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DEVELOPING A TOOL TO CHARACTERIZE THE ULTRADIAN RHYTHM IN DIPLOID SACCHAROMYCES CEREVISAIE USING THE REPORTER GENE GREEN FLUORESCENT PROTEIN

A Thesis

Submitted to the Graduate Faculty of the Louisiana State University and Agricultural and Mechanical College in partial fulfillment of the requirements for the degree of Master of Science in Chemical Engineering in

The Gordon A. and Mary Cain Department of Chemical Engineering

by

Imran Chiragh
B.S. University of New Mexico, Albuquerque, NM 2007
May 2011
DEDICATION

To my incredible parents Dr. Abdul-Latif Chiragh and Wajahat-un-Nisa Chiragh who have and continue to inspire, enlighten, and motivate me everyday
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ABSTRACT

Biological rhythms control many temporal behaviors of organism, such as the sleep cycle, hearts rhythms, seasonal animal migrations etc. Understanding these rhythms would provide insight into the temporal process of living organisms. *Saccharomyces cerevisiae*, a budding yeast, is an ideal model organism to study biological rhythms in eukaryotic cells because of its sequenced genome and discerned processes. By characterizing the biological rhythm in budding yeast, insight can be gained into more complex organisms.

Previous studies have exhibited oscillatory behavior of oxygen consumption and determined that deletion of the GTS1 gene dissipates this rhythm. However, to further understand the specific behavior of this gene, GTS1 needs to be simultaneous monitored as it is expressed. In this study to monitor this ultradian rhythm regulating gene, a promoter-reporter construct was inserted through homologous recombination to track the expression of GTS1 in a diploid yeast strain, BY 4743. The promoter-reporter construct replaced one copy of the GTS1. As the GTS1 was expressed, the construct was expressed and detected by its reporter gene, green fluorescent protein (GFP). Synchronization of the cell cycle and ultradian rhythm was achieved by addition of hydroxyurea and nocodazole to the growth media. GFP levels were quantified by flow cytometry, with samples taken every 10 minutes.

The results showed GFP expression level from the transformed yeast strain exhibiting a 3.33-fold increase relative to the non-transformed yeast strain. GFP expression yielded a biological rhythm with two identifiable periods, each with a 70 minute period. The first oscillation began at time zero and had a GFP expression maximum of 2.96 times the control level and a minimum of 2.62. The second oscillation began at 70 minutes had a GFP expression maximum of 3.09 times the control and a minimum of 2.76. The biological rhythm observed was
shorter than its own cell cycle, roughly 111 minutes. Oscillatory behavior was observed as long as the culture remained synchronous.

This study characterized the behavior of GTS1, an ultradian rhythm gene. By characterizing the behavior of this gene in *S. cerevisiae*, homologous genes in more complex organisms such as rodents or humans can be better understood. By extrapolating temporal behavior in yeast to humans, a cost effective drug prescreening can be implemented to evaluate possible biological rhythmic changes.
1.1 Motivation and Objective

All organisms, from unicellular to complex multicellular organisms, respond to environmental pressures. This ability to sense and change in reply to these environmental cues is crucial to species survival. Two of these environmental changes are the daily rhythms of light and temperature caused by the earth’s rotation. These variations affect food availability, predator activity, and biochemical processes. Due to the daily environmental pressures like UV radiation, organisms must be careful in replicating and expressing their DNA to avoid possible mutations. Therefore, it is easy to envision that the behavior and metabolism of most organisms by an endogenous clock would follow a 24-hour schedule.

Coinciding with the 24-hour schedule, living systems produce periodic oscillations in a variety of physiological variables. These oscillations better optimize the organism’s viability through a complex control system or internal clock. The physiological system responsible for measuring time and synchronizing an organism’s internal processes with the daily events in its environment is known as the circadian clock (Moore-Ede, et al., 1982). A wide variety of organisms display periodic behavior, ranging from fractions of a second to years. Examples of cycles longer than a 24-hour schedule, infradian rhythms, are ovarian cycles, annual rhythms, and ecological oscillations (Goldbeter). Ultradian rhythms, rhythms shorter than a 24-hour schedule, are neural rhythms, cardiac rhythms, and the yeast metabolic cycle.

Circadian and ultradian systems share numerous similarities. Both systems exhibit temperature compensation of periods, indicating a strong likelihood that the prime functions of both rhythms have timing roles (Lloyd, et al., 2007). Organisms live under conditions in which
temperature can suddenly change; therefore, an accurate robust clock must not run faster or slower as temperatures fluctuate. (Lloyd, et al., 2007). The circadian and ultradian systems share sensitivity to the Li\(^+\) concentration (Lloyd, et al., 2007) (Manji, et al., 2000). Li\(^+\) inhibits enzymes in circadian and ultradian rhythm organisms, lengthening the period of the rhythm (Yin, et al., 2006) (Dokucu, et al., 2005) (Lloyd, et al., 2007). Periodic alterations of circadian time scale have led to the discovery of organisms’ ability to shift a system to an ultradian time scale (Lloyd, et al., 2007). Circadian and ultradian systems are not only similar in the structure of timing keeping and external time cues such as light, food, temperature, and sound for alteration but are occasionally dependent on one another. Ultradian rhythms can also be utilized to create circadian rhythms by various methods, such as two frequency beats in which independent oscillators with different frequencies and a common output produce complex waveforms (Lloyd, et al., 2007).

