The Genetics of Circadian Rhythms in *Neurospora crassa*: Do Multiple Oscillators Exist?

A Senior Honors Thesis

By

KAREN KORTUM

Submitted to the Office of Honors Programs
& Academic Scholarships
Texas A&M University
In fulfillment of the requirements of the

UNIVERSITY UNDERGRADUATE
RESEARCH FELLOWS

April 2001

Group:

Biomedical Sciences

The Genetics of Circadian Rhythms in *Neurospora crassa*:

Do Multiple Oscillators Exist?

A Senior Honors Thesis

By

KAREN KORTUM

Submitted to the Office of Honors Programs
& Academic Scholarships
Texas A&M University
In fulfillment of the requirements of the

UNIVERSITY UNDERGRADUATE
RESEARCH FELLOWS

Approved as to style and content by:

Deborah Bell-Pedersen
(Fellows Advisor)

Edward A. Funkhouser
(Executive Director)

April 2001

Group: Biomedical Sciences

ABSTRACT

The Genetics of Circadian Rhythms in *Neurospora crassa*:

Do Multiple Oscillators Exist? (April 2001)

Karen Angela Kortum
Department of Biochemistry and Biophysics
Texas A&M University

Fellows Advisor: Dr. Deborah Bell-Pedersen
Department of Biology

Circadian biological clocks are found in virtually all organisms and function to generate daily rhythms. The circadian clock (built by one or more oscillators) controls rhythmicity in a wide range of processes, ranging from sleep/wake cycles in humans, photosynthesis in some plants, to reproduction in fungus and other eukaryotes. One organism, *Neurospora crassa*, displays an easily observed and assayable circadian rhythm in asexual spore production, conidiation, and is an excellent model for understanding the molecular and biochemical basis of circadian rhythms. Studies in *Neurospora* have identified the *frequency* (*frq*) gene as a central component of the fungal clock. Under most growth conditions, rhythmic development is absent in strains lacking a functional FRQ protein. However, under some conditions, rhythmic conidiation can be observed in a FRQ-null strain. This residual rhythmicicty was noticed early on, but went essentially ignored until recent experiments demonstrated rhythms in cultured FRQ-null strains grown in 12 hour temperature cycles in constant darkness. These data suggested that the FRQ-based oscillator is not the only oscillator in the cell and led to our hypothesis that the clock system is composed of more than one oscillator that can function to generate rhythms. To test this hypothesis, I have created random mutations in a FRQ-null strain and assayed for loss of rhythmicity in the temperature cycles. Fourteen mutant strains were identified that met these criteria. It is expected that some of these mutations will identify genes that function in the temperature-dependent oscillator.

Table of Contents

List of Figures

Chapter 1: Introduction

Daily rhythms were first described in 1729 by the French astronomer, Jean Jaques d'Ortous de Marian. He noticed that certain plants opened and closed their leaves with the changing of day to night. In his pioneering experiments, he placed some of these plants in the dark for several days, and the cycle of leaf movement continued without the sunlight changes. He concluded that this cycle did not depend on the rising and setting of the sun, but was in fact an endogenous characteristic of the plant (reviewed in Edery 2000). This work is where the science of the circadian (meaning about one day) biological clock began.

Circadian rhythms are daily rhythms that generated by the circadian clock and are present in almost all organisms, from the most complex and multi-cellular eukaryotes down to some single-celled prokaryotes. Some examples of these daily rhythms include sleep/wake cycles in humans, opening and closing of plants' leaves to harvest light, fungal reproduction cycles, and bees that fly once a day to flowers to harvest nectar at the time of day it is produced.

Although the rhythmic processes that are observed in nature are diverse, all circadian rhythms share defining characteristics, and they even share some similarities at the molecular level. For example, most eukaryotic organism have clock-associated proteins that contain the so-called PAS domains involved in protein/protein interactions, thus providing speculation regarding the evolutionary history of circadian clocks (Kay 1997). At a broader level of similarities, all circadian rhythms are defined by the following four essential characteristics (Edmunds 1988):

1. Self-sustained oscillation under constant conditions
2. A period of approximately 24 hours
3. The ability to be entrained by environmental stimuli, such as light or temperature pulses
4. Temperature compensation of the period of the cycle.

