ISOLATION OF CIRCADIAN CLOCK MUTANTS OF *NEUROSPORA CRASSA*[1]

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ABSTRACT

Three mutants of *Neurospora crassa* have been isolated which have altered period lengths of their circadian rhythm of conidiation. The strains, designated "frequency" (*frq*), were obtained after mutagenesis of the band (*bd*) strain with N-methyl-N'-nitro-N-nitrosoguanidine. In continuous darkness at 25° *bd* has a period length of 21.6 ± 0.5 hours; under the same conditions the period length of *frq-1* is 16.5 ± 0.5 hours; *frq-2*, 19.3 ± 0.4 hours; and *frq-3*, 24.0 ± 0.4 hours. Each of the mutants segregates as a single nuclear gene. All three mutants appear very tightly linked to each other, but it has not yet been determined whether the mutants are allelic. No major changes in the responses to light and temperature have been observed in any of the mutants. It is suggested that these mutants represent alterations in the basic timing mechanism of the circadian clock of Neurospora.

CIRCADIAN rhythms have now been observed in nearly all phyla of eukaryotes, including microorganisms such as algae, fungi, and protozoa. Recent interest has developed concerning the molecular basis of circadian rhythmicity, but the apparent complexity of the biological clock (PITTENDRIGH 1960), coupled with the vast array of biochemical parameters which are rhythmic (e.g., HALBERG 1960, 1969), has presented the biochemist with a difficult task. In the past, biochemical analysis of complex processes has been facilitated by a genetic approach, in which the isolation of single-gene mutants has allowed the investigator to focus on one element of the process at a time. This approach has been most successful in elucidating biochemical pathways (e.g., SRB and HOROWITZ 1944), genetic control mechanisms (JACOB and MONOD 1961), bacteriophage assembly (WOOD *et al.* 1968), and now even behavioral phenomena (BENZER 1967). For this reason we have begun a search for biological clock mutants in *Neurospora crassa* (FELDMAN and WASER 1971), which has a well-defined circadian rhythm of conidiation (SARGENT, BRIGGS and WOODWARD 1966; SARGENT and BRIGGS 1967) and a vast backlog of genetic and biochemical techniques and information.

A number of mutants in various organisms have been isolated which affect the expression of a circadian rhythm. However, these mutants—e.g., *acylic* in Paramecium (BARNETT 1966, 1969), and *patch* (STADLER 1959) and *band* (SAR-

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GENT, BRIGGS and WOODWARD 1966) in Neurospora—do not alter the timing mechanism itself but only determine whether the underlying rhythmicity is expressed in a particular morphological or physiological process. More recently, however, mutants which alter the period length of a rhythm and which therefore do affect the timing mechanism have been isolated in Drosophila (KONOPKA and BENZER 1971) and Chamydomonas (BRUCE 1972). We now report the isolation of three mutants which alter the period length of the circadian clock of Neurospora with a preliminary description of their genetic and physiological characteristics.

MATERIALS AND METHODS

Strains: Wild-type strains *74-OR23–1A* and *74-OR8–1a*; *bd; nic-3, me-7; thi-3;* and *alcoy* were obtained from the Fungal Genetics Stock Center, Humboldt State College, Arcata, California. However, it was necessary to reisolate each of these strains, including wild type, in order to eliminate a series of slow-growth genes that interfered with the scoring of conidial banding.

Media: Stock cultures were maintained on HOROWITZ (1947) complete medium and stored on silica gel (DAVIS and DE SERRES 1970). Crosses were carried out by standard procedures (DAVIS and DE SERRES 1970) on WESTERGAARD and MITCHELL's (1947) crossing medium.

Scoring of banding: Each culture was inoculated at one end of a "race" tube (RYAN 1950) containing VOGEL's (1964) salts, 1.2% sodium acetate and 0.5% Difco casamino acids. The tubes were placed at 25° in constant light overnight and then transferred to constant dark at 25° unless otherwise noted. The position of the growth front was marked on the tube at the time of transfer to darkness and thereafter at 24-hour intervals in red light (GE BCJ, 60 Watt); this light does not affect the expression or timing of banding (SARGENT and BRIGGS 1967). The time at which each band occurred was determined by interpolation between the growth fronts, the period length for each culture was determined by averaging the period lengths between successive bands, and the mean period length for each strain and its standard deviation were calculated by averaging the results of the individual cultures.

