CHARACTERIZATION OF A CIRCADIAN RHYTHM MUTANT IDENTIFIED IN A GENETIC SCREEN IN *NEUROSPORA CRASSA*

A Senior Honors Thesis

by

VICTOR VASCO KEASLER IV

Submitted to the Office of Honors Programs
& Academic Scholarships
Texas A&M University
in partial fulfillment of the requirement of the

UNIVERSITY UNDERGRADUATE
RESEARCH FELLOWS

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ABSTRACT

Characterization of a Circadian Rhythm

Mutant Identified in a Genetic Screen

in *Neurospora crassa*. (April 2002)

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Most organisms display daily rhythms in biochemical, physiological, and behavioral events. Examples of these rhythms include photosynthesis in plants and sleep/wake cycles in birds and mammals. These daily rhythms are controlled by an internal timekeeper called the circadian clock. In the filamentous fungus *Neurospora crassa*, asexual spore formation, known as conidiation, is under control of the circadian clock. *Neurospora* is a premier organism for the study of the circadian clock because the fungus is easy to manipulate, both genetically and biochemically, and the conidiation rhythm is easily monitored using specialized growth tubes called race tubes. One property of the circadian clock is that it can be reset by an environmental stimulus, such as a light or temperature pulse. This resetting is easily viewed on a race tube as a change in the phase of the conidiation rhythm. To identify genes involved in clock resetting, a genetic approach has been undertaken. In this approach we use ultraviolet (UV) light to mutate *Neurospora* conidia. Following germination, the mutagenized cells

are tested on race tubes for a loss of rhythmicity or failure to reset their clock in response to a light pulse. To date, 1300 mutagenized cells have been tested and twenty mutant strains with an altered developmental rhythm have been identified. I am currently characterizing one of the mutant strains, which is arrhythmic. The goal of this project is to genetically map the mutation that causes arrythmicity and clone the respective gene.

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INTRODUCTION

Circadian rhythms are biological rhythms observed in bacteria, fungi, plants, and animals. The term circadian is derived from the Latin words *circa diem* meaning "about a day" (Halberg et al., 1959). Several examples of biological phenomena under control of a circadian clock are sexual and asexual reproduction, bioluminescence, hormonal levels, and activity/rest cycles (Lakin-Thomas et al., 1990). Other examples that illustrate the time of day specificity of the circadian clock include a marine dinoflaggelate glowing at night, opening of a plants' leaves to capture the sunlight during the day, insect emergence from a pupal case in the early evening, and fungal spore development early in the morning (Bell-Pedersen, 1998).

The characterization of circadian rhythms can be traced back as far as the 1700's to a French Astronomer named Jean Jaques d'Ortous De Marian. He made a seminal observation about these daily rhythms. He observed that plants would open and close their leaves with a 24-hour period that persisted under constant environmental conditions (deMarian, 1729). It was later found that the rhythm could be reset in a time-dependent manner by environmental signals and that the period remains unchanged over a broad physiologically relevant temperature range - termed temperature compensation (Pittendrigh, 1960).

These characteristics are interesting because the environment is a variable place

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This thesis follows the style and format of *Cell*

due to the earth's rotation. There are constant changes in the temperature, illumination, humidity, UV irradiation, color of ambient illumination, magnetic field strength, as well as the activity of other organisms (Feynman et al., 1964). However, organisms use their clock to keep in synchrony with the changing environment. This allows an organism to anticipate environmental change and coordinate activities to the appropriate time of day.

The next important step is to identify an organism in which to study mechanisms of circadian rhythms. It has been shown that Seasonal Affective Disorder, as well as certain types of depression are linked to a malfunction of the circadian clock. Therefore, the ideal way to study the clock is in humans. However, human experimentation is difficult to do both legally and ethically. That is why Stephan and Zucker's research in identifying independent self-sustaining clocks in the suprachiasmatic nucleus (SCN), retina, and non-mammalian pineal gland has been such a breakthrough (Stephan and Zucker, 1972). Tissues used to derive primary cell cultures have shown circadian rhythmicity, which establishes the cell-autonomous nature of the clock in multicellular organisms. Similar demonstrations in unicellular eukaryotes and prokaryotes of circadian rhythms that share the same defining properties of the clock with that of multicellular organisms indicates that these simple organisms are useful as model systems for unraveling the mechanism of the clock. Therefore, a simple organism such as *Neurospora crassa* provides an appropriate model organism for studying the circadian clock.