An important feature of circadian rhythms is that they are not driven by 24-hour environmental cycles imposed by the Earth's rotation, but instead are generated by an endogenous clock that maintains rhythmicity, even under constant and to temporally coordinate and partition activities to the appropriate times of the day. Not surprisingly, a circadian clock whose intrinsic period closely matches the environmental light/dark cycles or temperature cycles improves the fitness of cells. These data demonstrate that circadian rhythms provide a selective advantage to organisms (Ouyang et al., 1998). Because the function of the circadian is to provide an internal measure of external time, the free-running period needs to be reset each day to precisely 24 hours. This is accomplished by sensing environmental time cues and shifting the phase of the rhythm appropriately. The two most important time-givers are the daily light/dark and temperature cycles. To entrain to external time, the oscillator responds differently to the cues when they are perceived at different times within the circadian cycle. For example, in the fungus *Neurospora crassa*, light signals perceived in the early night are interpreted as dusk and the clock delays to dusk. Light signals given during the late night are interpreted as dawn and the clock advances to dawn. This dependable mechanism can assist the organism in recognizing warmth of daylight, as is important for photosynthesizing plants, and cooler night-time, as is important for nocturnal owls. Finally, all circadian rhythms are temperature compensated which means that they maintain a normal period within their physiological temperature ranges. This prevents the rhythm from having an accelerated, shorter period in heat or a slowed, longer period in cooler temperatures. Therefore, it provides normality of the period length for rhythmic organisms when changing from sun to shade, night to day or winter to spring. However, because a circadian clock can be entrained by temperature transitions (pulses or cycles), the system is not entirely temperature independent (reviewed in Lakin-Thomas et al., 1990).

The circadian system is most often considered to containing the following three elements as depicted in Figure 1 (Roenneberg 1998);

1. Input pathways to receive environmental stimuli, such as light and temperature

2. The central clock or oscillator that keeps time
3. Rhythmic output pathways that act downstream of the oscillator

These elements only show a very simplistic view of the circadian clock, but they do not describe how the clock works. The actual workings of the clock are proving to be much more complex. For example, multiple oscillators that regulate diverse output pathways have been described in single cells (Roenneberg and Morse, 1993). Second, feedback from the oscillator to the input pathways and from output to both the oscillator and the input has been described (reviewed in Roenneberg and Mittag, 1996). Understanding these complexities in their entirety constitutes a major biological puzzle, one that relates not only to simple eukaryotic model systems, but also to human physiology and mental well-being.

FIGURE 1: Simple diagram of a circadian clock

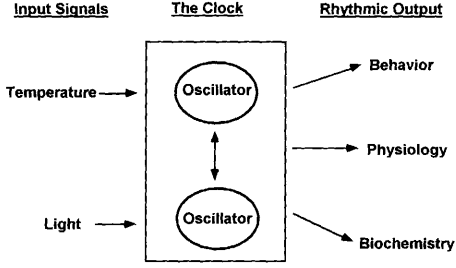

Figure 1 Legend: This figure displays a simple view of a circadian clock. Each clock is controlled by a central oscillator that receives input from the environment and responds with a type of rhythmic output.

It was not until the advent of genetics that circadian rhythms began to expose their intricate design. By 1994, only two clock genes had been discovered and described: *period* (*per*) in *Drosophila* (Konopka 1971) and *frequency* (*frq*) in Neurospora (Feldman 1973). Today, clock genes have been discovered in many organisms, including humans, rodents, fish, frogs, insects, plants, and cyanobacteria.

With all of this new knowledge, the science surrounding circadian rhythms has a great deal of potential for uses to help humanity. For example, the most recognizable human circadian rhythm is wake/sleep cycles, but when disrupted, such as in so-called jet lag, this can cause many problems to the affected individual. In response to this problem and other sleep related disorders, researchers sought out and located cycling hormones involved in sleep. From this research, doctors can now administer melatonin, a circadian regulated hormone, to relieve the disorders related to sleep disruption (Brzezinski 1997).

There is potential in other research for aiding humanity in alleviating other problems related to circadian rhythms. Some of these disorders include chronic sleep disorder, manic-depression, and SAD, or winter depression (Copinschi 2000). Cell division has also been linked to the circadian clock, and due to the connection between mitosis and cancer, this could lead to a new perspective in fighting cancer.

Studying circadian clocks in simple model organisms has lead to insights in these important areas. Because clocks share common characteristics and mechanisms, the study of the clock system of a simple eukaryotic model organism has and will likely continue to provide important clues into the aforementioned human disorders, but it would not have the same difficulties in assaying the test subjects. One of the most intensively studied simple eukaryotic systems is the fungus *Neurospora crassa* and the discovery of the essential clock gene *frequency* has led to information regarding how a circadian clock is built within the cell (reviewed in Bell-Pedersen, 2000).

Chapter 2: *Neurospora crassa* as a model organism for the study of circadian rhythms.