Mutagenesis: Following the method of MALLING and DE SERRES (1970), conidia from a five-day-old slant of band (*bd*) were filtered through glass wool and suspended in 10 ml of phosphate buffer (5.3 g KH_2PO_4 + 16.3 g $Na_2HPO_4 \cdot 7\ H_2O$ into 1 liter H_2O adjusted to pH 7.0) at a concentration of 2×10^7 conidia/ml. N-methyl-N′-nitro-N-nitrosoguanidine (Aldrich Chemical Co., Inc., Milwaukee, Wisconsin) at a final concentration of 100 μM was added and the culture shaken for one hour. The conidia were then washed three times in buffer and plated on sorbose-complete medium (DAVIS and DE SERRES 1970). The treatment resulted in a killing rate of 30–40%.

Mutant screening: Initial screening for mutants with abnormal banding frequencies was aided by the observation that race tube conditions could be simulated by using 150 × 16 mm screw cap tubes containing 5 ml of medium in a horizontal position. Individual colonies from the sorbose-complete plates were inoculated at the open end of the tube, the tubes plugged with cotton, and the cultures allowed to grow to the "bottom". After inoculation, cultures were placed at 25° in the light overnight and then transferred to constant dark at 25°. After about 5 days the tubes were removed and inspected visually for abnormal banding patterns. About 6,000 colonies were screened in this way, of which about 15 appear to have reproducible alterations in the banding frequency. The first three isolated whose period lengths were more than two hours shorter or longer than band have been analyzed in detail and will be discussed in this paper.

RESULTS

Initial characterization of mutants: Each of the three mutants, designated "frequency" (*frq*), has a period length which is either shorter or longer than the

TABLE 1

Period lengths of mutants at 25°

bd	21.6 ± 0.5
frq-1	16.5 ± 0.5
frq-2	19.3 ± 0.4
frq-3	24.0 ± 0.4

parent band strain. It should be remembered that all *frq* strains, unless otherwise stated, are actually *bd frq* double mutants, since the *bd* gene is necessary to observe the clear expression of conidial banding. Table 1 shows that *frq-1* and *frq-2* have period lengths shorter than band while the period length of *frq-3* is longer than band.

Segregation of mutant phenotype: Each of the mutants segregates as a single nuclear gene. Crosses were made between *bd frq*$^+$ (i.e. band) and each of the mutants. In no case was any difference observed between reciprocal crosses. For both ordered asci and random spores, cultures from individual spores were tested on growth tubes to determine period lengths.

In the cross between *bd frq-1* and *bd frq-1*$^+$, five asci were obtained. In all cases half of the ascus showed normal period length and half showed the mutant period length (Table 2)—i.e., spores were either normal or mutant; no spores had intermediate period length nor was there any deviation from the 4:4 segregation. Among the random spores the distribution of period lengths is clearly bimodal, with about half of the progeny showing wild-type period lengths and half showing *frq-1 period* (Figure 1). The absence of intermediate phenotypes and the 1:1 ratio confirms single-gene segregation. Finally, the width of the distribution of period lengths within each peak in Figure 1 is not due to genetic variability, since the standard deviation of each peak is no greater than the standard deviation obtained by multiple determinations of each of the parents. All of these results strongly support the conclusion that the *frq-1* phenotype segregates as a single-gene nuclear mutation which shortens the period length of the circadian rhythm of conidiation by about five hours.

TABLE 2

Period lengths of progeny—bd frq-1 × bd frq-1+
Parents: *bd frq-1* —16.5 ± 0.5
bd frq-1+ —21.6 ± 0.5

Spore	Ascus 1	2	3	4	5
1	15.7	21.4	...	...	21.4
2	15.9	21.6	21.7	16.0	21.1
3	15.5	21.2	16.0	14.3	...
4	15.9	21.5	16.5	15.8	21.3
5	21.4	16.4	16.3	21.1	16.4
6	21.7	16.3	16.1	21.3	17.1
7	21.4	16.5	21.0	21.1	...
8	21.4	16.5	21.4	21.1	...