In studying the molecular components of the clock in multiple organisms, it has become apparent that some of the components have been well conserved throughout evolution (reviewed by Bell-Pedersen, 1998). Despite some variety in the details, the scheme of a molecular transcription/translation-based feedback loop at the basis of circadian timing is seen in organisms from cyanobacteria to fungi to flies to rodents. Therefore, advances in any of these systems will likely have a broad impact on our understanding of the circadian clock in all organisms (Bell-Pedersen, 1998).

N. crassa is known as the orange bread mold. It grows as branching, thread-like cells called hyphae, which grow together and form vegetative mycelium. *N. crassa* has perforated cell walls that allow for cytoplasm and nuclei to travel between compartments (Springer, 1993). The organism has three basic sporulation pathways including ascospore formation, microconidiation, and macroconidiation. Ascospores are produced when there is a limited amount of nitrogen available for use. The vegetative hyphae initiate the sexual sporulation pathway by forming protoperithecia, which are the female reproductive structures (Westergaard et al., 1947). The protoperithecia then fuse with cells of the opposite mating type, and the fertilized cell undergoes one round of meiosis and two rounds of mitosis to produce four pairs of meiotically-derived ascospores in each perithecia (Bistis 1981; Raju, 1980). The ascospores are dispersed by forcible ejection into the air, and will germinate upon heat shock or exposure to certain chemicals.

Microconidia are small and uninucleate (Lowry et al., 1967). They are formed asexually within vegetative hyphae, have a grayish brown color, and germinate at low

rates. Macroconidia, on the other hand, are larger than microconidia and are multinucleate. The macroconidiation pathway is induced by desiccation or carbon source deprivation. Vegetative hyphae receive a signal to induce development, at which time aerial hyphae emerge and through a series of budding steps produce bright orange spores (Ricci et al., 1991). The signal can be from environmental cues or from the endogenous circadian clock. In the absence of environmental signals spore development is time-of-day specific and occurs in the early morning. The advantage of producing macroconidia at this time may be because this is when the wind will disperse them the best. The conidiation rhythm is the circadian phenotype studied in my experiments.

N. crassa provides a good model for study of the circadian clock for several reasons. First of all, it produces a large amount of ascospores in a fairly short period of time. This allows for genetic crosses to be scored quickly and completely. Also, there are a large number of genetically mapped mutant strains in *N. crassa* (Perkins et al., 1982). The genome was sequenced by the Whitehead Institute approximately one year ago and is available for research purposes (http://www-genome.wi.mit.edu/annotation/fungi/neurospora/). The genome contains 4.3×10^7 base pairs encoding about 10,000 genes. *N. crassa* is haploid for most of its life, and it has seven chromosomes, which are also referred to as linkage groups. Heterokaryons can also be created in *N. crassa* that allow for research on otherwise dominant and lethal recessive alleles.

In order to visualize clock-regulated conidiation, *N. crassa* is inoculated onto a petri plate or race tube. Petri plates were used originally, but it was determined that a petri plate does not allow for long enough periods of visualization. At that time the

"race tube" was used to examine growth rates of auxotrophs, but it was determined that it would also be effective for use in studying clock-regulated conidiation. Race tubes are approximately 30-40 cm cylindrical glass tubes with their ends curved up at a 45° angle (Figure 1). The ends of the tubes are plugged with cotton.

Figure 1. Race Tube

This is an example of the design used for the race tubes.

To grow *N.* crassa cultures for analyzing the conidiation rhythm, an agar-based, minimal medium is used in the race tube and a small amount of conidia are inoculated at one end. The tube is left in the light for approximately 24 hours, the growth front is marked, and then the tube is placed into a temperature-controlled dark room. Multiple race tubes can be transferred simultaneously into the dark room in order to set their clocks to subjective dusk, allowing for studies to be done on strains that are all set to the same circadian time. The race tubes are marked at the same time everyday for approximately 7-10 days using a red safety light, which has no resetting effect on the clock. After this period of time they are removed from the dark room and can be analyzed for rhythmicity, period, phase, growth rate, and other visual phenotypes.

