Circadian clock genes frequency and white collar-1 are not essential for entrainment to temperature cycles in Neurospora crassa

Patricia L. Lakin-Thomas*

Department of Biology, York University, Toronto, ON, Canada M3J 1P3

Edited by J. Woodland Hastings, Harvard University, Cambridge, MA, and approved January 18, 2006 (received for review December 2, 2005)

The fungus Neurospora crassa is a model system for investigating the mechanism of circadian rhythmicity, and the core of its circadian oscillator is thought to be a transcription/translation feedback loop involving the products of the frq (frequency), wc-1 (white-collar-1) and wc-2 (white-collar-2) genes. Several reports of rhythmicity in frq and wc null mutants have raised questions about how central the FRQ/WC loop is to the circadian system of Neurospora. Several research groups have attempted to answer this question by looking for entrainment of the conidiation banding rhythm in frq null mutants. Because the frq mutants are blind to light and cannot be entrained to light/dark cycles, these groups have used symmetric temperature cycles of equal-duration cool and warm phases to entrain the rhythm. Under these conditions, the direct effects of temperature on conidiation (masking effects) can compromise observations of the endogenous rhythm. I have reexamined this question by using short heat pulses to clearly separate masking from endogenous rhythms, and I have assayed entrainment in both frq and wc-1 null mutants. I found similar patterns of entrainment in the wild-type and double mutant strains. Strong masking effects were found in the frq mutant but not in the wc-1 mutant. I concluded that a rapidly damping temperature-entrainable oscillator is present in the null mutants. A single temperature-entrainable oscillator may drive the conidiation rhythm in all strains, and additional properties such as light sensitivity and temperature compensation may be conferred by the intact FRQ/WC loop in the WT strain.

Circadian (24-h) rhythmicity is a nearly ubiquitous property of living things found in all eukaryotes and some prokaryotes. The filamentous fungus Neurospora crassa has been used for many years as a model organism for studying both the formal properties and the molecular mechanism of circadian rhythmicity (1–3). Neurospora produces asexual conidiospores under appropriate conditions, and the timing of spore production is controlled by one or more oscillators. When cultures are grown on solid agar medium, the growing front lays down regions of spore-producing hyphae alternating with nonsporulating regions in a pattern of “bands” and “interbands” as the growth front advances across the agar surface. This banding pattern has been used since the beginning of Neurospora circadian research as the primary indicator of the state of the internal oscillator. The banding pattern is described as the “output” of the oscillator, just as the hands of a clock indicate the state of the hidden clockworks.

Studies of the molecular mechanism of rhythmicity in Neurospora have focused on a small set of “clock” genes, frq (frequency), wc-1 (white-collar-1) and wc-2 (white-collar-2), whose gene products affect the period and expression of the banding rhythm. Molecular analysis of the control of expression of these genes has suggested a feedback loop in which the white-collar (WC) proteins, as a white-collar complex (WCC), activate transcription of the frq gene, whereas the frq protein (FRQ) inhibits the activity of the WCC. This feedback loop has been proposed as the core of the circadian oscillator (1, 4).

Several laboratories have observed free-running, persistent rhythmic banding in the absence of a functional FRQ/WC feedback loop by using mutant strains carrying null mutations of frq, wc-1, or wc-2 (3, 5–9). The conidial banding rhythm in these strains is often not as robust as in wild type and does not conform to the standard definition of a “circadian” rhythm, which should be robust, have a period length of ~24 h at different temperatures (temperature compensation), and be sensitive to light. The term FLO (frq-less oscillator) has been coined to describe rhythmicity in the absence of a functional FRQ/WC feedback loop (10).