Perturbations to this biological clock can affect health and lead to disease. Many drugs show biological rhythms in both toxic and therapeutic effects and can be manipulated to provide the most effective treatments (Moore-Ede, et al., 1982). Moreover, many illnesses are caused by a malfunction of the circadian timing system, including insomnia, affective disorders, testosterone levels, anorexia nervosa, Alzheimer’s disease, depression, and cancer. By understanding the process of the circadian rhythm, therapeutic methods can be develop to help individuals with these types of illnesses and to create drug prescreens evaluating a drug’s effect on biological rhythms.

To investigate biological rhythms, studies have employed *Saccharomyces cervisiae*, a budding yeast, because of its fully sequenced genome and highly adaptable DNA transformation system. Also, budding yeast cells are ideal organisms for this research due to their rapid growth
and ease of transfer to different media (Sherman, 2002). Therefore, budding yeast is a model eukaryotic system for diverse biological studies. In a study by Murray (Murray, et al., 2001), the oxygen consumption by budding yeast exhibited an ultradian rhythm when grown in a continuous flow reactor with acidic medium. Murray deleted the gene GTS1 and found a halving of the periodicity of oxygen consumption. GTS1 was found to be associated with the ultradian rhythm. However, further studies to understand the specific behavior of this gene in standard conditions has not been done. Moreover in the study by Murray, synchronization of the ultradian rhythm between cells was done by cell starvation, involving depletion of endogenous glycogen and trehalose. For cell starvation to occur, this technique requires many days and an acidic medium, with a pH of 3.4. In the study by Murray, GTS1 was recognized to be associated with the ultradian rhythm; however, expression levels of this gene have not been quantified. By quantifying the specific expression of GTS1, the ultradian rhythm can be directly monitored at the gene level. Tracking GTS1 will provide insight into the mechanics of the ultradian rhythm.

To accomplish this task, GTS1 must be monitored as it is expressed.

To address this issue, this study characterized the ultradian rhythm in diploid budding yeast, BY 4743, by monitoring the expression level of GTS1. To accomplish this task, first a novel yeast strain was created with a promoter-reporter construct that continuously monitored the expression of GTS1. The promoter-reporter construct was inserted into BY 4743 through homologous recombination. The construct acted as a knockout cassette by replacing one copy of GTS1. As GTS1 was expressed by the cell, the construct was expressed concurrently and detected by a reporter gene, green fluorescent protein (GFP). GFP is a common reporter gene utilized for its ability to be expressed in non-homologous species. Also, GFP has no physiological effect on cell operation and is easily detected using fluorescence microscopy.
Second to achieve the best GFP expression output, culture conditions were optimized by growing budding yeast in asynchrony and synchrony. Cell cycle synchronization of yeast populations was reached in 6 hours and used standard medium by a block-and-release method. Third, the GFP expression was observed in batch and continuous flow systems. GFP expression was quantified using flow cytometry to analyze expression levels over time. By applying the aforementioned method, GTS1 was directly monitored through GFP expression.

1.2 History of Biological Rhythms

Circadian is derived from Latin words, circa “about” and dies “day,” and was first defined by Franz Halberg in 1959 for the approximately 24-hour cycles that are endogenously generated by an organism. However, a biological clock study was accomplished far earlier and is documented in the marches of Alexander the Great in the fourth century B.C. Recorded in the marches are the daily movements of leaves and flower petals (Bretzl, 1903). However, Jean Jacques d’Ortous de Mairan in 1729 conducted the first conclusive experiments to determine if biological rhythms were a consequence of a periodic environment (Figure 1) (Moore-Ede, et al., 1982). de Mairan investigated leaf movement in a sensitive heliotrope plant which opens its leaves and pedicels during the day and folds them at night (Moore-Ede, et al., 1982). de Mairan moved the plant into a place away from sunlight, and he found the plant continued its daily schedule of opening during the day and folding at night. Therefore, the determination of the circadian rhythms in the absence of environmental time cues was first demonstrated.