Neurospora crassa, commonly known as the orange bread mold, is a filamentous fungus that is a model genetic organism. Its seven chromosomes, referred to as linkage groups, have been completely sequenced only months ago by the Whitehead Institute (http://www-genome.wi.mit.edu/annotation/fungi/neurospora/). The complete genome of *Neurospora crassa* contains 4.3×10^7 base pairs, and it holds approximately 12,000 genes (Orbach 1988). This fungus usually exists as a haploid organism making the occurrence of mutations easily noticeable and selectable. It also is capable of creating heterokaryons, which provide researchers with details on dominance and lethal recessive alleles. One feature that makes *Neurospora* a premier organism for the study of the circadian clock is that the clock controls the daily production of asexual conidiodiospores. This developmental rhythm provides an easy rhythm to observe and measure through the use of race tubes, as shown in figure 2.

A race tube is a cylindrical glass tube approximately 30 - 40cm long with agar-based medium poured inside the bottom and its ends curved upward at a 45 degree angle (Sargent 1966). Conidiospores are inoculated at one end of the race tube, and the sample is then placed in constant light for 24 hours at room temperature. At the end of this daylong incubation, the growth front is marked, and all race tubes are transferred to a temperature-controlled dark room. This transfer from constant light to constant dark sets the circadian clock of the organism to dusk and synchronizes all cells to the same circadian time. While incubating in the dark room, the fungus extends its hyphae across the tube's medium, and once a day it produces readily visible bands of fluffy orange conidia. Between each of these bands, the fungus displays growth across the race tube as undifferentiated vegetative hyphae. This display of banding is usually aided by the presence of the *band* gene to inhibit the elevation of CO_2 usually seen in wild type. The presence of the CO_2 in the race tube will cause the masking of this banding effect (Sargent 1966). Because the *band* gene displays such difference in the conidia bands and

Figure 2: Diagram of a race tube

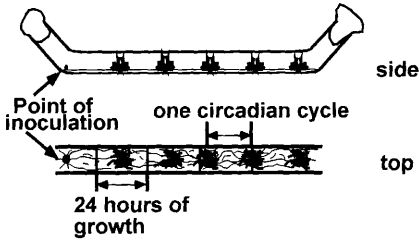

Figure 2 Legend: This diagram shows the race tubes used for assaying *Neurospora crassa's* conidiation cycle. When inoculated at one end of the tube and placed in the dark to grow, the fungus will expand across the medium, leaving patches of conidia. This banding pattern allows the determination of whether or not the cultures are rhythmic and the calculations of phase and period.

the vegetative hyphae, the period of the rhythm can be determined by the distance between the conidial bands.

The first step in calculating the period involves marking the growth front of the fungus in the dark room every 24 hours. This can be done by working under a "safe" red light while marking the incubating race tubes. Red is considered safe because the clock components are only responsive to blue light. After approximately five days, the race tubes can be removed, and the distances between the growth fronts, as well as the distance between the centers of the conidiation bands are measured. By comparing these two distances, researchers can infer the period and the phase of the rhythm.

Using these methods, scientists have determined that the conidiation cycle of *Neurospora* is indeed controlled by circadian clock components. The cycle will

continue in complete darkness at a temperature of 24°C with a period of 21.5 hours (Pittendrigh 1959). This fulfills the requirements of proceeding at constant conditions and of maintaining a period of approximately 24 hours. It is also temperature compensated between the temperatures of 18°C and 30°C, as per another characteristic of circadian rhythms (Nakashima 1982). Abiding by the final requirements, this rhythm can be reset by light pulses (Sargent 1966) or by temperature cycles (Gooch 1994).

Through old and new technologies of genetics and biochemistry, scientists have searched for the genes that make up the oscillator, and several clock related have been discovered. One of the vital genes of *Neurospora*'s conidiation cycle is the *frequency* gene, or *frq*. The *frq* gene displays cyclic behavior throughout the day, in tandem with the conidiation cycle of *Neurospora*. Its mRNA and gene product, the FRQ protein, cycle in concentration throughout the day, as shown in figure 3. The gene's transcript reaches it peak around midmorning (Aronson 1994). Following this accumulation of the *frequency* transcript, the FRQ protein begins to rise in concentration, and it reaches its peak approximately 4 - 6 hours after the mRNA (Dunlap 1998). After translation, the proteins begin to become phosporylated, and then they enter the nucleus to suppress their own transcription, resulting in a decrease in the *frq* mRNA (Aronson 1994). Once FRQ protein is heavily phosphorylated, it is degraded, and this takes place after approximately 12 hours. In the absence, or minimized presence of FRQ protein to limit transcription, mRNA concentration again rises, and the cycle starts once more.

