2* and *frq-4*, although, again, all three of these mutants were isolated in separate mutagenesis experiments.

As an independent confirmation of the map location of *frq-6*, *frq-7* and *frq-8*, each was crossed to the double mutant *nic-3, met-7*. All three mutants showed linkage to these markers of linkage group VII in the map order *nic-3–met-7–frq*, with recombination frequencies of 10 to 15% between *met-7* and *frq*.

Identification of a new clock locus: A new mutant with a period length of 23.5 hr has been designated chrono (*chr*) and maps to the left arm of linkage group VI. A three-point cross of *chol-2, chr*$^+$, *pan-2*$^+$ × *chol-2*$^+$, *chr, pan-2* yielded the gene order *chol-2–chr–pan-2*, with a recombination frequency of about 10% between *chol-2* and *chr*. *chr* shows single-gene segregation in random spore analysis (Figure 2) and this result was confirmed in four ordered tetrads.

Dominance relationships of the mutants: Each of the new *frq* mutants shows incomplete dominance in heterocaryons with *frq*$^+$. In all cases, heterocaryons had period lengths that were intermediate between mutant and wild type (Table 1); in this respect they are similar to the other *frq* mutants. We have previously shown (FELDMAN and HOYLE 1976) that the change in period length in *frq-1/frq*$^+$heterocaryons was proportional to the fraction of *frq-1* nuclei; *i.e.*, there was a gene dosage effect of the *frq-1* mutation over a wide range of nuclear ratios.

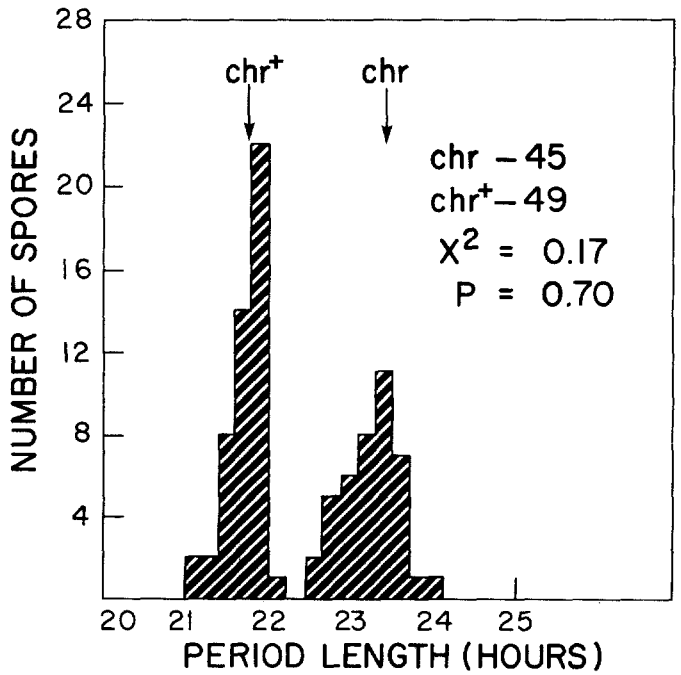

FIGURE 2.—Distribution of period lengths among progeny of a cross between *chr* and *chr*+. Position of arrows indicates period lengths of parents. Mean period lengths of progeny were 21.5 ± 0.2 for *chr*+, 23.2 ± 0.3 for *chr*.

TABLE 1

Incomplete dominance of clock mutants in heterokaryons

Genotype	Nuclear ratio	Observed period length*	Predicted period length†
frq-6, pan-2; *frq*+, *inos*	52:48	20.0 ± 0.4 (8) hr	20.3 hr
frq-7, inos; *frq*+, *pan-2*	48:52	24.4 ± 0.7 (6)	25.1
frq-8, inos; *frq*+, *pan-2*	25:75	23.8 ± 0.7 (6)	23.4
chr, inos; *chr*+, *pan-2*	68:32	22.7 ± 0.6 (5)	22.7

* Values in hours ± S.D. Numbers in parentheses indicate number of replicate race tube cultures examined.

† Assuming a linear gene dosage model. Period lengths of parents: *frq-6*, 19 hr; *frq-7*, 29 hr; *frq-8*, 29 hr; *chr*, 23.5 hr; wild type, 21.6 hr.

The expected period lengths for the *frq-6*, *frq-7* and *frq-8* heterocaryons based on a similar gene-dosage model are listed in Table 1; there is reasonably good agreement between observed and predicted values, although there is only one nuclear ratio value for each heterocaryon.

Table 1 also shows that *chr* is incompletely dominant to *chr*+ and that the predicted period length of the heterocaryon is close to the observed value. However, the relatively small change in period length caused by the *chr* mutation makes it doubtful that any quantitative predictions about the gene dosage would be statistically significant.

Phenotypes of multiple mutants: In order to study possible interactions among clock mutants in different genes, double mutants were constructed using the *frq*, *chr* and *prd* mutations. As a working hypothesis, we assume that if the effects of the two mutations are additive, the genes affect different components of the circadian system; if the effects are not additive, the genes or gene products may affect the same process or interact with each other in some other way.

