COMPLEMENTATION ANALYSIS OF LINKED CIRCADIAN CLOCK MUTANTS OF *NEUROSPORA CRASSA*[1]

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ABSTRACT

A fourth mutant of *Neurospora crassa*, designated *frq-4*, has been isolated in which the period length of the circadian conidiation rhythm is shortened to 19.3 ± 0.3 hours. This mutant is tightly linked to the three previously isolated *frq* mutants, and all four map to the right arm of linkage group VII about 10 map units from the centromere. Complementation tests suggest, but do not prove, that all four mutations are allelic, since each of the four mutants is co-dominant with the *frq*+ allele—i.e., heterokaryons have period lengths intermediate between the mutant and wild-type—and since heterokaryons between pairs of mutants also have period lengths intermediate between those of the two mutants.

GENETIC analysis as a tool for dissecting biological clock mechanisms has recently been initiated in several organisms. In Drosophila two mutants with altered periodicities and one arrhythmic mutant all map to the same locus, and all three appear to be allelic (KONOPKA and BENZER 1971). In Neurospora three circadian clock mutants (two with short periods and one with a long period) are also tightly linked (FELDMAN and HOYLE 1973). On the other hand, four long-period mutants in Chlamydomonas map to separate loci and their effects on period lengthening are additive (BRUCE 1972, 1974). For the genetics of the Neurospora clock system to be of significant use in gaining insight into and testing molecular mechanisms, additional mutants and types of genetic studies are obviously necessary. This paper reports the isolation of an additional linked clock mutant in Neurospora and describes the behavior of all four linked mutants in heterokaryons.

MATERIALS AND METHODS

Strains: The following mutant strains with altered circadian clock periodicities were previously isolated in this laboratory (FELDMAN and HOYLE 1973)—*bd, frq-1* (period length (τ) = 16.5 hours); *bd, frq-2* (τ = 19.3 hours); *bd, frq-3* (τ = 24.2 hours). The wild-type *bd,*

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frq+ ($\tau = 21.6$ hours) and the other strains used—*nic-3*, *met-7*; *thi-3*, *pan-2* (allele Y153M66); and *inos* (allele 37401)—were obtained from the Fungal Genetics Stock Center, Humboldt State College, Arcata, California.

General procedures: Methods for maintaining stock cultures, carrying out crosses, scoring for the conidial banding rhythm on "race" tubes, mutagenesis, and mutant screening were all as previously described (FELDMAN and HOYLE 1973).

Heterokaryon analysis: Two markers, *inos* and *pan-2*, were used to construct forced heterokaryons. Each of these strains was crossed to *bd frq*+ and to each of the *bd frq* mutants in order to obtain two sets of marked strains, all heterokaryon-compatible with each other. Heterokaryons were then constructed by mixing conidia of two appropriate strains in minimal medium according to standard procedures (DAVIS and DE SERRES 1970). To obtain heterokaryons of varying nuclear ratios, conidia were mixed in ratios ranging from 1:100 to 100:1. Hyphal tips were isolated in all cases to eliminate the possibility of cross feeding. Stock cultures which grew up from the hyphal tip isolates were maintained on slants of VOGEL's (1964) minimal sucrose medium and were used for all further analysis. To determine the period length of the heterokaryons, cultures were grown on race tubes as previously described. It was confirmed that none of the marked strains used to construct the heterokaryons grew on the race tubes.

Nuclear ratios were determined using the formula of DAVIS and DE SERRES (1970, p. 121) after plating conidia on VOGEL's minimal sucrose medium either unsupplemented or supplemented with 0.1 mg/ml inositol or 0.01 mg/ml calcium pantothenate or both. In order to prevent growth of the *pan-2* colonies on unsupplemented plates, it was necessary to pre-wash the agar three times by suspending in distilled water, stirring for 30 minutes and decanting most of the water each time (suggested by D. STADLER). To ensure that the nuclear ratios did not change during growth on the race tubes, at the end of an experiment mycelium was collected from the "distal" end of the tube and transferred to slants of the same medium as in the race tubes (1.2% sodium acetate, 0.5% Difco casamino acids, VOGEL's salts). When these cultures grew up they were again inoculated onto race tubes to determine period lengths and plated to determine nuclear ratios. Data are reported only for those cases in which two successive determinations of nuclear ratios agreed within 10% and period lengths within 0.5 hours, since nuclear ratios often changed as much as 30%–40% between the first and second determination. Further analysis of this phenomenon has not been carried out.