There is currently a debate in the literature as to how to explain this frq-less rhythmicity, and there are several different points of view as to its source and significance (3). I will caricature four positions along the continuum as follows: (i) There is a single oscillator in Neurospora that produces the banding rhythm output. Frq-less rhythmicity is defective because the oscillator is missing an input pathway that provides light input and stability to the oscillator. The functions of FRQ and WC can be described as input pathway components rather than essential oscillator components (11–13). (ii) There are two equally important oscillators, the FRQ/WC loop and the FLO, that interact and mutually entrain under normal conditions; in frq null mutants, only the FLO is active and it lacks some of the standard circadian properties (9, 14). (iii) There are several or many FLOs that are normally coupled to the circadian FRQ/WC loop, but they do not make a critical contribution to circadian rhythmicity. In the absence of the FRQ/WC loop, these FLOs oscillate in a noncircadian manner (15, 16). (iv) Only the FRQ/WC oscillator is significant for circadian rhythmicity; the FLO is not normally present and the rhythmicity in frq null strains results from an “unnatural oscillatory state.” There may be many FLOs that appear in different unnatural conditions when FRQ is absent (15).

The question at issue is the following: How central is the FRQ/WC loop to circadian rhythmicity in Neurospora? Because the Neurospora clock is a model system that informs our understanding of the molecular basis of rhythmicity in other organisms, this question relates to the larger question of how central a transcription/translation feedback loop is to circadian rhythmicity in general. A series of three papers (11, 15, 17) has attempted to answer the Neurospora question by looking for entrainment of the conidiation rhythm in frq null mutants. Because frq null mutants are blind to the effects of light on the circadian oscillator (8, 11, 18), these authors have used temperature cycles to entrain the banding rhythm.

Roenneberg et al. (17) have succinctly summarized the principles of entrainment, and an excellent review by Johnson et al.

Conflict of interest statement: No conflicts declared.

This paper was submitted directly (Track II) to the PNAS office.

Abbreviations: FLO, frq-less oscillator; T cycle, entraining cycle; WC, white collar.

*E-mail: plakin@yorku.ca.

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www.pnas.org/cgi/doi/10.1073/pnas.0510404103

PNAS | March 21, 2006 | vol. 103 | no. 12 | 4469–4474

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(19) is also available. Briefly, an oscillator (such as the circadian oscillator) will adopt the same period as a repeated stimulus (such as the daily light/dark cycle) if several conditions are met: The period of the entraining stimulus must be close to the intrinsic period of the entrained oscillator, and the strength of the entraining stimulus must be sufficiently large. One of the properties of entrainment is that the phase relationship between the entrained oscillator and the entraining cycle will change, depending on the length of the entraining cycle (T cycle) and stimulus strength.

Entrainment experiments often reveal the presence of “masking” effects. Masking is defined (17, 20) as an acute effect of a stimulus, not mediated by any effects on an oscillator. An example is the activity rhythm of mammals: A circadian oscillator controls the rhythm of activity, and light resets the oscillator, but a pulse of light at night can immediately suppress the activity of a nocturnal animal independently of effects on the circadian oscillator. Although masking responses can be thought of as an important aspect of an organism’s adaptation to a cyclic environment (20), masking can potentially compromise an entrainment experiment by obscuring an entrained, endogenous rhythm.

In the original report of temperature entrainment in a frq null mutant, Merrow et al. (11) demonstrated that the phase angle between the temperature cycle and the conidiation rhythm changes as a function of the length of the T cycle, in both the wild type and a frq null mutant. These authors concluded that the same temperature-entrainable oscillator is functioning in both strains.

Pregueiro et al. (15) repeated these experiments and analyzed the data by using a different landmark to determine the phase of the banding rhythm. They concluded that the rhythm in the frq null strain is not entrained but rather is “driven.” Although these authors do not use the term “masking,” their use of “driven” seems to refer to the same process, that is, a direct response to the environment not involving an oscillator.

Roenneberg et al. (17) reanalyzed the data from the first two reports by using several different phase markers and concluded that the analysis of Pregueiro et al. did not take into account the masking effects of the temperature cycles. Roenneberg et al. also experimentally demonstrated masking in Neurospora by using rhythmic air flow and presented a model in which masking effects and an entrained oscillator both contribute to the waveform of the entrained conidiation rhythm.

These three reports (11, 15, 17) used symmetric temperature cycles, with equal duration of warm and cool phases. This experimental design makes it difficult to disentangle the potential endogenous oscillator effects from masking effects. Indeed, Roenneberg et al. (17) concluded that the data “suggest an entrainment pattern that is obfuscated by masking components.”