This negative feedback loop appears to follow the period of the conidiation cycle, somewhat implying its role in the cycle. The most meaningful evidence of *frq*'s importance to this circadian rhythm, however, appears when investigating FRQ-null mutant strains. Losing a functional FRQ protein does not completely abolish the conidiation rhythm, but it does lose qualities of a defined circadian cycle. For example, the FRQ-null strains frq^9 and frq^{10} are no longer able to be entrained by light, and they will begin to extend across the tube at a faster rate when the temperature rises (Chang 1997). When assayed on a race tube in constant conditions, these mutant strains will show a slight amount of conidiation across the length of the race tube in an arrhythmic

Figure 3: The Negative Feedback Cycle of the *frequency* Gene

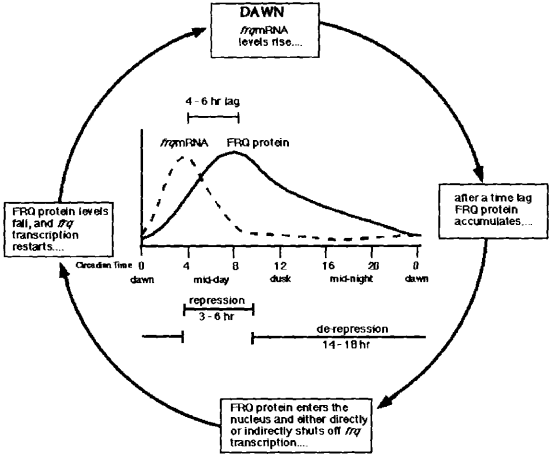

Figure 3 Legend: This figure illustrates the negative feedback loop of FRQ. At the beginning of the day, the mRNA levels start to rise. After four to six hours, the protein levels rise, and FRQ enters the nucleus to inhibit its own transcription. As FRQ protein disintegrates, the transcription restarts, mRNA rises again, and the loop has gone full circle.

form. Therefore, losing a functional FRQ protein does not abolish the development of conidia. However, it does cease to abide by the complete set of standards for circadian rhythms.

Another interesting revelation can be found when the *frq* gene becomes over-expressed. To see this, researchers placed the gene behind an inducible promoter. The construct was transformed into *Neurospora* wild type strains. What they found was that when the ectopic *frq* gene was induced it was not expressed rhythmically and the fungus lost its rhythmicity in the conidiation cycle. However, the protein continued to enter the nucleus to inhibit transcription at the endogenous *frq* promoter, demonstrating negative feedback. Apparently, this feedback inhibition is necessary for normal cyclic output.

Yet more evidence of FRQ's presence in the circadian clock lies in the reactions of FRQ to light and temperature. When pulsed with light after growing in the dark for some time, the fungus will display a quick rise in the *frq* mRNA, overriding the protein-driven inhibition (Crosthwaite 1995). If given a large enough amount of light at any given time of the day, both the transcript and the protein will alter in level enough to reset the phase of the rhythm. Temperature will also affect the cyclic nature of FRQ, but this happens through a different pathway. The mRNA of *frq* will encode two types of protein, a long form (LFRQ) and a short form (SFRQ) (Garceau 1997). At higher temperatures, more LFRQ is produced, but with enough of either protein, the cycle will continue. Therefore, within certain temperatures, the rhythm will maintain its cyclation (Liu 1997). A temperature change will reset the phase of the rhythm at first, but the system adapts soon to the new environment with no change in its period.

Two other proteins, known as WHITE COLLAR-1 (WC-1) and WHITE COLLAR-2 (WC-2), also participate in *Neurospora*'s conidiation cycle. They are part of a positive feedback cycle in the FRQ-oscillator and are believed to give positive input into FRQ's rhythmic expression. This is due to the requirement of both WC-1 and WC-2 in order to provide a cycle of FRQ in *Neurospora* (Crosthwaite 1997). WC-1 is found primarily in the nucleus, whereas WC-2 can be found in both the nucleus and the cytoplasm. The mRNAs of these two proteins are not rhythmically transcribed, but the WC-1 protein is found to cycle in concentration out of phase with FRQ protein accumulation. This is due to FRQ's activation at a post-transcriptional level to promote WC-1 accumulation (Lee 2000, et al). Both WC-1 and WC-2 interact with each other

through their PAS domains to make heterodimers. These PAS domains are found in many other organisms, and they are linked to the circadian response to environmental signals, such as light. Currently, researchers are uncertain as to how this works in the cell. Although not supported with evidence, it has been suggested that the proteins' phosphorylation when exposed to light alters their activity (Schwerdtfeger 2000, et al). This suggestion would provide even more evidence that WC-1 and WC-2 play a vital role in the conidiation cycle's circadian clock.