It has also been discovered that wild-type *N. crassa* does not conidiate in the race tubes. This is because *Neurospora* accumulates carbon dioxide near the surface of the

culture, which inhibits conidiation. One way to alleviate this problem is to blow fresh air across the colony, but this tends to contaminate the cultures. Instead, a mutant strain of *N. crassa* is used that allows for conidiation despite build-up of CO_2. This strain is called *band (bd)*, and in addition to its ability to grow despite high CO_2 levels, it also grows about 30 % slower than the bd^+ strain (Sargent et al., 1972). The mutation was termed *band* because once per circadian cycle there is a more dense zone of conidiation. The area of more dense conidiation is termed the "band" and the region between the bands where there is less dense mycelial growth and few conidia is termed the "interband." This alternating pattern provides a "fossil" record of the rhythm that can easily be studied (Figure 2).

Figure 2. Rhythmic and Arrhythmic Strains

This figure illustrates the difference between rhythmic and arrhythmic strains of *N. crassa*. Race tubes 1 and 4 show the band and interband pattern and numbers 2 and 3 illustrate an arrhythmic strain that conidiates constantly down the length of the race tube. The vertical lines represent 24-hour growth marks and the dots designate the centers of the conidial bands.

There are three major areas of the *Neurospora* circadian clock system that are currently under study (Figure 3). The first is the input pathway. This is the part of the

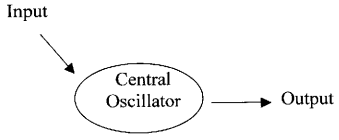

Figure 3. Simple View of the Clock
This is a basic design of the circadian clock. The three major components including the input, central oscillator, and the output are illustrated here.

clock that receives stimuli from the environment, such as temperature and light. Two input genes that have been characterized in *N. crassa* are *white collar* (*wc*-1) and *white collar* (*wc*-2). The products of these genes, WC-1 and WC-2, positively activate *frq* expression and are required for overt circadian rhythmicity (Harding et al., 1980). Experiments have also shown that mutation in *wc-1* completely block light induction of *frq*, and mutations occurring in *wc-2* allow only a transient *frq* photoresponse (reviewed by Bell-Pedersen, 1998). Also, *wc-2* mutant strains show a short-lived light and temperature-induced expression of *frq*, suggesting that *wc-2* acts as a positive element within the clock feedback cycle and is necessary for circadian clock function. Based on this data, it is believed that *wc-2* encodes a positive component of the oscillator and *wc-1*

is a positive-acting clock-associated gene that provides an important link between the light photoreceptor and a component of the oscillator (reviewed by Bell-Pedersen, 1998).

The second major part of the circadian clock is the central oscillator. It processes the information that is received from the input pathway. The *frq* gene is a major component of the central oscillator and has been well characterized. The *frq* gene was identified through chemical and UV mutagenesis; mutations of *frq* resulted in strains that had periods that were shorter, longer, or that even abolished the wild-type 22-hour conidiation rhythm. It has also been shown that both *frq* mRNA and FRQ protein cycle with a 22-hour period in wild-type strains grown in constant darkness. Appropriate changes in oscillation have also been seen in both short- and long-period mutant strains (reviewed by Bell-Pedersen, 1998). It takes approximately 1 day to complete a cycle of the *frq* feedback loop. This begins at dawn when both *frq* mRNA and protein levels are low. *Frq* transcript is the first to rise and it reaches a peak accumulation level about 4-5 hours later (just before noon). A delay of 4-6 hours is seen in the maximal FRQ protein levels as compared to *frq* mRNA such that *frq* message levels begin to fall prior to FRQ protein reaching maximal accumulation. It is therefore believed that FRQ protein enters the nucleus and acts rapidly in an indirect manner to repress the levels of *frq* mRNA by inhibiting the activity of the positive components WC-1 and WC-2. FRQ protein remains at a high enough level for the rest of the day and into the early evening in the nucleus to keep *frq* turned off. However, once FRQ protein begins to turnover and the levels fall below a critical mass, *frq* is no longer efficiently repressed and the cycle can

be restarted by positive factors encoded by *wc-1* and *wc-2* (reviewed by Bell-Pedersen, 1998).