I have used an experimental design that is improved in two aspects: I have used short heat pulses to separate the effects of masking from the endogenous rhythm, and I have used two different high temperatures to explore the effects of changing stimulus strength. I have also assayed entrainment in an additional “clock null” strain, wc-I, as well as a frq null strain, frq10.

**Results and Discussion**

**Time Series.** The density traces for each set of race tubes (six replicate tubes) were averaged and the resulting time series were plotted against the total hours of culture time in constant darkness. Representative experiments are shown in Fig. 1. The data are included in Fig. 4, which is published as supporting information on the PNAS website. Fig. 1A shows the control cultures...
without heat pulses, grown at 22°C. The wild type has a clear endogenous rhythm of conidiation peaks, as expected. No consistent rhythm can be seen in frq10 or wc-1 except for a few low-amplitude peaks that are sometimes seen in frq10.

Fig. 1 B–D shows entrainment of all three strains to T cycles of 16, 24, and 28 h. Additional examples of entrainment can be found in Fig. 4. In Fig. 1C, direct responses to the heat pulses (masking peaks) can be seen in the wild type and frq10 as well as entrained peaks. Masking peaks were also found in these two strains in other T cycles (Fig. 4). In agreement with Roenneberg et al. (17), robust masking responses were found in the frq null strain, as large or larger than the masking responses in wild type. No clear masking peaks were seen in wc-1 under any of the tested conditions. The reason for this lack of masking in wc-1 is not known.

In Fig. 1D, the long 28-h T cycle induced two peaks per cycle in all three strains. It is difficult to tell whether the peaks coinciding with the heat pulses are masking peaks or a second endogenous peak that begins before the heat pulse, as might be expected for a T cycle much longer than the intrinsic periods of the strains.

Fig. 1E demonstrates a “release” experiment in which the heat pulses were discontinued after entrainment was established. The wild type continued to produce peaks as expected. The two mutant strains produced one additional peak after the last heat pulse and then damped to arrhythmicity. In a release experiment using T = 28 h and pulses of 30°C (Fig. 4M), frq10 produced one peak and wc-1 produced two peaks after release from entrainment. This result suggests that the oscillator in these mutant strains is rapidly damping and cannot drive a noticeable rhythmic output for more than a few cycles after entrainment, as proposed in ref. 17.

Fig. 1F demonstrates failure of entrainment. In this short-period T cycle of 8 h with a weak pulse strength of 0.5 h at 30°C, none of the three strains entrain. The peaks in wild type are ragged and uneven compared to control and entrained cultures, suggesting a masking influence on conidiation. The frq10 strain shows a clear masking response to every heat pulse. The wc-1 strain shows no masking or entrained responses to the pulses, with the exception of a few small peaks beginning at ~85 h. Very poor entrainment was also found by using T = 24 h and pulses of 1 h at 37°C (Fig. 4O). In T cycles of 8 h with pulses of either 0.5 h or 1 h at 27°C, masking responses were not seen and entrainment was poor (data not shown).

All three strains demonstrated a tendency for frequency demultiplication in short T cycles. In theory, oscillators are capable of entraining to periodic stimuli in frequencies that are small-integer multiples of the entraining frequency (17, 21). In Fig. 4G, the WT strain entrained to a 12-h T cycle with a pattern of alternating large and small peaks, indicating that the oscillator repeated a pattern every 24 h. Fig. 5, which is published as supporting information on the PNAS web site, shows examples of the null mutants in 8-h T cycles. In Fig. 5A, one race tube of six replicates of the frq10 strain entrained to an 8-h cycle with a 24-h period. Note that no masking or entrainment responses were seen in frq10 in other replicate tubes in this experiment (Fig. 5B). In Fig. 5C, one race tube of six replicates of the wc-1 strain entrained to an 8-h T cycle with a period of 16 h.