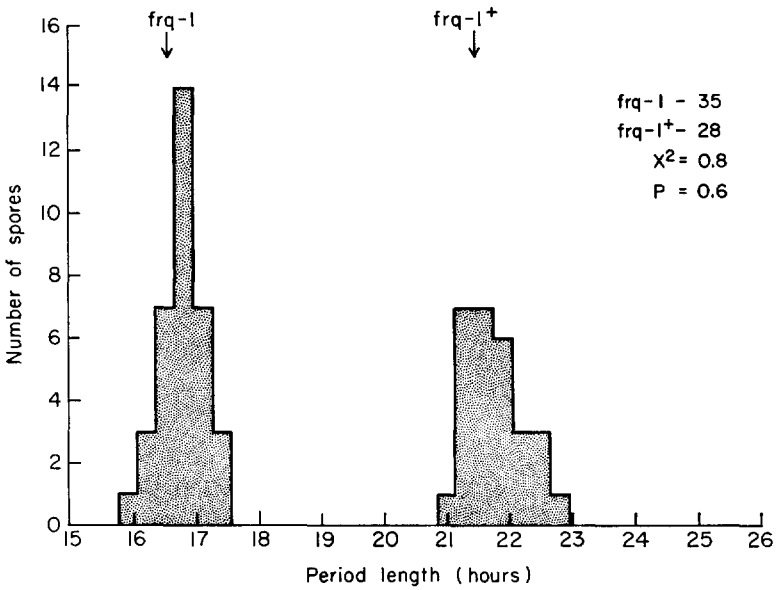

FIGURE 1.—Distribution of period lengths among random spore progeny of a cross between *bd frq-1* and *bd frq-1*+. Position of arrows indicates period lengths of parents. The mean period length and standard deviation for the *frq-1* progeny was 16.8 ± 0.4; for the *frq-1*+ progeny, 21.8 ± 0.5.

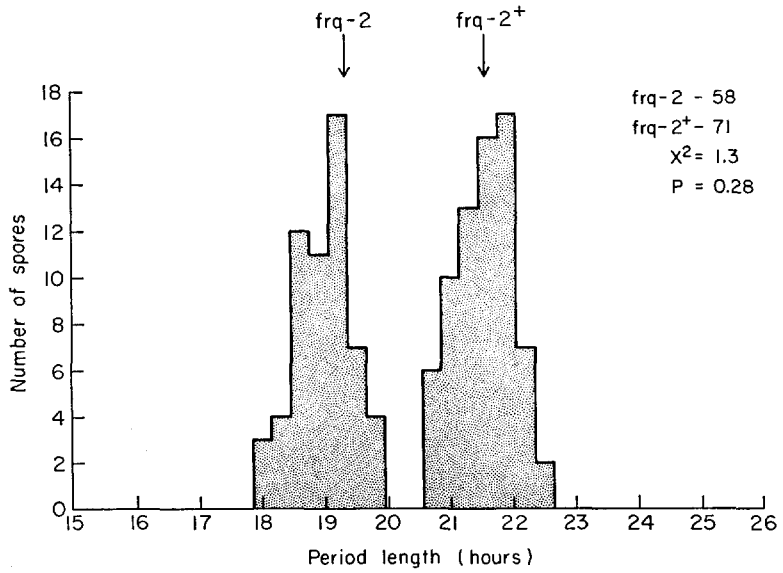

FIGURE 2.—Distribution period lengths among random spore progeny of a cross between *bd frq-2* and *bd frq-2*+. Position of arrows indicates period lengths of parents. The mean period length and standard deviation for the *frq-2* progeny was 19.0 ± 0.5; for the *frq-2* progeny, 21.5 ± 0.7.

frq-2 was analyzed in a similar way. Even though the difference in period length between *bd frq-2* and *bd frq-2+* is less than in the case of *frq-1*, segregation of the *frq-2* phenotype in six ordered asci and random spores (Figure 2) is clear and is also consistent with single-gene segregation.

frq-3, a mutation causing lengthened periodicity, also shows single-gene segregation in five ordered asci and random spores (Figure 3).

Linkage between the mutants: All three mutants appear tightly linked to each other. Pairwise crosses were made between the mutants and both ordered asci and random spores were collected. Among a total of 11 asci (*frq-1* × *frq-2*, 4; *frq-1* × *frq-3*, 4; *frq-2* × *frq-3*, 3), no recombination was observed between any of the mutants; all asci recovered were parental ditypes. In addition no wild-type recombinants were recovered from a total of 453 random spores (*frq-1* × *frq-2*, 333; *frq-2* × *frq-3*, 65; *frq-1* × *frq-3*, 55), and in each cross the distribution of period lengths among the progeny followed a bimodal distribution with approximately one-half of the progeny expressing the period length of one mutant and one-half expressing the period length of the other (Figure 4).