FRQ-null strains do not display rhythmic conidiation on standard growth media in constant temperature at approximately 25°C and constant darkness (Figure 4 top). However, under some growth conditions, some residual rhythmicity has been reported (Loros et al., 1989). The conidiation rhythm did not appear in all cultures, its period

FIGURE 4: Rhythmicity in FRQ-null strains

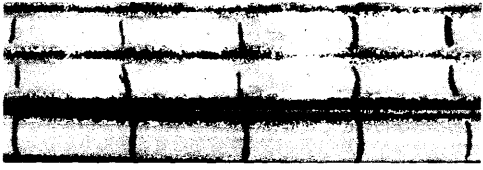

Figure 4 Legend: This picture shows two different types of race tube assays, both with FRQ-null mutants. The top two race tubes were placed in a temperature cycle of 22°C for twelve hours and 27°C for twelve hours. The bottom race tube was placed in the dark room at a constant temperature of 25°C. The lighter areas on the top tube are bands of conidia, and the dark lines on the race tubes signify the 24-hour growth fronts. Obviously, the race tubes assayed in the temperature cycle display a rhythmic output of conidiation, whereas the tube placed in constant conditions did not.

varied from 12 hours to 35 hours, it cannot be entrained by light, and it lacks the temperature compensation (Loros et al., 1989; Loros 2001). However, in recent experiments, when FRQ-null cultures were placed in a temperature cycle of twelve hours at 22°C and twelve hours at 27°C, these mutants began to exhibit a robust rhythm. The rhythm was demonstrated to not be driven by the temperature cycles, but rather had characteristics of a circadian rhythm. These data suggest that a secondary oscillator is present in the cell that relies on temperature for its input. Without a functional FRQ-based oscillator, this putative secondary oscillator can be uncovered. Based on the demonstration of rhythms in FRQ-null mutants, I hypothesize that there are other oscillators controlling rhythmicity in Neurospora crassa that do not completely depend on the FRQ-based oscillator.

In order to test this hypothesis, I sought to create and identify mutant strains that had lost the ability to display developmental rhythms in a temperature cycle. In overview, the *frq*^{10} strain was mutated, in the hopes of identifying mutations that affected genes in the hypothesized secondary oscillator. Following mutagenesis, the simple race tube assay was used to screen for mutations that are arrhythmic in the temperature cycle. This simple assay would provide the ability to differentiate between mutant samples that had a mutation marking a gene required for function of the second oscillator and those that displayed the *frq*^{10} behavior. Once such mutations are identified, other experiments can provide information as to the location and sequence of the mutated gene. Further research may include discovery of the functions of the gene and its gene product. Thus, this simple assay could open the door to a wealth of new information regarding the circadian clock system in fungi and higher eurkaryotes.

Chapter 3: Materials and Methods:

To begin the project, we first cultured *mcm*; *frq*^{10} strains on minimal media slants, containing 2% sucrose. The *mcm* mutant was used due to its ability to produce

microconidia with only one nucleus. This is helpful in mutagenesis because microconidia with a single nucleus will display mutations, but those with multiple nuclei would mask recessive mutations. The *frq*^{10} strain was used for mutagenesis. This strain contains a hygromycin-resistance gene in place of the *frq* open reading frame. Hygromycin can be used to select for colonies that still contain the FRQ-null mutation. Upon growth, the conidia were resuspended in liquid Vogels minimal media (Davis and DeSerres, 1970) containing 2% sucrose, and cultured to isolate microconidia. After incubating the fungus at 22°C for 24 hours, we then harvested the microconidia. In this step, the liquid media was drained through a double layer of cheesecloth into a sterile tube in order to separate the suspended microconidia from the macroconidia, which do not flow through the cheesecloth. The concentration of the microconidia was determined through the use of a hemocytometer, and the microconidia was diluted to 500 cells/mL. We then plated the sample onto media containing 2% sorbose, 0.05% glucose, 0.05% fructose, and 200ug/ml hygromycin to maintain the selection for the *frq*^{10} allele and rule out contamination of the cultures from wild type strains. The sorbose was added to the media to allow for tight colony growth on plates.

The cells were mutated using ultraviolet light exposure. In preliminary work, we tested both a control plate that was exposed to no ultraviolet (UV) light, as well as several experimental plates exposed to the ultraviolet light for various lengths of time. This type of mutagenesis can create a variety of mutations, such as inversions, deletions, etc. After a few tests, we concluded that exposure for 12 seconds killed approximately half of the colonies in comparison to the colonies growing on the control plate. For the remainder of the experiment, we irradiated our samples with UV light for 12 seconds and then covered them in foil to grow in 34°C for 48 hours. These samples were kept in the dark in order to eliminate light-induced repair systems. Our ideal was to create stains with one mutation per sample, and this is often achieved using procedures that result in 50% survival.

The mutated cultures were plated on sorbose plates to form colonies and were incubated at 30°C for two days. Colonies were transferred to minimal agar slants to

eliminate metabolic mutants. The slants were incubated at 30°C for several days and stored at –20°C. Conidia from the cultures were then examined on race tubes containing 0.017% arginine and 0.01% glucose. The race tubes were placed in the dark room in a temperature cycle of twelve hours at 22°C and twelve hours at 27°C. After reaching the end of the race tube in approximately five to seven days, the mutant strains were checked for loss of rhythmicity and normal growth. The arrhythmic mutants were assayed again on the race tubes to verify their phenotype.