The double mutants were identified from ordered tetrads, either tetratype or nonparental ditype asci. Examples of double mutants involving *chr* are shown in Table 2, which contains ordered tetrad data for the crosses of *frq-1* × *chr*,

TABLE 2

Identification of frq chr *double mutants in tetratype asci*

Spore	Period lengths of progeny*		
	frq-1 × *chr*	*frq-3* × *chr*	*frq-7* × *chr*
1	23.2 hr	23.3	32.8†
2	23.2	22.9	32.5†
3	—	26.5†	27.5
4	16.4	26.3†	28.9
5	—	23.8	23.8
6	17.1†	23.9	23.8
7	21.7	21.7	21.8
8	22.0	—	21.3

* Period lengths of parent strains: *frq-1*, 16.5 hr; *frq-3*, 24.0 hr; *frq-7*, 29.0 hr; *chr*, 23.5 hr. Wild type is 21.6 hr.

† Identifies double mutants in each tetrad.

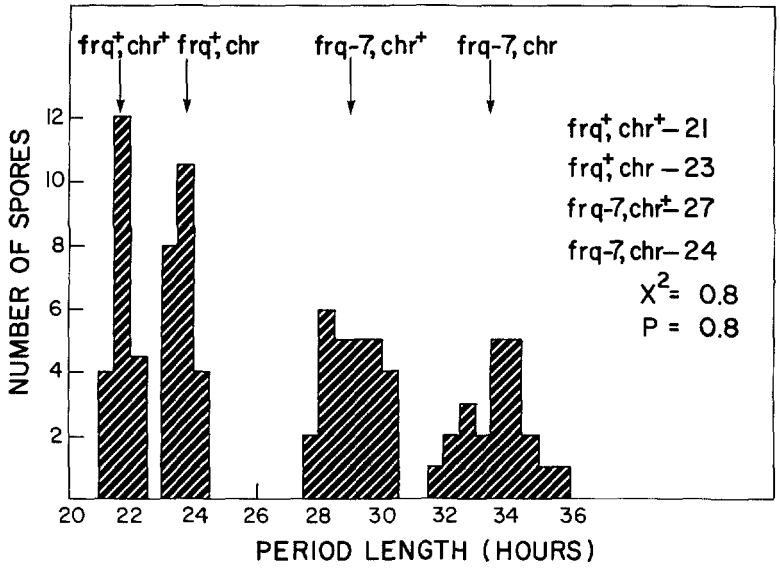

FIGURE 3.—Distribution of period lengths among progeny of a cross between *frq-7 chr*+ and *frq*+ *chr*. Position of arrows indicates the mean period lengths of the progeny in each of the genotypic classes. These values were as follows: *frq*+ *chr*+, 21.5 ± 0.2; *frq*+ *chr*, 23.2 ± 0.6; *frq-7 chr*+, 29.0 ± 0.8; *frq-7 chr*, 33.3 ± 1.0.

frq-3 × *chr* and *frq-7* × *chr*. Each of the four genotypic classes can be identified from these tetratype asci. Additional data needed to determine accurately the period lengths of the double mutants were obtained from random spore analysis of each of these crosses. Figure 3 shows the segregation of random spores for the cross *frq-7* × *chr* and the identification of each of the four genotypic classes. The period lengths of the *frq-7 chr* double mutants ranged from 30.8 to 35.2 hr, with a mean of 33.2 hr. In a similar way, identification of the double mutants *frq-1 chr* and *frq-3 chr* were made from random spores.

Table 3 contains ordered tetrad data for *chr* × *prd* and *frq-7* × *prd*. In the

TABLE 3

Identification of chr prd *and* frq-7 prd *double mutants in ordered tetrads*

Spore	Period lengths of progeny	
	chr × *prd**	*frq-7* × *prd**
1	25.9 hr	21.0 hr
2	25.4	21.4
3	27.7†	21.0
4	27.2†	21.3
5	23.4	32.9†
6	23.2	31.5†
7	21.7	33.2†
8	21.9	—

* Period lengths of parents: *chr*, 23.5 hr; *prd*, 26.0 hr; *frq-7*, 29.0 hr. Wild type is 21.6 hr.
† Identifies double mutants.

TABLE 4

Summary of period length of double mutants

Strain	Observed period length*	Predicted period length†
frq-1 prd‡	19.3 ± 0.4 (8)	20.7 hr
frq-2 prd‡	22.8 ± 0.1 (5)	23.5
frq-3 prd‡	28.5 ± 0.5 (6)	28.2
frq-7 prd	34.5 ± 1.0 (8)	32.1
frq-1 chr	17.0 ± 0.2 (8)	18.5
frq-3 chr	26.0 ± 0.8 (4)	26.0
frq-7 chr	33.2 ± 1.0 (55)	30.7
chr prd	28.4 ± 0.3 (10)	28.7

* Observed period lengths in hours ± S.D. (numbers of spores examined).
† Assuming additivity of period length changes. Short-period mutants (*frq-1,2*) cause a negative change in period length, while long period mutants (*frq-3,7*, *chr*, *prd*) cause a positive change.
‡ From FELDMAN and ATKINSON 1978.

chr × *prd* cross, the double mutant was identified from the one tetratype ascus obtained, while in the cross of *frq-7* × *prd*, a nonparental ditype ascus was used, since no complete tetratype asci were obtained due to poor germination of the spores. In both of these crosses, strains carrying the *prd* mutation could be identified independently by their slow growth rate (see FELDMAN and ATKINSON 1978). In addition, double mutants were also identified from random spore analysis as described above. As a final confirmation of the presence of both mutant alleles in the double mutants, several presumed double-mutant progeny were backcrossed to wild type, including two isolates of *frq-7 chr*, and one each of *frq-7 prd* and *chr prd*. In all cases, each of the single mutants was recovered.