Average Days. The average days were calculated as the mean density of all cycles in all replicate race tubes for one strain and T cycle and are plotted in Fig. 2. The beginning of each pulse is plotted at 0 h, the end of each pulse is at 2 h, and the density traces end when the next pulse begins. Masking effects can be seen as an increase in density preceding the beginning of the pulse (that is, near the end of the trace), followed by an abrupt decrease in density during and after the pulse (between 0 and

Fig. 2. Average days. The mean of the density traces for all days in all replicate tubes was calculated to give the average day for that set. Each T cycle on each race tube was treated as an individual day. Mean densities are plotted against hours after the beginning of the pulse. Vertical line at time = 2 h indicates the end of the pulse. Small arrows indicate examples of masking responses. T cycles are indicated by the legend. (A–C) 30°C pulses, (D–F) 37°C pulses. (A and D) Wild type. (B and E) frq10. (C and F) wc-1.
second cycle of an endogenous oscillator. For example, in Fig. 2 demonstrate changes in the timing, shape, and amplitude of the peaks and their onsets can be seen to shift with changes in the type of the preexisting mycelium behind the growth front. In the previous reports (15, 17), conidiation tended to occur during the cool phase of the temperature cycles, which is in agreement with the decrease in conidiation density during and after the heat pulse. Masking effects are particularly clear in the 24-h T cycles for the WT and frq10 strains with both 30°C and 37°C pulses. Aside from the masking effects, the average days plotted in Fig. 2 demonstrate changes in the timing, shape, and amplitude of the entrained endogenous peaks. For example, in Fig. 2B, both the peaks and their onsets can be seen to shift with changes in T-cycle in frq10. Changes in peak shape for wc-1 are particularly clear in Fig. 2F.

Peak Identification. Peaks were identified in the individual race tube density traces as described in Methods, and the times (in hours) and phases (in degrees) of peaks are listed in Table 1, which is published as supporting information on the PNAS web site. In traces with two clear peaks per cycle, the masking peak was identified as the peak closest to the time of the heat pulse and is included in Table 1 as the “secondary” peak. The other peak was identified as the “principal” peak. When only one peak was found, it was identified as the “principal” peak.

Clear masking peaks were found in the frq10 cultures in all 37°C cycles and in the longer 30°C T cycles (20, 24, and 28 h). Masking peaks were also found in the WT cultures for 24-h 37°C cycles and both 24-h and 28-h 30°C cycles. A small shoulder on the mean day traces for WT 37°C T cycles of 12, 16, and 20 h represents a small peak that was not always resolved from the principal peak in the individual traces. This small peak is listed in Table 1 as the secondary peak for these cultures. No masking peaks were found in any wc-1 cultures. A secondary peak was found in the 28-h 30°C T cycle for wc-1, which may represent a second cycle of an endogenous oscillator.

Principal peak phases (in degrees) are plotted against T cycle in Fig. 3 A and B. For all strains, the entrained phase changes with the T cycle, and the relationship is not linear, as predicted for an entrained oscillator (13, 17). The Watson-Williams test for multiple samples (22) was used to test the hypothesis that all samples in each set are from the same distribution. That is, that the principal peaks occur at the same phase. A set is defined as all T cycles for one strain at one pulse temperature. The null hypothesis was rejected at $P < 0.0005$ for all six sets. In other words, the peak phases change significantly with changing T cycle.

For an entrained oscillator, it is predicted (17) that the slope of the phase vs. T cycle plot will change with the strength of the entraining signal: A strong signal will produce a shallower slope. It was demonstrated in ref. 23 that in phase-resetting experiments with single heat pulses, the amount of phase shift increased with either increased temperature or increased pulse length. It is predicted, therefore, that entraining pulses of 37°C will decrease the slope of the T cycle plot as compared to 30°C pulses, and this prediction was fulfilled by the data. The slopes of the phase vs. T cycle plots were calculated over the range of T cycles from 12 h to 24 h, excluding the 28-h T cycle at 30°C for which there is no counterpart at 37°C. For wild type, the slopes of the 30°C and 37°C plots are $-11.8$ and $-7.6$; for frq10, $-18.7$ and $-6.2$; and for wc-1, $-9.6$ and $-6.9$. The wc-1 strain appears to entrain more strongly to the 30°C pulse than the other strains, based on the slope of the T cycle plots. Although the analysis of peak times in degrees appears to provide evidence that the principal peaks are genuinely entrained, the possibility remains that the peaks are produced not by an entrained self-sustained oscillator but by an “hourglass” process. An hourglass is defined (24) as a process that is started by a stimulus, completes one cycle at a constant rate, and does not restart until stimulated again, in the way that an hourglass is not a self-sustained oscillator but must be turned over each time it is used. In this case, the peaks would always occur at the same number of hours after the heat pulse. Peak times in hours are analyzed by one-way ANOVA, and the null hypothesis was rejected at $P < 10^{-5}$ for all six data sets. This result indicates that peaks within a set do not occur at the same time after the pulse, and an hourglass mechanism is unlikely to account for the results.