Characterization of frq-4: A fourth circadian clock mutant *frq-4*, isolated independently from the three previously reported mutants after mutagenesis of the band strain, has a period length of 19.3 ± 0.5 hours, about 2½ hours shorter than the wild-type *frq*+. As in previous studies all *frq* strains also carry the *bd* (band) gene unless otherwise noted, since the latter facilitates clear expression of conidial banding. In crosses to *frq*+, *frq-4* segregated as a single nuclear gene among 6 asci and 71 random spores (Figure 1). *Frq-4* also shows normal growth rates, as do *frq-1*, *-2*, and *-3*, on VOGEL's minimal medium with sucrose or acetate as carbon source or on HOROWITZ (1947) complete medium.

Linkage relationship of frq-4: *Frq-4* is located in the same region as *frq-1*, *-2*, and *-3*. In crosses of *frq-4* × *frq-1* and *frq-4* × *frq-2*, no wild-type recombinants were recovered among a total of 29 and 38 random spores, respectively. In the case of *frq-4* (19.3 hours) × *frq-2* (19.3 hours), there was a unimodal distribution of period lengths among the progeny (Figure 2), while for *frq-4* (19.3 hours) × *frq-1* (16.5 hours) the period lengths of the progeny followed a bimodal distribution with about half the progeny having the *frq-1* period length and half that of *frq-4* (Figure 3). Although these numbers are not large, they bring to

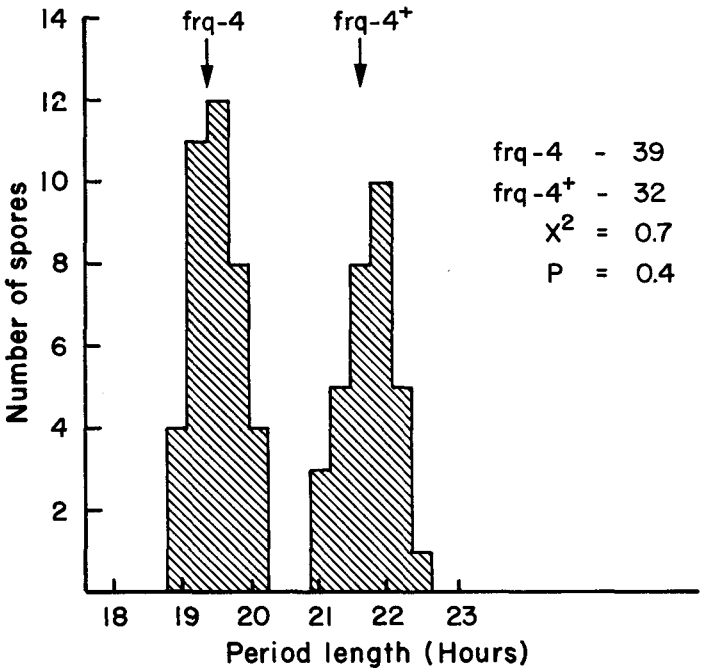

FIGURE 1.—Distribution of period lengths among progeny of a cross between *bd frq-4* and *bd frq-4+*. Position of arrows indicates period lengths of parents. The mean period length for the *frq-4* progeny was 19.4 ± 0.3 hours; for the *frq-4+* progeny, 21.7 ± 0.4 hours.