An alternative method was used to mutate the cultures involving random insertional mutagenesis. This process would provide insertions throughout the genome of the fungus. Because the sequence of the plasmid insert is known, locating the disrupted gene from this mutagenesis would be greatly simplified. In this procedure, the microconidia were cultured and harvested, as described above, and then the microconidia were diluted to 1×10^9 cells/mL by adding sorbitol to the solution. The benomyl resistance plasmid, pMLSBN #134, was added to the cells, and incubated on ice for five minutes. We then performed electroporation on the suspension, added more sorbitol, and quickly mixed with melted top agar for pouring onto plates containing 2% sorbose, 0.05% glucose, 0.05% fructose, and 200ug/ml hygromycin. The cultures were allowed to grow at 34°C for 48 hours, and then benomyl resistant colonies were picked to minimal slants containing benomyl for storage and the cultures were later assayed on race tubes, as described above.

Chapter 4: Results

As described in Materials and Methods (Chapter 3), my project began by first investigating the appropriate strains and conditions to identify mutations that affect rhythmicity in temperature cycles. We discovered that exposing *Neurospora* cells to ultraviolet light for a length of 12 seconds resulted in a survival rate of 50%. Under these conditions, we expect that the survivors have a high frequency of single mutations. Higher kill rates often result in multiple mutations. When optimizing the race tube

assays, we concluded that a temperature cycle of 12 hours at 22°C and 12 hours at 27°C provided the most reliable banding pattern in the *FRQ-null* strains. Therefore, this protocol was used to identify arrhythmic mutants. We also concurred that the UV mutagenesis method provided an easier means to generate high numbers of mutant strains as opposed to insertional mutagenesis using the transformation procedure. Therefore, we focused on the UV mutants for subsequent screening. During the course of these experiments, we found more robust rhythms were detected in double mutant frq^{10}/wc-2 strain. Since WC-2 is required for normal oscillations as well as normal light responses, this strain lacks the FRQ-based oscillator and is also deficient in blue-light responses. Thus, one additional advantage of this strain is that we can rule out any contribution of light to the observed rhythms in the temperature cycles.

Over 1500 mutant colonies were picked from the sorbose-containing plates to agar slants for long-term storage, and of these, 1225 mutant strains were examined on race tubes in constant darkness at 25°C and in the temperature cycles. As expected, the majority of the strains displayed the normal frq^{10} behavior of arrhythmicity in constant temperature and rhythmicity in the temperature cycle. Some of these mutants grew poorly on the minimal slants. Once transferred to race tubes, however, the mutants with poor growth did not always continue to develop poorly, possibly due to the change in environment. The mutants that did not grow well on race tubes (36) or showed rhythms in the temperature cycles were not studied further.

Importantly, of the 1225 mutant strains analyzed, 22 arrhythmic strains were identified in the temperature cycles (Figure 5). Eight of these had poor development and are not being further studied. However, 14 of the mutant strains grew and developed normally. Therefore, of the 1225 strains we screened, 1.14% have the desired phenotype.

Figure 5: Results of Temperature Cycle Assays

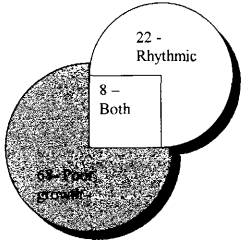

Figure 5 Legend: This graph shows the outcome of our research on the FRQ-null mutant strains. After mutating these samples, they were placed in temperature cycles in the dark room and observed for loss of rhythmicity. Only 22 out of 1225 lost their rhythmicity, but eight of those showed poor development. The remaining 14 will continue to be tested.

Chapter 5: Discussion

The major goal of this study was to test the hypothesis that additional oscillators, those that function without well-characterized FRQ-based oscillator, exist in *Neurospora* cells. Through the identification of mutant strains that abolish the residual rhythms in FRQ-null cells, I have made major progress towards this goal. The idea is that the mutations will identify genes that are required for the function of the alternative oscillator. The mutations could conceivably be in genes that are required for the oscillators' function, or in the genes that are involved in the temperature resetting of the oscillator. Future experiments to test where within the circadian system the gene functions could include placing the gene of the mutation behind a constituitive promoter and assaying these samples in temperature cycles. Should the new FRQ-null strain

continue to display a rhythm in the temperature cycle, this experiment would imply the location of the gene controlling the oscillator's funtion. If the mutant strain instead displayed a constant, arrhythmic output of conidia, the results would imply the finding of the locus of the temperature-resetting gene.