Table 4 summarizes the double mutant results from this and one previous study (FELDMAN and ATKINSON 1978). In all cases, the multiple mutants showed cumulative effects. For example, double mutants consisting of two long-period mutants had period lengths longer than either of the two single mutants, while a long- and a short-period mutation gave a double mutant with an intermediate period length. In fact, in most cases, the period length change in the double mutant is the simple additive effect of each of the single mutants. However, in the two double mutants involving *frq-7*, the period length of the double mutant is several hours greater than that predicted from a simple additive model, but the long period and wide range of period lengths among these double mutants make it difficult to conclude that these differences are significant.

DISCUSSION

The three new *frq* mutants analyzed in this paper bring to seven the number of independently isolated circadian clock mutants that map to this locus. Thus, seven of the nine Neurospora clock mutants isolated after nitrosoguanidine mutagenesis are *frq* mutants. It is interesting that experiments using other mutagens, such as UV, ethyl methanesulfonate and diepoxyoctane, have failed to

yield any *frq* mutants, although mutations at other loci that alter clock periodicity have been isolated (FELDMAN, GARDNER and DENISON 1979; and unpublished results). Nitrosoguanidine is believed to act on DNA in growing cells primarily at replicating forks (CERDA-OLMEDO, HANAWALT and GUEROLA 1968), but since little or no DNA synthesis occurs during the first hour of conidial germination (SERNA and STADLER 1978), the significance of this fact is not clear. Since it is also known that nitrosoguanidine induces multiple, closely linked mutations in some stationary phase cells (BOTSTEIN and JONES 1969), *frq* mutations might require two or more closely linked "hits" that would not be detected without fine-structure analysis of this region.

All seven mutants have several properties in common: they show incomplete dominance, they have normal growth and development (GARDNER, unpublished data) and each alters the free-running period of the circadian clock by a reproducible and discrete amount. In fact, examination of the period lengths of the *frq* mutants—16.5, 19, 24 and 29 hr—reveals that each differs from the wild-type value of 21.6 hr by approximately 2.5, 5.0, or 7.5 hr. Thus, period length alterations at the *frq* locus are not random and appear to effect a 2.5-hr quantum element in clock organization. It may be that such an element can be repeated a variable number of times during each circadian cycle and that the *frq* locus controls the number of times it occurs.

It is also interesting that both fast and slow mutations arise with about equal frequency, since it is somewhat unusual to find both "up" and "down" mutations occurring with equal frequency in a gene. Since the mutations effect a quantum change in period length and since there is a gene dosage effect in heterokaryons, it could be that in the mutants the number of copies of the *frq* locus is altered and that each copy of the gene is somehow responsible for one 2.5-hr segment. Indeed, in *Drosophila melanogaster*, changing the number of copies of the wild-type allele at the *per* locus does alter clock periodicity, with each extra copy shortening the period by about 0.5 hr (R. KONOPKA, personal communication).

An interesting finding is the recent observation (DIECKMANN and BRODY 1980) that oligomycin-resistant (*oli-r*) mutants map close to the *frq* locus and have a shortened circadian period length. The tight linkage to *frq* has led the authors to suggest that *frq* and *oli* may be allelic. If so, that would focus attention on the role of mitochondrial ATPase, since the *oli* gene is believed to code for one of the subunits of that protein (SEBALD, GRAF and LUKINS 1979).

The *chr* mutation with a 23.5 hr clock represents the third locus that affects clock periodicity, unlinked to those previously identified—*frq* and *prd* (formerly called *frq-5*). The *chr* mutation also shows incomplete dominance and adds to the unusual result that only a small fraction of mutants affecting clock periodicity in all three organisms studied—Drosophila, Neurospora and Chlamydomonas—are recessive. The interpretation of incomplete dominance has been discussed previously (FELDMAN and HOYLE 1976; FELDMAN and ATKINSON 1978), and we have no new insights to add at this time.

The availability of *chr* has increased the possibilities for examining gene interaction among different clock loci. The data in Table 4 show that in double mutants all clock mutations examined have cumulative effects on period length, and in most cases the period length of the double mutant is close to the value predicted by an additive, noninteracting model of gene function. There are a few cases where the additive prediction is one or two hours shorter or longer than the observed value, but these small differences are often within the limits of variability of the system, and no obvious pattern of gene interaction has emerged. These results are similar to those observed by BRUCE (1974), who found additivity among four clock mutants in Chlamydomonas in multiple mutants with period lengths as long as 36 to 38 hr.

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