Conclusions. By using short heat pulses, I have been able to clearly separate two peaks per cycle in some T cycles. The peaks coinciding with the heat pulses have been identified as masking responses by several criteria: (i) They maintain a relatively constant phase relationship with the heat pulses (Table 1), (ii) they are seen coinciding with heat pulses in cultures that are not entrained (Fig. 1F), and (iii) they disappear when the heat pulses are discontinued (Fig. 1E).

The nonmasking peaks in the null mutants appear to be produced by an entrained endogenous oscillator, and to conform to the expectations of entrainment, by several criteria: (i) The peaks do not occur at a fixed time after the heat pulse. Peak phases change with the T cycle (Fig. 3). (ii) Peak shapes also change with T cycle (Fig. 2), which would not be predicted if the peaks were directly driven by the heat pulses. (iii) The slopes of the phase vs. T cycle curves decrease with increasing stimulus strength (30°C to 37°C) as expected for an entrained oscillator. (iv) The oscillation continues for at least one cycle after release from entrainment (Figs. 1E and 4M). (v) Combinations of T cycles and stimuli can be found that are outside the range of entrainment although they are still capable of producing masking effects (Fig. 1F). I conclude that the null mutants frq10 and wc-1 have (one or more) rapidly damping oscillator(s) that are...
capable of entraining to heat pulses in a manner similar to the WT strain. These results do not resolve the question of whether this heat-entrainable oscillator is the same as “the circadian oscillator.” Pregueiro et al. (15) suggest that the finding of “normal circadian entrainment in frq null strains...” would have profound implications. Whether these results demonstrate “normal circadian entrainment” is not a meaningful question: Any oscillator should be capable of entrainment under appropriate conditions, and the existence of entrainment does not identify an oscillator as circadian. Free-running rhythmicity in these null mutants, demonstrated under other conditions (3, 5–9), does not show all of the properties of an intact circadian system, such as temperature compensation and light entrainability. In that sense, a frq-less oscillator is not “circadian.” However, the oscillator revealed here by temperature entrainment may be the oscillator used by the circadian system, and the circadian properties may be supplied by pathways that are defective in these mutants. In a complex system, we expect to see a change in the properties of the system as a whole if one of the components of the system is removed or altered by mutation. The fact that the system’s behavior is now different does not mean that we are now looking at an entirely different system. The simplest hypothesis is that there is a single oscillator that is used by the organism to generate rhythmicity, and this rhythmicity has circadian properties only when the entire system is intact in the WT strain.

Methods

Strains. All strains of N. crassa used in this study carry the bd mutation, which prevents inhibition of conidiation by high CO₂ concentrations in closed culture vessels, and the csp-1 mutation, which prevents the release of conidiospores and reduces contamination. The WT strain is therefore a double mutant csp-1; bd. The frq⁻¹⁰ strain (csp-1; bd; frq⁻¹⁰) carries a null mutation at the frq locus created by targeted gene disruption (6). The wc-1 strain (csp-1; bd; wc-1) carries a mutation at the wc-1 locus (allele ER53) that produces a small amount of a truncated protein product (25). A trace amount of FRQ protein is produced in the dark, and light induction of FRQ is absent in this strain (25). Strain construction was described in ref. 8.