Prior to any additional experimentation, each strain is being back-crossed using the sexual reproduction cycle of *Neurospora crassa* on order to eliminate any extraneous mutations caused by the mutagenesis procedures. After reexamining the progeny for normal growth and arrhythmicity in the temperature cycles, the arrhythmic progeny will be examined in heterokaryon tests to determine allelism and dominance. This will be accomplished by crossing each mutation into two strains, each containing a different auxotropic marker (such as *his* and *arg*). The double mutant strains will then be cultured together (each containing a different marker), and all combinations will be tested for growth on minimal medium. Only heterokaryons will survive. The survivors will then be assayed on racetubes in the temperature cycles. If the mutations reside within the same gene, or if one is dominant to the other, we expect to see arrhythmic heterokaryons. If the mutations reside at different loci and are recessive, then they should complement each other and result in rhythmic heterokarons. Strains containing mutations in different loci will be crossed to a multiply marked strain in order to map the mutation to a particular linkage group. The alcoy strain contains markers on each of *Neurospora*'s seven linkage groups. Once a linkage group is determined for each mutation, additional markers on that linkage group will be examined for linkage. In this way, the mutation can be mapped to particular regions of the chromosomes. Once the mutations are genetically mapped, the information will be used in attempts to locate a cosmid that contains the gene of interest using the physical map. When a candidate cosmid is located, it will first be used to test for rescue of the mutation. Rescue can be accomplished by transformation of the cosmid into the mutant strain. Typically, DNA insertion in *Neurospora* is by random ectopic insertion, and this usually results in rescue frequencies of approximately 20%. If rescue occurs with a particular cosmid, the sequence of the cosmid will be examined for potential open reading frames that could

encode the gene of interest. Each of the cosmids has been sequenced. Educated guesses will be made to determine which open reading frame might encode the protein of interest. This will be base in part on the known functions of the encoded proteins. Once a candidate gene is determined, primers will be made and used to generate a PCR product of the gene from the mutant genomic DNA. This PCR product will be sequenced. If the chosen gene is correct, we could expect to identify one or more alterations in the PCR product as compared to the wild type sequence. All of the mutants that were identified as having the desired phenotype were from UV mutagenesis, and the genes specified by the mutation could contain transitions, transversions, deletions, or inversions. If sequence alterations are observed in the candidate gene, the wild type gene will be subcloned from the cosmid and then used to rescue the mutation. Both rescue using the open reading frame and the mutated sequence will be evidence to suggest that the gene of interest has been determined. Once the genes identified by the mutations are determined, we can then attempt to discover the function of these genes through experiments described earlier in this section.

If these mutant strains are not indicators of a secondary oscillator, they could also indicate a metabolic mutant. This would provide an explanation for the poor growth seen on race tube assays in a little less than half of the arrhythmic mutants. Perhaps when assayed on a different medium with other metabolites and at varying concentrations, we might see a return to the temperature entraining rhythm. This could still provide interesting new data with further research, such as what organic molecules are required for the temperature-dependent rhythm and where the genes for their metabolic pathways lie on the chromosomes.

Should further research on these arrhythmic mutant strains not support my hypothesis, it would certainly not mean the end of the search for evidence of a secondary oscillator in *Neurospora crassa*. Future assays could screen for extragenic suppressors of FRQ protein under growth conditions in which the *frq* null allele is normally arrhythmic. This could be done by creating more mutants in a FRQ-null strain and

screening them in a constant temperature in constant darkness. Should they show a return of rhythm, this would imply that an extragenic suppressor has affected the temperature-driven cycle. These extragenic suppressors may lead to more information on circadian rhythm of the conidiation of *N. crassa*.

On the other hand, our mutant strains may prove to be genuinely important in providing evidence of my hypothesized temperature dependent oscillator. The future experiments explained in the previous paragraphs may provide support for this belief. Should this not prove to be true, our mutants will still be of value to the research community in order to look into these mutants that have lost the function of this mysterious oscillation. Therefore, this experiment has provided the research community with 14 mutant strains that can be used to study this hypothesis of the multiple oscillators in *Neurospora crassa*.

References:

Aronson, B. D., K. A. Johnson, J. J. Loros, and J. C. Dunlap. "Negative feedback defining a circadian clock: Autoregulation of the clock gene *frequency*." *Science* 263: 1578-1584, 1994.

Bell-Pedersen, D. "Understanding circadian rhythmicity in *Neurospora crassa*: From behavior to genes and back again." *Fungal Genetics and Biology* 29: 1-8, 2000.

Brzezinski, A. "Melatonin in humans." *N Engl J Med* 336: 186-195, 1997.

Chang, B. and H. Nakashima. "Isolation of temperature-sensitive rhythm mutant in *Neurospora crassa*." *J. Plant Res.* 110: 449-453, 1998.

Copinschi, G., K. Spiegle, R. Leproult, and E. Van Couter. "Pathophysiology of human circadian rhythms." *Novartis Found Symp* 227: 143-157, 2000.