In 1832, Augustin de Candolle found that the Mimosa pudica’s daily leaf movements not only continued in constant darkness but also that the opening of the leaves started an hour to two hours earlier each day. “Free-running” was first demonstrated for the circadian clock with its own endogenous period when it was no longer synchronized to a 24-hour light-dark cycle. This
discovery indicated that circadian rhythms are directed by autonomous clocks inside the organism and are not the result of undetected environmental stimulus. De Candolle described his discovery as “an inherent tendency of plants to show periodic movements” (Moore-Ede, et al., 1982).

Figure 1: Representation of de Mairan’s experiment. A heliotrope plant was exposed to the environment and a second heliotrope plant was placed inside a box during sunlight hours (on left) and during night hours (on right). de Mairan found that both plants persisted in their daily schedule of opening during the day and folding at night. (Moore-Ede, et al., 1982).

In 1929, von Frisch conducted experiments to demonstrate that organisms could measure the passage of time. von Frisch marked bees individually and then offered them sugar water at an artificial feeding location for several days at the same time during the day. On the day of the study, no food was placed in the dish and the time of arrival of each bee was observed (Moore-Ede, et al., 1982) (Beling, 1929). Results showed that the bees arrived within approximately the same time as they had in the training. Von Frisch conducted the study in salt mines 180 meters
below the surface, yet the bees were able to maintain their capacity for timekeeping. Entrainment, synchronization to an external cue, was possible when the food was placed at intervals close to 24 hours. For food placed at 19 or 48 hour intervals, the bees could not maintain their capacity to recognize the pattern and therefore were unable to be entrained. Von Frisch demonstrated that food was an external cue which also entrained the circadian rhythm. With evidence from this experiment and others like it, the scientific emphasis shifted from circadian rhythms as a biological marvel to circadian rhythms as a critical time-measuring device (Moore-Ede, et al., 1982).

In the twentieth century, Erwin Büning and Colin Pittendrigh did extensive work to persuade biologists of the circadian clock’s importance to all organisms. Büning demonstrated that plants (Büning, 1932) and insects (Büning, 1935) exhibited circadian rhythms after they or their parents were raised in constant conditions such as constant light or constant dark. He also illustrated that the free-running period was genetically inherited when strains from parents with different endogenous periods were crossed. Moreover, he discerned that circadian clocks measure the length of the day including the total circadian cycle (Moore-Ede, et al., 1982). Büning indicated the ability for an organism to detect seasonal changes in day length was advantageous for its vitality. Pittendrigh demonstrated the time of day that the fruit fly (*Drosophila*) emerged from its pupa was controlled by a circadian clock with diminutive influence by variations of environmental temperature (Pittendrigh, 1960). He educed the circadian clocks are temperature-compensated which makes the clock a viable timekeeping device (Moore-Ede, et al., 1982).

In 1866, William Ogle was the first to observe rhythmic changes in daily body temperature in humans. He found in the early morning, there is a rise in body temperature while
men are still asleep, and a fall in body temperature in the evening while men are still awake (Moore-Ede, et al., 1982) (Ogle, 1866). Ogle’s results were corroborated in 1906 by Simpson and Galbraith’s study of body temperature in monkeys. In their study, they measured rectal and auxiliary temperature of five monkeys every two hours for 60 days (Moore-Ede, et al., 1982). Simpson and Galbraith confirmed not only the presence of body temperature rhythm but that the rhythm prevailed in constant darkness or light with an endogenous periodicity (Simpson, et al., 1906). As the schedule of light and dark was reversed, the rhythm progressively became inverted, illustrating synchronization with 24-hour light-dark cycle.

In the 1960s, circadian rhythms were investigated in a variety of organisms, leading to documentation of rhythms in various organisms and human physiological systems. Moreover, it was no longer significant to find a circadian rhythm for a physiological variable but, on the contrary, it was significant to find physiological variables lacking a circadian rhythm. For highly rhythmic animals in which multiple control systems show circadian rhythms, an explicitly elaborate physiological strategy was required to preserve a variable constant throughout day and night. Rhythmic systems must compensate each other to achieve the non-rhythmic function (Moore-Ede, et al., 1982). An example is in the blood pressure of squirrel monkeys. The circadian rhythm is not detected; however, it is regulated by endocrine systems and renal salt and water excretion mechanisms that are highly rhythmic in their function (Moore-Ede, et al., 1982).

1.3 Criteria of a Biological Rhythm

There are two criteria of a circadian clock. First, the structure must measure the passage of time independently of any periodic input from the environment (Moore-Ede, et al., 1982). Therefore, this structure must convert a non-periodic source of energy into a self-sustaining
periodic output. Tissues within the body that exhibit a circadian rhythm but are unable to sustain circadian oscillation on their own do not contain a clock. Therefore, these tissues are driven by a circadian clock elsewhere in the organism. The circadian clock can be found in the smallest entity, whether it is an organ or a subcellular fraction that can measure circadian time in the absence of time cues in the environment (Moore-Ede, et al., 1982). Second, this structure must be utilized to time biological events such as replication, cell division, or the yeast metabolic cycle. The circadian clock is a good timekeeper because it has relatively high resolution and uniformity. The resolution of a clock is the ability to recognize the temporal order of two events closely spaced in time. The uniformity of a clock is a measure of the regularity of its period and the clock’s ability to predict the occurrence of other regularly timed phenomena in its surroundings (Moore-Ede, et al., 1982). Measuring the uniformity of a circadian clock is different from measuring a circadian rhythm. Even if a circadian clock transmitted perfectly regular signals, the physiological transmission which processes the observed rhythm from the clock might have a delayed response.