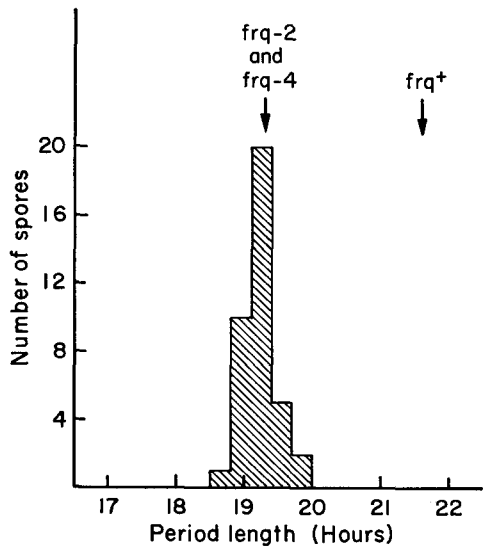

FIGURE 2.—Distribution of period lengths among progeny of a cross between *bd frq-2* and *bd frq-4*. Position of arrows indicates lengths of parents or wild type. The mean period length of the progeny was 19.4 ± 0.3 hours.

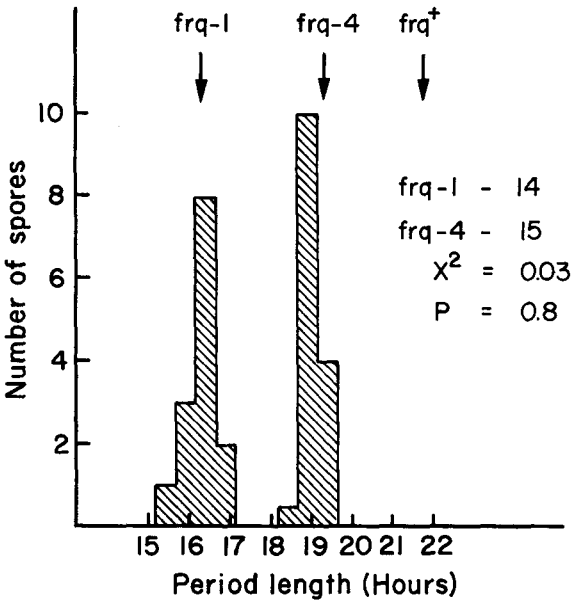

FIGURE 3.—Distribution of period lengths among progeny of a cross between *bd frq-1* and *bd frq-4*. Position of arrows indicates period lengths of parents or of wild type. The mean period length for the *frq-1* progeny was 16.3 ± 0.4 hours; for the *frq-4* progeny, 19.0 ± 0.3 hours.

more than 600 the total number of progeny of pairwise crosses among the four mutants we have examined with no wild-type recombinants observed.

Mapping of the frq *mutants*: Each of the four mutants has been mapped by a 3- or 4- point cross to the right arm of linkage group VII approximately 10 map units from the centromere (Table 1).

TABLE 1

Mapping of frq *mutants by three- or four- point crosses*

Zygote genotype and percent recombination	Parentals	Numbers of progeny: Single crossovers R1	R2	R3	Double crossovers R1&2	R1&3	R2&3
+ *thi-3* + *frq-1*	41	10	0	6	0	0	0
nic-3 + *met-7* +	40	13	1	3	0	2	0
22 1 9							
+ *thi-3* + *frq-2*	34	6	1	5	0	1	2
nic-3 + *met-7* +	21	6	1	2	0	0	0
18 4 13							
+ + *frq-3*	18	8	3	—	0	—	—
nic-3 *met-7* +	11	8	2	—	0	—	—
32 10							
+ + *frq-4*	26	3	3	—	4	—	—
nic-3 *met-7* +	26	17	3	—	1	—	—
38 12							

Standard conventions are followed: The top number of each pair of complementary classes represents progeny carrying the *nic-3*+ allele. Regions (R) are numbered from left to right.