Crosthwaite, S. K., J. J. Loros, and J. C. Dunlap. "Light-induced resetting of a circadian clock is mediated by a rapid increase in *frequency* transcript." *Cell* 81: 1003-1012, 1995.

Crosthwaite, S. K., J. J. Loros, and J. C. Dunlap. "*Neurospora wc*-1 and *wc*-2: ranscription, photoresponses, and the origins of circadian rhythmicity." *Science* 76: 763-769, 1997.

Davis, R. H. and F. J. deSerres. "Genetic and microbiological research techniques for *eurospora crassa*." *Methods Enzymol.* 17A: 79-143, 1970.

Edery, Isaac. "Circadian rhythms in a nutshell." *Physiol Genomics* 3: 59-74, 2000.

Edmunds, L.N. Jr. *Cellular and Molecular Bases of Biological Clocks. Models and Mechanisms for Circadain Timekeeping.* New York: Springer, 1988.

Feldman, J.F. and Hoyle, M.N. "Isolation of circadian clock mutants of *Neurospora crassa*." *Genetics* 75: 605-613, 1973.

Garceau, N., Y. Liu, J. J. Loros, and J. C. Dunlap. "Alternative initiation of translation and time-specific phosphorylation yield multiple forms of the essential clock protein FREQUENCY." *Cell* 89: 469-476, 1997.

Gooch, B. D., R. A. Wehseler, and C. G. Gross. "Temperature effects on the resetting of the phase of the *Neurospora* circadian rhythm." *J. Biol Rhythms* 9: 83-94, 1994.

Kay, S.A. "PAS, present, and future: clues to the origins of Circadian clocks". *Science* 276: 753-754, 1997.

Kondo, T., and M. Ishiura. "Circadian rhythms of cyanobacteria: monitoring the biological clocks of individual colonies by bioluminescence." *J. Baceriol.* 176: 1881-1885, 1994.

Lakin-Thomas, P. L., G. G. Coté, and S. Brody. "Circadian rhythms in Neurospora crassa: biochemistry and genetics." Critical Rev. Microbiol. 17: 365-416, 1990.

Lee, K., J. Loros, and J. C. Dunlap. "Interconnected feedback loops in the *Neurospora crassa* circadian system." *Science* 289: 107-110, 2000.

Loros, J. J., S. A. Denome, and J. C. Dunlap. "Molecular cloning of genes under control of the circadian clock in *Neurospora*." *Science* 243: 385-388, 1989.

Loros, J. J. and J. C. Dunlap. "Genetic and molecular analysis of circadian rhythms in *Neurospora*." *Annu. Rev. Physiol.* 63: 757-794, 2001.

Liu, Y., N. Y. Garceau, J. J. Loros, and J. C. Dunlap. "Thermally regulated translational control of FRQ mediates aspects of temperature responses in the *Neurospora* circadian clock." *Cell* 89:477-486, 1997.

Nakashima, H. "Phase shifting of the circadian clock by diethylstilbestrol and related compounds in *Neurospora crassa*." *Plant Physiol* 70: 982-986, 1982.

Orbach, M., D. Vollrath, R. Davis, and C. Yanofsky "An electrophoretic karyotype for *Neurospora*." *Mol. Cell Biol.* 8: 1469-1473, 1988.

Ouyang, Y., C. R. Andersson, T. Kondo, S. S. Golden, and C. H. Johnson. "Resonating circadian clocks enhance fitness in cyanobacteria " Proc. Natl. Acad. Sci. USA 95: 8660-8664, 1998.

Perkins, D. D. "*Neurospora*: The organism behind the molecular revolution." *Genetics* 130: 687-701, 1992.

Pittendrigh, C. S., B. G. Bruce, N. S. Rosensweig, and M. L. Rubin. "Growth patterns in *Neurospora*." *Nature* 184: 169-170, 1959.

Roenneberg, T. and M. Merrow "Molecular circadian oscillators: an alternative hypothesis." *J Biol Rhythms* 13: 167-179, 1998.

Ronneberg, T. and M. Mittag. "The circadian program of algae." Semin. Cell Dev. Biol. 7: 753-763, 1996.

Roenneberg, T. and D. Morse. "Two circadian oscillators in one cell." *Nature* 362: 362- 364, 1993.

Sargent, M.L., W.R. Briggs, and D.O. Woodward. "Circadian nature of a rhythm expressed by an invertaseless strain of *Neurospora crassa*." *Plant Physiol* 41: 1343-1349, 1966.

Schwertfeger, C. and H. Linden. "Localization and light-dependent phosphorylation of white collar 1 and 2, the two central components of blue light signaling in *Neurospora crassa*." *Eur. J. Biochem.* 267: 414-421, 2000.