The final component of the clock is the output. Very little is known about the output and to date there has been no component to link the oscillator and output together like the *wc* genes and the *frq* gene link input to the oscillator. Even though we know that organisms have a clock to temporally control a wide range of activities, we know very little about how the regulation takes place or of the clock proteins responsible for signaling time information to the rest of the cell. (reviewed by Bell-Pedersen, 1998). One strategy for studying the output is to identify genes that are rhythmically expressed, but have no effect on oscillator function. Currently 11 clock-controlled genes (ccgs) have been identified in *Neurospora* that have a variety of roles. For example, *ccg*-2 encodes a characteristic fungal hydrophobin (Bell-Pedersen et al., 1992; Lauter et al., 1992), *ccg*-12 encodes copper metallothionein (CuMT) (Bell-Pedersen et al., 1996b), *ccg*-7 encodes glyceraldehyde-3-phosphate dehydrogenase (GADPH) (Shinohara et al., 1998), and *al*-3 encodes geranylgeranyl pyrophosphate synthase (GGPPS) (Arpaia et al., 1995b).

EXPERIMENT

The first step was to create mutant strains that showed altered circadian rhythmicity. There are several ways to do this including UV mutagenesis, X-rays, and chemical mutagens. These types of mutagenesis can induce several different types of mutations such as single base-pair alterations, several to many base-pair deletions, deletions of two or more consecutive genes, and chromosomal rearrangements. For this project, UV mutagenesis was chosen because it is probably the most convenient, all-purpose mutagen. Several different time intervals of exposure of *bd* cells to UV light were tested in order to provide for approximately a 25 % survival. After UV irradiation, the plates with the *N. crassa* conidia were placed in complete darkness. This was done in order to prevent photoreactivation from repairing the induced mutations. After several days, the colonies that grew were picked onto minimal media in order to eliminate auxotrophic mutants. From here the individual strains were tested on race tubes in constant darkness (DD), or following a 1-hour light pulse, and those with altered conidiation rhythms were identified. The desired mutant strains fell into two major categories. Category I included strains that produced no "bands" as they grew on the race tubes. These strains are referred to as arrhythmic because they do not show the typical pattern of bands and interbands. Instead, they either strictly grow as mycelia or conidiate constantly down the length of the race tube. The second category (II) included strains that did not respond to a one-hour light pulse.

While in the dark room, the race tubes were given a one-hour light pulse in order to cause a phase shift in their conidiation pattern. This is manifested in the *bd* strain as either a phase advance or delay depending on when the light pulse is given. A light pulse given in the late night to early morning will produce a phase advance causing a premature band of conidia. On the other hand, a light pulse given in the late afternoon to early evening will produce a phase delay that is seen as a band of conidia further down the race tube than the control that did not receive the light pulse. Putative mutants were those that did not respond to the light pulse, suggesting that they were unable to recognize it. Further analysis of category II mutants was inconclusive; no mutant strains gave the desired results. Therefore, one strain from category I was chosen for further classification. This strain has been labeled LR75.6A. When LR75.6A is grown on a slant and compared to *bd*, little difference can be seen. The arrhythmic strain does have a slower growth rate both in a slant and on a race tube, but it does produce conidia in the slant. Therefore, the mutation does not preclude development entirely, only clock related development.

My first step to characterize LR75.6A was to ensure that only one mutation was responsible for the arrhythmic phenotype. This was accomplished by setting up a backcross of the mutant strain (LR75.6A) to the *bd* strain. The *bd* strain was used as the female and LR75.6A was used as the male. Their progeny were picked onto minimal slants and scored on race tubes. If there is only one mutation responsible for the arrhythmicity of LR75.6A, then there should only be two different phenotypes observed in all of the progeny. This is because the *Neurospora* progeny are haploid and so there

is no masking of alleles. The expected ratio was 1:1 for the arrhythmic phenotype and the *bd* phenotype. On race tubes, the arrhythmic phenotype is characterized by mycelial growth down the race tube, but no bands of conidia. The *bd* phenotype is an alternating pattern of bands and interbands down the length of the race tube. The period of conidiation should be approximately 22 hours and is measured by the following formula:

$$\frac{\text{Growth Rate}}{\text{Band Rate}} * 24 \text{ hours}$$

Sixty-seven progeny were tested on race tubes. Fifty-four percent had an arrhythmic phenotype and 46% showed the 22-hour *bd* period of rhythmicity. Table 1 shows the 1:1 expected ratio and only two phenotypes suggesting that there is only one mutation that segregates with one locus.