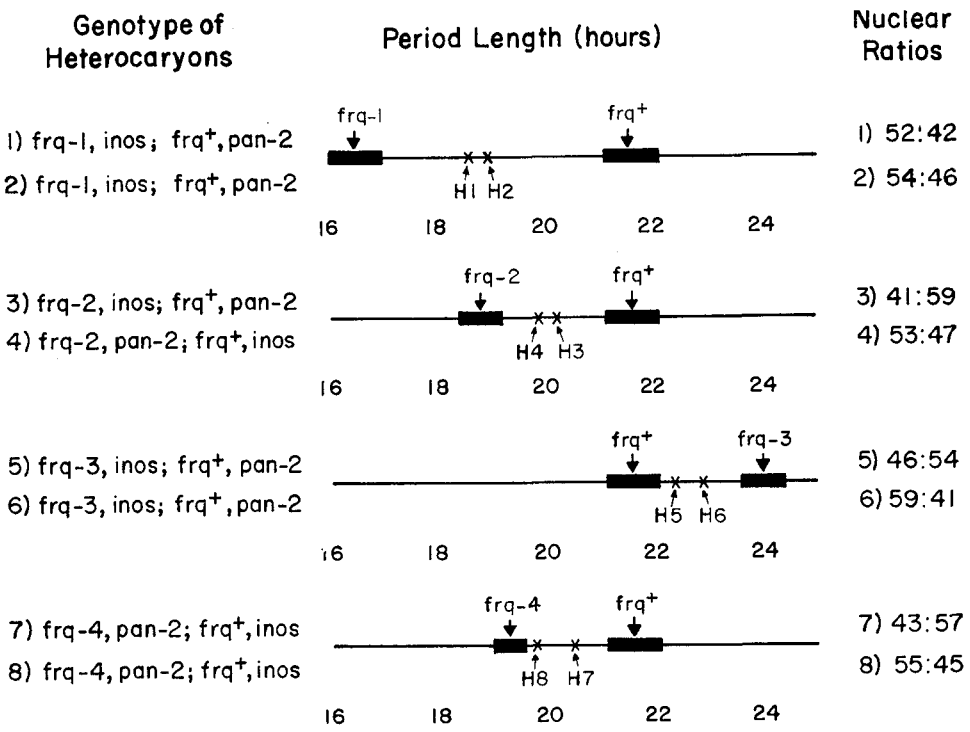

FIGURE 4.—Co-dominance of *frq* mutants with wild type. The solid bars include the mean ± std. dev. of period length for the parents in the heterokaryons. The *X*'s indicate the period length of the particular heterokaryon (H) whose genotype is given to the left of the diagram and whose nuclear ratio is given to the right.

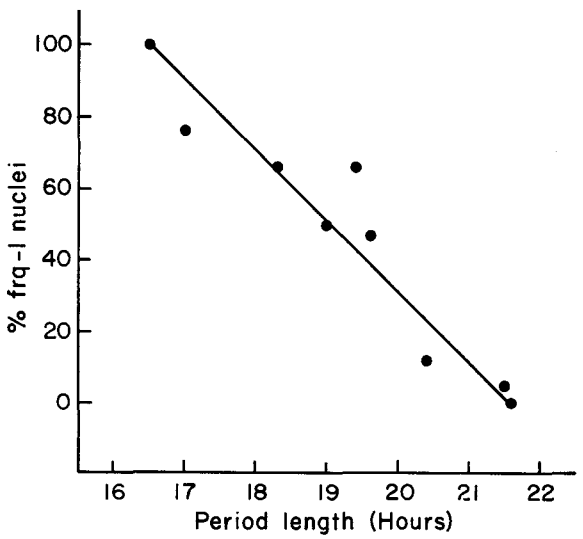

FIGURE 5.—Relationship between period length and nuclear ratio in *frq-1/frq+* heterokaryons.

Dominance relationships of the frq *mutants*: Each of the four *frq* mutants is co-dominant with the *frq*$^+$ allele in heterokaryons. This is evident from Figure 4, which shows that the period lengths of heterokaryons containing a *frq* mutation in one nucleus and *frq*$^+$ in the other are intermediate between the two parental strains; in fact, with nuclear ratios close to 1:1, the period length of the heterokaryons are approximately equal to the average period length of the parents. In