Mapping the mutants: As an independent confirmation of the linkage of the three mutants to each other, each has been shown to map at approximately the same position on linkage group VII. In crosses to PERKINS' *alcoy* strain (see DAVIS and DE SERRES 1970), no linkage was found between the *frq* mutations and any of the three markers in alcoy. As a control, the *bd* gene showed tight linkage to *cot* and confirmed its position on linkage group IV (SARGENT and WOODWARD 1969). Each of the *frq* strains was then crossed to the double mutant

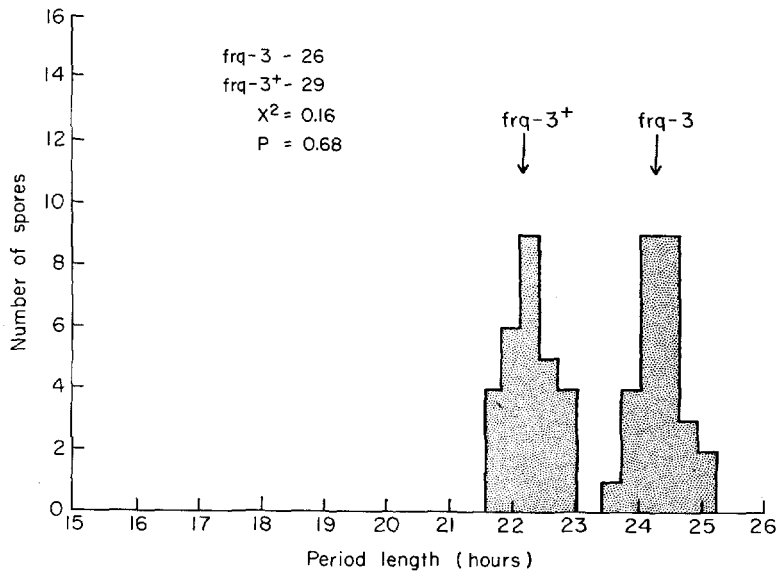

FIGURE 3.—Distribution of period lengths among random spore progeny of a cross between *bd frq-3* and *bd frq-3+*. Position of arrows indicates period lengths of parents. The mean period length and standard deviation for the *frq-3* progeny was 24.5 ± 0.4; for the *frq-3+* progeny, 22.2 ± 0.4.

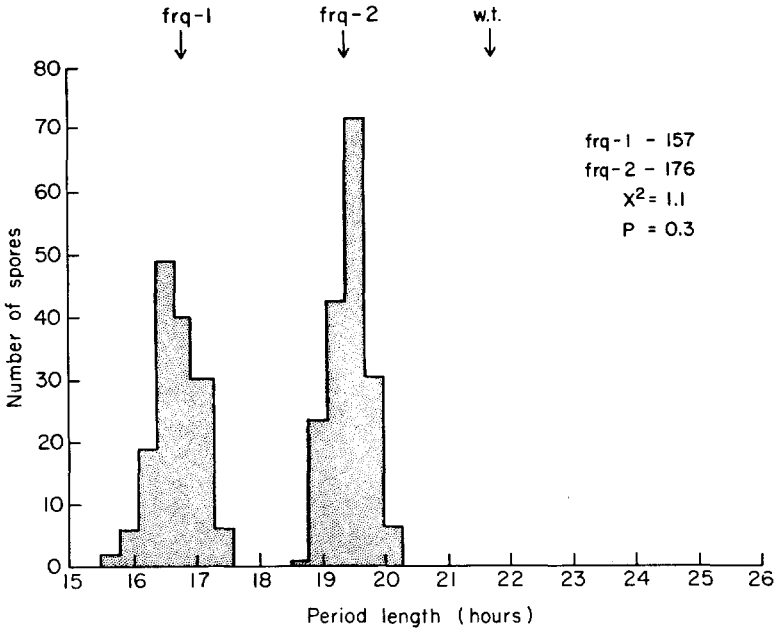

FIGURE 4.—Distribution of period lengths among random spore progeny of a cross between *bd frq-1* and *bd frq-2*. Position of arrows indicates period lengths of parents. The mean period length and standard deviation for the *frq-1* progeny was 16.7 ± 0.4; for the *frq-2* progeny, 19.4 ± 0.3.

nic-3, *me-7*, and to *thi-3*. Linkage to all three of these markers on linkage group VII was approximately the same for all three *frq* mutants (Table 3). Ordered asci from these crosses will be necessary to determine whether the *frq* mutants are on the left or right arm.