Culture Conditions and Heat Pulses. Cultures were grown in 30-cm glass growth tubes (“race tubes”) on Vogel’s minimal medium, with 2% agar/0.01% arginine/0.5% maltose. Race tubes were inoculated at one end with small plugs of mycelium from Petri plate cultures grown in constant light at 22°C for 2 days (8). Race tubes were transferred to constant darkness at 22°C at the time of inoculation with plugs. Cultures were grown in Percival Scientific (Perry, IA) environmental chambers, model 1-36LL, with Advanced Intellus Controller. In most experiments, temperature pulses were delivered as steps up from 22°C to either 30°C or 37°C for 2 h followed by a step down to 22°C. The length of the T cycles was varied in 4-h increments in different experiments; for example, a 20-h T cycle indicates 2 h at high temperature plus 18 h at 22°C, whereas a 28-h T cycle indicates 2 h at high temperature plus 26 h at 22°C. In a few experiments, the high temperature was 27°C, or shorter heat pulses of 1.0 or 0.5 h were used. Delivery of temperature cycles was confirmed with a temperature recorder [Dickson (Addison, IL) TempTrace] inside the environmental chamber. A set of six replicate race tubes was used for each strain and condition.

Density Traces. During growth of the cultures, the position of the growth front was observed under red safelight and marked approximately every 24 h, and the exact time of marking was recorded. After growth was finished (~7 days), the bottom of each race tube was scanned into a TIFF file at 300 dpi (“marked scans”). The growth marks were then removed from the tubes (leaving the first mark for orientation), and the tubes were scanned again (“clean scans”). The marked scans were opened in IMAGEJ software for Macintosh (http://rsb.info.nih.gov/ij/index.html), and the positions of the growth marks were recorded (as pixel number counted from the first growth mark) by using the built-in Measure procedure. This procedure gave a data set of growth mark position vs. time at which the growth front was marked. Time was calculated as cumulative hours from the first growth mark (time = 0). The clean scans were opened in IMAGEJ, and a density profile was recorded starting from the first growth mark by using the built-in PlotProfile procedure, averaging pixel densities across the entire tube width. This procedure gave a data set of pixel densities vs. pixel numbers (proportional to position along the tube). The pixel density vs. pixel number data were analyzed as follows: Pixel number was converted to time by using the growth mark data with linear interpolation between the two nearest growth marks. Pixels were then lumped into bins of 0.1 h, averaging the corresponding pixel densities within bins. Pixel densities vs. time were plotted for each individual race tube. A set of six replicate race tubes was then averaged to give mean density vs. time. Areas of high density correspond to “bands” of conidiation, and areas of low density correspond to “interbands” with little conidiation.

Peak Identification. Peaks were identified in the density traces of the individual race tubes. Each density profile was smoothed with a 41-point moving average. Peaks were identified as maximum density values, beginning after the second heat pulse in all experiments except the 28-h T cycle, in which peaks were identified beginning after the first heat pulse. If the smoothed trace did not yield a clear principal peak, the moving average was applied again before searching for maxima. The peak time was calculated in hours after the beginning of the most recent heat pulse. Values in hours were converted to degrees by dividing by the T cycle length and multiplying by 360.

Statistical Analysis. Statistical calculations using degrees were carried out according to the statistical methods for circular distributions of Zar (22). Mean angles (°) in degrees were calculated from the individual peaks identified on all replicate race tubes and are reported as peak phases in Table 1; n values ranged from 18 to 60 individual peaks, depending on T cycle (Table 1). Mean angular deviation (s) in degrees is a measure of dispersion (22) similar to standard deviation for linear data and is reported in Table 1 as “dev.” Mean angles in degrees were converted to peak times in hours by dividing by 360 and multiplying by the T cycle in hours.

To determine whether peaks occurred at different phases in different T cycles, the Watson-Williams test for multiple samples (22) was used to analyze the phases calculated in degrees. This test for circular distributions is similar to one-way ANOVA for linear data. To determine whether peaks always occurred at the same time in hours after the pulse in different T cycles, the peak times in hours were analyzed by using standard one-way ANOVA. It was assumed that the peak times in hours are closer to a normal linear distribution than a circular distribution. In practice, there was very little difference in the statistical measures calculated by either circular or linear methods for the data in hours (results not shown).

We thank Donna Moon, Sarah Farooq, and Diana Muñoz, whose assistance is gratefully acknowledged, for collecting data; Nicholas Mrosovsky and the members of the Toronto Clock Club for stimulating discussions of the data; and Stuart Brody, Martha Merrow, and Tilly Roenneberg for many constructive comments. This work was supported by Discovery Grant 250133-02 from the Natural Sciences and Engineering Research Council.