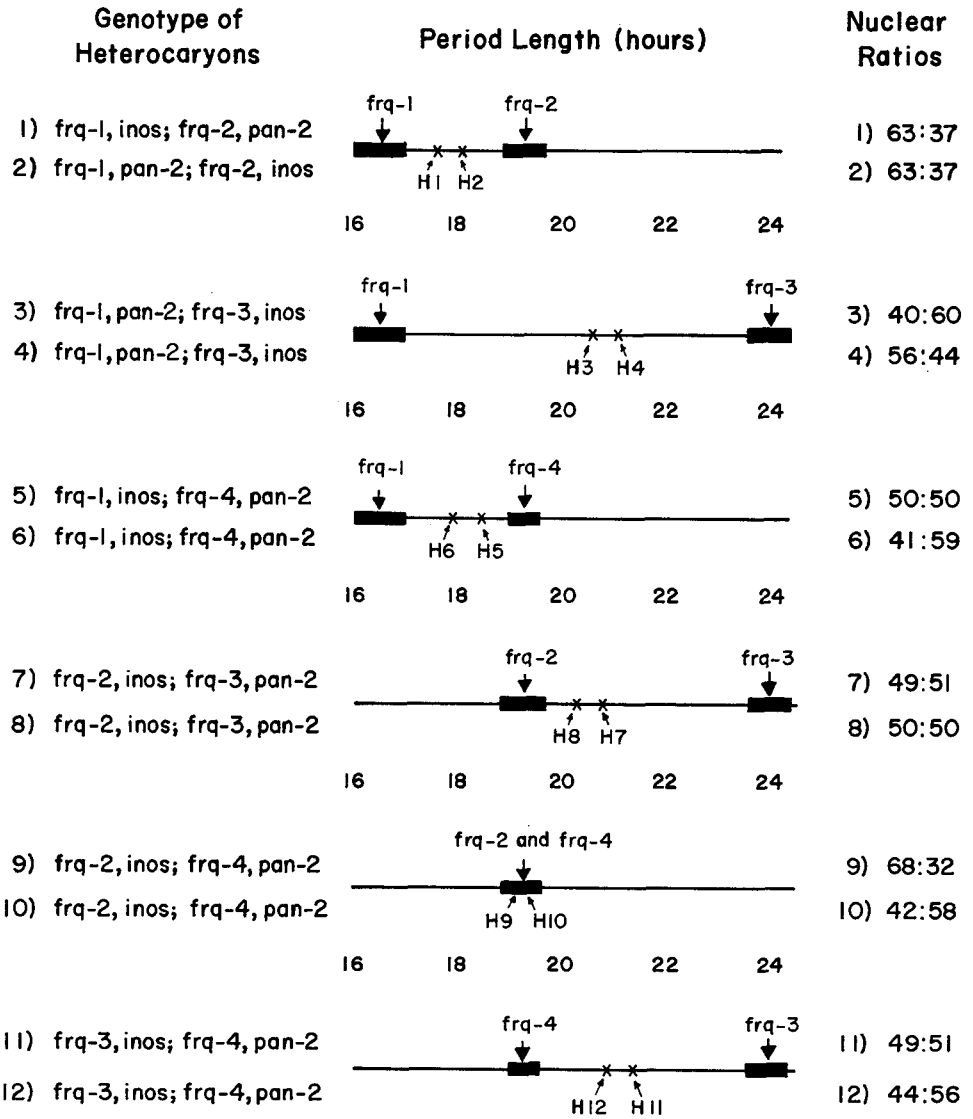

FIGURE 6.—Complementation tests between *frq* mutants. Solid bars include the mean ± std. dev. of period length for each of the parents in the heterokaryon. The *X*'s indicate the period length of the particular heterokaryon (H) whose genotype is given to the left of the diagram and whose nuclear ratio is given to the right.

addition, in the case of *frq-1/frq*$^+$, heterokaryons were constructed with varying nuclear ratios; there was an approximately linear relationship between period length and nuclear ratio (Figure 5).

Complementation tests of the frq *mutants*: Each of the four *frq* mutants is co-dominant with each of the other mutants. In heterokaryons in which one type of nucleus contained one *frq* mutant and the other type another *frq* mutant, the period length of the heterokaryon was intermediate between the two parental periodicities (Figure 6).

DISCUSSION

There are now four independently isolated mutants of *Neurospora crassa* which alter the period length of the ciraadian clock and which are located in a region of the genome probably less than 0.5 map units long. Since we do not yet have a rapid technique for scoring period length nor a procedure for selecting rare recombinants in crosses between mutants, fine structure mapping of this region has not yet been carried out. All four mutants appear to have normal characteristics of growth and development—i.e., the only altered phenotype we have so far detected is the altered circadian periodicity. Since *frq-2* and *frq-4* have the same period lengths, they may represent independent but identical mutational events.