Table 1. Data from backcross of LR75.6A with the *bd* strain

	LR75.6,A X *bd,a*
Arrhythmic Progeny #/(%)	36 (54%)
Rhythmic Progeny #/(%)	31 (46%)

This table illustrates the 1:1 ratio seen from the progeny of the *bd,a* X LR75.6A cross.

The second step in the characterization process was to test for any linkage to the *frq* locus. Several alleles of the *frq* gene have already been well characterized, and we

do not wish to do any further characterization on this locus. In order to test for linkage, another cross was set up that involved *bd,frq7,a* as the female crossed with LR75.6A as the male. Progeny were picked and tested on race tubes. The defining characteristic of the *bd,frq7,a* strain is a 29-hour period as compared to the wild-type 22-hour period. This strain is, therefore, known as a long-period mutant. If there is not a close linkage between the *frq* locus and the location of the LR75.6A mutation, a 2:1:1 ratio will be seen in the progeny. That is because 50% will be arrhythmic, 25% will have the 22-hour *bd* period and another 25% will have a 29-hour long-period. The results did not seem to indicate that there was a close linkage between LR75.6A and the *frq* locus. Out of the fifty total progeny tested, 40% were arrhythmic and 60% were rhythmic. Of the rhythmic ones, 36% (of the total progeny) showed the wild-type 22-hour period and 24% showed the 29-hour long-period (Table 2). Furthermore, the phenotype of LR75.6A suggested that it is not an allele of WC-1 or WC-2.

Table 2. Data from cross of LR75.6A to *bd,frq7,a*

Period Length (#)	**22 hour** (18)	**29 hour** (12)
Number of Arrhythmic Progeny	20	

This table shows the period length and phenotype of progeny from *bd,frq7,a* X LR75.6A. The progeny in the top row are rhythmic and the progeny in the bottom row are arrhythmic.

Together, these initial analyses suggested that LR75.6A identifies a novel component required for normal conidiation rhythms. To genetically map the mutation, I set up a cross with a strain called alcoy. The alcoy strain has three chromosomal aberrations, which allow several chromosomal arms to be tested conveniently and simultaneously. Linkage groups I and II are marked with the *albino* (*al*) mutation. Progeny that are al^- will have a white color instead of their normal orange. The *colonial*(*cot*) mutation provides a temperature-sensitive marker for linkage groups IV and V. Strains that are cot^- do not grow at 37°, whereas strains that are cot^+ grow at this temperature. This phenotype can be tested easily by picking progeny from the cross and incubating all progeny at 37° immediately. After three or four days, those that do and do not grow are separated. The ones that did not grow can now be incubated at room temperature and within five to seven days they too will begin to grow. Also, linkage groups III and VI are joined with *yellow* (*yl*), a mutation that produces yellow progeny.

This mutation is the most difficult to work with because often the orange and yellow progeny look fairly similar. The best way to differentiate is to find one *yl*$^{-}$ progeny and one *yl*$^{+}$ progeny and compare all the rest of the progeny to those two. Each progeny is then examined for their race tube phenotype and linkage of arrhythmics to the markers is determined.

I determined that the LR75.6A mutation lies on either linkage group I or II because it was found to be linked to *al*$^{-}$. In order to determine to which of the two chromosomes the linkage is to, another genetic cross was used. I used *bd,his3eas,a* as the female and LR75.6A as the male. Progeny from this cross were picked and 108 were screened. The *bd,his3eas,a* strain has two convenient markers on it for linkage groups I and II. The first is the *his* mutation, which is an auxotroph that requires histidine to be present in the media in order to permit growth. The *his* mutation can easily be assessed by inoculating all candidate progeny onto minimal media slants with or without a 2.0mL stock/100mL of media concentration of histidine. The *his* marker is located on linkage group I. The other marker is *eas*, which stands for "easily wettable." The phenotype of this mutation is exactly what it sounds like. When inoculated on a slant, the progeny have a very moist look to them and the conidia stick to the walls of the test tube. The *eas* mutation is located on linkage group II.