Independence of frq *and* bd: In order to unequivocally demonstrate that the *frq* mutations are independent of the band gene, an experiment was carried out to measure periodicity in strains whose genotype is *bd*$^+$ *frq*. This is possible since SARGENT and KALTENBORN (1972) showed that most *bd*$^+$ strains will show conidial banding if a continuous stream of air is passed through the growth tube during growth. Therefore, a cross was made between *bd frq-2* and *bd*$^+$ *frq-2*$^+$

TABLE 3

Linkage of frq *mutants to* nic-3, thi-3, *and* me-7

Mutant	Percent recombination (no. of recombinants/total) *nic-3*	*thi-3*	*me-7*
frq-1	24 (9/38)	25 (5/20)	37 (7/19)
frq-2	25 (3/12)	21 (8/39)	42 (5/12)
frq-3	27 (4/15)	26 (10/38)	40 (6/15)
Total	25 (16/65)	24 (23/97)	39 (18/46)

In each case the cross was bd frq aux$^+$ × bd$^+$ frq$^+$ aux$^-$. *Frq* was scored only among the *bd* progeny. In all three cases, *bd* was unlinked to *nic-3*, *thi-3*, *me-7*, or *frq*.

TABLE 4

Tetratype ascus from a cross of bd frq-2 × bd+ frq-2+

Genotype	Period length (in hours)	
	—air	+air
bd+ *frq-2*+	no banding	21.8
bd *frq-2*+	22.5	22.0
bd+ *frq-2*	no banding	19.4
bd *frq-2*	19.6	18.9

(i.e., wild type). Asci were dissected and scored for period length on growth tubes with and without air. The results of a tetra-type ascus (Table 4) show that even among the *bd*+ progeny the *frq-2* and *frq-2*+ spores are easily distinguishable.

Responses to light and temperature: Circadian rhythms, including those in Neurospora, can be synchronized to external light-dark cycles and their period lengths are relatively constant over a wide range of physiological temperatures (SARGENT, BRIGGS and WOODWARD 1966). All the *frq* mutants have retained both of these characteristics. In a cycle of 12 hours light: 12 hours dark each entrained normally, even though in the case of *frq-1* the natural, or free-running, period length is eight hours shorter than the external cycle.

Band also shows a temperature-compensation of its period length with a Q_{10} about 1 between 20° and 30°, and a Q_{10} of about 1.2 above 30° (SARGENT, BRIGGS and WOODWARD 1966). Each of the mutants shows similar temperature compensation.

DISCUSSION

Each of the three *frq* mutants described segregates as a single-gene nuclear mutation which alters the period length of the circadian clock of Neurospora. In this regard, these mutants are different from patch or band, which instead determine whether or not the clock is overtly expressed as conidial banding. Since period length, or frequency, is an intrinsic property of the timing process, it seems reasonable to conclude that the *frq* mutations have resulted in an alteration in the clock mechanism itself. In this regard, then, these mutants are similar to those reported in *Drosophila* (KONOPKA and BENZER 1971) and Chlamydomonas (BRUCE 1972), in which a change in oscillation frequency was also observed.

Although the exact map position of each of the mutants has not yet been determined, it is clear that they are all very closely linked—no recombination between the mutants has been detected among more than 400 progeny, and each maps approximately the same distance from *nic-3* and *thi-3* on linkage group VII. It should be emphasized that these are the first three of our mutants which we analyzed and that our screening procedure did not favor mutations on any particular chromosome. Therefore, this apparent clustering of clock mutants does not seem fortuitous and strongly suggests that at least for the class of mutants for which we screened—namely, those with relatively normal growth and conidiation and altered timing of the conidial banding— there are few regions

of the genome that can produce such non-lethal abnormalities. This conclusion is reinforced by the similar observation made with the Drosophila mutants, although in that case the selection procedure was restricted to mutations on the *X* chromosome. Of course, we are not suggesting that the biological clock is determined by only one gene. We have not yet determined whether our mutants are allelic or not, and in addition, many functions necessary for the clock may be necessary for normal conidiation, normal growth, or even survival; all such mutants would have been ignored in this initial screening.

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