Tight linkage among the Neurospora mutants is similar to the case of the three linked clock mutants in Drosophila (KONOPKA and BENZER 1971) but is in striking contrast to the situation in Chlamydomonas, where four long-period mutants are all unlinked (BRUCE (1974). It is presently unclear whether this difference represents a fundamental difference in clock mechanisms or an idiosyncracy of the selection and screening procedures used to isolate the mutants. However, the latter seems more likely, since in our original screening for Neurospora mutants we purposefully ignored strains in which scoring of conidial banding was difficult and as a result would have omitted any mutants in which conidiation was inhibited or in which growth was severely restricted. Indeed, we have recently isolated a new mutants which is unlinked to the four described in this paper and which does have a significant alteration in its growth rate (FELDMAN and ATKINSON 1975).

The complementation tests, designed to test whether the mutants are allelic, have given somewhat equivocal results. Usually such analysis is carried out with recessive mutants and complementation is said to occur if the *trans* heterokaryon shows the wild-type phenotype. In our case none of the mutants is recessive to wild type; each is co-dominant with the *frq*$^+$ allele such that the period length of the heterokaryon is intermediate between mutant and wild type. While this in itself is an interesting result, it complicates interpretation of the complementation tests, in which the period length of heterokaryons between the mutants was also intermediate between the two parents. Complementation tests can be carried out with mutants that are not recessive by comparing the *cis* heterokaryon with the *trans* heterokaryon (LEWIS 1963). However, since we

have been unable to construct the *cis* heterokaryons, we cannot draw any definitive conclusions as to whether the mutants are all in the same cistron.

Complementation tests with the three Drosophila clock mutants were also somewhat unclear (KONOPKA and BENZER 1971). Both the arrhythmic and long-period (28.6 hours) mutants appeared recessive to wild type (24.4 hours), since heterozygotes of each had period lengths (25.2–25.5 hours) close to wild type. On the other hand, the heterozygote between the short period mutant (19.5 hours) and wild type had an intermediate period length (21.9 hours). Heterozygotes between the long and short period mutants had an intermediate (22.9 hour) period length. Since, as pointed out by the authors, this intermediate value is close to the wild-type period length, and since the *cis* heterozygotes were not analyzed, again it could not be determined for certain that the mutants are allelic, although that seemed the most likely possibility.

It is striking that until now, in both Neurospora and Drosophila, all mutations which alter clock periodicity without causing other obvious abnormalities map to the same locus. While this does not imply that circadian clocks are under the control of a single gene, it does suggest that only a relatively small number of gene functions affecting the clock can be altered without significantly affecting the growth and development of the organism. It also suggests that the identification of the function of such a gene would be an important step in understanding molecular mechanisms underlying the clock.

The co-dominance of the *frq* mutants suggests that each *frq* allele is producing a functional but quantitatively altered gene product. While incomplete dominance does occur in some cases between wild type and auxotrophic mutant alleles, maximal growth rates of these heterokaryons are usually maintained unless the wild-type allele is present in fewer than 5% of the nuclei (PITTENGER and ATWOOD 1956). It is conceivable that mutations in structural genes resulting in altered but functional gene products would behave as co-dominants in heterokaryons. However, the most carefully documented examples of co-dominant mutations in Neurospora are mutations in known regulatory genes. For example, in the regulation of quinic acid degradation certain constitutive *qa-1*^{c} mutants are semi-constitutive in heterokaryons (VALONE, CASE and GILES 1971), while in the case of the *UW-6* mutant, which regulates the synthesis of alkaline phosphatase, it has even been shown that the fractional derepression of the enzyme in the *UW-6/UW-6*$^{+}$ heterokaryons corresponds with the observed nuclear ratios (LEHMAN *et al.* 1973). This latter result is quite similar to our results with *frq-1/frq*$^{+}$ heterokaryons in which period lengths correspond with nuclear ratios. It is tempting to speculate that the *frq* locus regulates the level of some gene product(s) important for clock function and that mutations in the *frq* locus that alter these levels produce changes in the oscillation frequency of the clock.

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