Table 3. Data from cross of LR75.6A to *bd,his3eas,a*

	#/Total Screened	% of Total Screened
LR75.6A-, *eas*+	40/108	37.0%
LR75.6A+, *eas*-	46/108	42.6%
LR75.6A-, *eas*-	12/108	11.1%
LR75.6A+, *eas*+	10/108	9.3%
LR75.6A-, *his*+	24/108	22.20%
LR75.6A+, *his*-	31/108	28.70%
LR75.6A-, *his*-	28/108	25.90%
LR75.6A+, *his*+	25/108	23.20%

This data illustrates the suggested linkage between the LR75.6A and *eas* loci. Notice that the progeny do not separate in a 1:1 ratio as is seen between LR75.6A and *his*.

In order to determine linkage we must look at the percentage of the progeny as they segregated with the two markers. The first ones discussed in Table 3 are LR75.6A and *eas*. If there is no linkage we would expect to see the parentals and the recombinants segregate with a 1:1 ratio. However, if there are more parentals than recombinants, this would suggest that there is linkage. Also, the lower the percentage of recombinants obtained, the closer linked the two markers are. As one can see from the data, the two parental strains LR75.6A-, *eas*+ and LR75.6A+, *eas*- comprised almost 80% of the total progeny. The two recombinants LR75.6A-, *eas*-, and LR75.6A+, *eas*+ were only found

in approximately 20% of the progeny. This suggests strong linkage, but we also must consider the linkage between LR75.6A and the *his* marker. According to the data shown in Table 3, we observed almost a 1:1 ration between the parental progeny (LR75.6A-, *his*+; LR75.6A+, *his*-) and the recombinant progeny (LR75.6A-, *his*-; LR75.6A+, his+). One final comparison that needs to be made is how each individual marker segregated to demonstrate that the cross was successful. For example, *eas*+ and *eas*- should segregate in a 1:1 ratio.

Table 4. Expected ratios from cross of LR75.6A to *bd,his3eas,a*

	#/Total Screened	**% of Total Screened**
***eas*+**	50/108	46.3%
***eas*-**	58/108	53.7%
***his*+**	49/108	45.4%
***his*-**	59/108	54.6%
rhythmic	56/108	51.9%
arrhythmic	52/108	48.1%

This table shows that each individual loci separated in approximately a 1:1 ratio.

The data collected is very close to the expected ratio for *eas*, *his*, and rhythmic/arrhythmic progeny (Table 4), suggesting that there is a linkage between

LR75.6A and *eas*. The percentage of *eas*: LR75.6A recombinants indicate that LR75.6A lies 20 map units from *eas*. Further testing must now be done to determine on which side of the *eas* locus LR75.6A lies.

In order to make this determination, a three-point cross must be set up. This involves crossing one of the progeny from the *bd,his3eas,a* X LR75.6A cross with two other strains. One will be selected on either side of the *eas* locus. The test will again be for linkage and the one that shows the strongest linkage to LR75.6A will allow us to determine on which side of *eas* the LR75.6A mutation lies. The two markers to be used in this experiment are *arg*-5 and *trp*-3. The *arg*-5 strain is an auxotroph for arginine and will not grow without arginine supplementation. It is located approximately 49 centiMorgans (cM) from the *eas* locus (closer to the centromere). The *trp*-3 strain is also an auxotroph, and requires tryptophan supplemented in the media to grow. *Trp-3* is located approximately 12 cM from the *eas* locus (closer to the right end of the chromosome). Just like in the last cross, a 1:1 segregation is expected if there is no linkage between two loci.

Table 5. Data from cross of LR75.6A to *bd,arg-5,a*

	#/Total Screened	% of Total Screened
LR75.6A+, *arg*+	*10/101*	9.9%
LR75.6A-, *arg*-	*13/101*	12.9%
LR75.6+, *arg*-	33/101	32.7%
LR75.6A-, *arg*+	*45/101*	44.5%
***eas*+, *arg*+**	12/103	11.7%
***eas*-, *arg*-**	20/103	19.4%
***eas*-, *arg*+**	43/103	41.7%
***eas*+, *arg*-**	28/103	27.2%

The data illustrates linkage between the LR75.6A and *arg* loci. Notice that the majority of the progeny obtained were parentals. This was seen somewhat in the *eas* and *arg* loci, but not to the same degree.

In this cross, 103 progeny were analyzed and again the results were fairly clear. LR75.6A shows a strong linkage to the *arg* locus suggesting that our mutation lies somewhere between the *arg* locus and the *eas* locus. According to the data, approximately 77% of the progeny were parentals and only about 23% of the progeny were recombinants, suggesting that the distance between LR75.6A and *arg* is approximately 23 cM (Table 5). The data also shows that there is a small amount of linkage between the *arg* and the *eas* locus. This is expected as they are both on the same chromosome. However, the 69% parental progeny and the 31% recombinant progeny

are not significant. Again, examination of the percentages with which *eas*, *arg*, and rhythmicity segregated demonstrates the expected 1:1 ratio (Table 6).

Table 6. Expected ratios from cross of LR75.6A to *bd,arg-5,a*

	#/Total Screened	**% of Total Screened**
Eas+	41/104	39.4%
Eas-	63/104	60.6%
Arg+	55/103	53.4%
Arg-	48/103	46.6%
rhythmic	41/98	41.2%
arrhythmic	57/98	58.2%

This table shows that each individual loci separated in approximately a 1:1 ratio.

Now that we know a specific area in which the mutation lies, we can begin to search on a smaller scale for the exact location of the mutation using the known *Neurospora* sequence. This process involves looking at the genes that fall in the area between *eas* and *arg*-5. There are several genes in this area, but we suspect that the *female fertility* (*ff*) gene might be responsible for the mutation. This is because past crosses have shown an inability for LR75.6A to serve as the female. This is consistent with the *ff* characteristics, which include the inability to act as a female as well as a

reduced amount of total protoperithecia production. In order to test this hypothesis, an experiment was done involving growth of LR75.6A on a special media that prohibits growth in strains that have a non-functional *ff* gene. The media contains ascorbic acid, which is vitamin C. In order to test our hypothesis, both a *bd* strain and the LR75.6A strain were inoculated onto slants with this media and incubated at room temperature. The growth was monitored after two and three days and the results seemed variable. After two days, the *bd* strain showed healthier growth, but by three days both the *bd* strain and LR75.6A showed similar amounts of growth. A subsequent test will be done in order to compare the growth of both the *bd* strain and LR75.6A to the *ff* strain. Only after this is completed can further assessments be made.

Based on the *N. crassa* physical map, we have identified nine cosmids that cover the area where we believe the LR75.6A mutation lies. Each of these cosmids will be individually transformed into the LR75.6A strain and the transformants tested for an ability to restore the rhythmic phenotype. Once a cosmid is identified that restores the rhythm, DNA segments containing each open reading frame will be subcloned and the resulting plasmids transformed into the LR75.6A arrhythmic strain. Once the gene is identified, it will be carefully studied and characterized as to how it affects the clock.

Preliminary studies have looked at RNA production via a Northern blot in the LR75.6A strain in order to begin to ask which parts of the clock system are affected by the LR75.6A mutation. We first isolated RNA from LR75.6A every four hours over two consecutive days and asked if a known ccg was rhythmic. We probed LR75.6A RNA with *ccg-2* and it looks rhythmic (Figure 4). Along with the blot of LR75.6A is a second

blot using the *bd* strain. This blot was also probed with *ccg-2* and we are using it as a control. As we expect, a rhythm is observed in this blot (Figure 5) and approximately a 20-hour period is observed between the peaks at 12 and 32 hours. These results suggest, assuming that the clock operates in a linear fashion, that the mutation in LR75.6A is not affecting the central oscillator or the input since clock regulation of gene expression is still apparent in the mutant. Therefore, we believe that the LR75.6A mutation is manifested in an output component. However, it is also possible that this mutation is acting on the developmental pathway. In order to test this, a developmental induction experiment needs to be done. Cultures will be harvested after development is induced by desiccation after 0 and 30 minutes, as well as 1, 2, 4, and 8 hours. The RNA will be probed with a gene such as *fl* (*fluffy*) that is known to be developmentally regulated. This is done to test if normal developmental induction occurs in the mutant. We will also do several more Northern blots probed with *frq* as well as several other ccgs. The results of these Northern blots will allow us to determine where in the developmental pathway or in which clock component the mutation is acting.

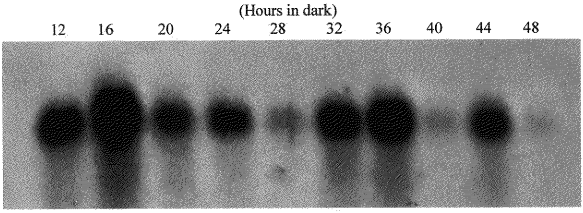

Figure 4. Northern Blot of LR75.6A

This is a Northern Blot of LR75.6A RNA probed with *ccg-2*. There are 10 time points on this blot representing samples that were taken every four hours from 12 to 48 inclusive. The two peaks are at 16 hours and 36 hours, suggesting approximately a 20-hour period. Excluding the point at 44 hours, there is evidence of a nice rhythm that cycles to a peak and then a trough.

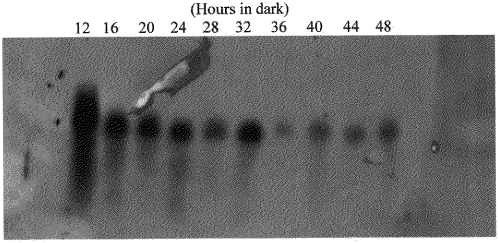

Figure 5. Northern blot of *bd*

This is a Northern Blot of *bd* RNA probed with *ccg-2* and used as a control. There are 10 time points on this blot representing samples that were taken every four hours from 12 to 48 inclusive. The two peaks are at 12 hours and 32 hours, suggesting approximately a 20-hour period.

SUMMARY

This project is not yet complete, but has accomplished much so far. We started by UV mutagenizing wild-type conidia in order to obtain a collection of mutant strains of *N. crassa*. The mutagenesis was done using different time periods in order to obtain approximately a 25% survival. From there we were able to test the strains on race tubes for altered growth phenotypes such as an inability to respond to a light pulse or arrhythmicity. This arrhythmic growth was observed as either myceilial growth or constant conidiation down the entire length of the race tube. These mutants were isolated and one was selected for further characterization. That strain has been labeled LR75.6A.

The LR75.6A strain produces conidia when grown in a slant, but does not do so when grown on a race tube. It also has a slightly slower growth rate than the *bd* strain when examined on a race tube. The growth rate of LR75.6A is approximately 2.43 cm/day and the growth rate of *bd* is about 3.36 cm/day. The first step in the process of mapping the mutation was to backcross LR75.6A to the *bd* strain in order to ensure that only one mutation was responsible for the arrhythmic phenotype. From this cross only two phenotypes were recovered; one was the *bd* phenotype and the other was the arrhythmic phenotype. Next a cross was done to check whether LR75.6A was closely linked to *frq*. The data showed that there was no close linkage to *frq*, and it was also observed that LR75.6A did not have the characteristics that would link it to *wc-1* or *wc-2*. From here, several more crosses were performed in order to determine on which

linkage group the LR75.6A mutation existed. I have determined that it is on linkage group II, between *arg-5* and *eas*.

At this point we believe that the mutation might be in the *ff* (*female fertility*) gene because the mutant strains shows two general characteristics that are true with *ff* strains. The first is a reduced amount of total protoperithecia produced and the other is the inability to serve as a female in a cross. In order to further test this hypothesis, I am transforming a series of cosmids into the LR75.6A strain. There are seven total cosmids that cover almost the entire area of the female fertility gene. Once I am able to complement the mutation with a wild-type copy, I should be able to see restored rhythmicity on a race tube.

Once I am able to restore rhythmicity the next project will be to determine where the mutation acts. It might be in one of the clock components or it might be in a developmental pathway. This will be determined by doing a series Northern blots with *frq*, several of the ccgs, and developmental mRNAs (such as *fl*).

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