The circadian clock normally resets each day, entrainment, by cues in the environment. Therefore, errors occurring from slight deviations throughout the day are not cumulative. In natural environments, multiple external time cues known as zeitgebers exist and can be used to entrain an organism. The zeitgeber can be introduced and removed, and their ability to transmit temporal information can be assayed. Four criteria must be met for an environmental variable to be considered a zeitgeber (Moore-Ede, et al., 1982). The first criterion is the absence of other time cues. The circadian rhythm must be free-running with an independent period before the time cue is imposed upon the organism. The organism must resume the free-running rhythm after the time cue has been removed. Second, there must be period control. Once the organism is
exposed to the zeitgeber, the period of the circadian rhythm must tune itself so that it is equal to the period of the zeitgeber. The third criterion is the time cue must have a stable phase relationship. A stable and reproducible relationship must emerge and be preserved between the timing of the observed rhythm and the timing of the zeitgeber. The occurrence of the rhythm must be independent of clock time and dependent only on the imposed time cue. Fourth, the time cue must have phase control. If the time cue is removed, the rhythm must start to free-run from a phase induced by the zeitgeber and not by the rhythm preexisting to entrainment.

It is important to differentiate a zeitgeber, which can entrain a cycle, from other environmental influences, which may only influence the waveform of the circadian rhythm. An example of an environmental influence that influences only the waveform but does not act as a zeitgeber is masking. In a study by Patricia DeCoursey (DeCoursey, 1960), flying squirrels were subjected to a 24-hour temperature cycle (12 hours at 25°C and 12 hours of 15°C). The rhythm exhibited an apparent entrainment with activity mostly in the cold phase; however, a slight amount of activity was found each day at a time predicted by the free-running clock. When the animal was released from the temperature cycle, the phase of the activity rhythm was better predicted by the previous free-running rhythm rather than the applied temperature cycle. In this example, the temperature cycle cannot be considered an effective zeitgeber.

The circadian clock is a timekeeper for biological processes within the body. The two criteria for a circadian clock are the ability to measure time independent of environmental input and to time biological events such as replication. Zeitgebers such as light or food are utilized to entrain an organism.
1.4 Summary

By a change in behavior and metabolism through an endogenous clock, all organisms can optimize temporal processes when environmental pressures are present. This ability is crucial for the organism to survive in response to environmental cues. Perturbations to this biological clock in humans can affect health and lead to disease such as insomnia, affective disorders, testosterone levels, anorexia nervosa, Alzheimer’s disease, depression, and cancer. Many drugs show biological rhythms and can be manipulated to optimize drug effectiveness. By understanding the circadian rhythm, therapeutic methods can be developed to patients with these illnesses and drug prescreens can be created to evaluate a drug’s effect on biological rhythms.

To investigate biological rhythms, studies have employed *Saccharomyces cervisiae*, a budding yeast, because of its fully sequenced genome, highly adaptable DNA transformation system, rapid growth, and ease of transfer to different media. Therefore, budding yeast is a model eukaryotic system for diverse biological studies. Moreover, budding yeast holds many homologous genes to more complex eukaryotes such as plants, rodents, and human.

In this study a tool was developed to characterize the ultradian rhythm in diploid budding yeast, BY 4743, by monitoring the expression level of the ultradian rhythm gene, GTS1. This was achieved by first creating a novel yeast strain with a promoter-reporter construct which monitored the expression of GTS1. The promoter-reporter construct was inserted through homologous recombination and expressed concurrently with GTS1. The promoter-reporter was detected by the common reporter gene, green fluorescent protein (GFP). Second to attain the best output of GFP, budding yeast was grown in asynchrony and synchrony cultures. A block-and-release method was employed to synchronize the cell cycle of budding yeast in standard medium. Third, the GFP expression was examined in batch and continuous flow systems. GFP
expression was quantified using flow cytometry to analyze expression levels over time. By tracking the expression of GFP, GTS1 was directly monitored.
CHAPTER 2 – LITERATURE REVIEW OF THE CIRCADIAN RHYTHM

As biological rhythms are analyzed, research can investigate diseases associated with the misalignment of the circadian rhythm in humans. Moreover, studies can provide insight into the relationship between disease progression and malfunctions in the circadian rhythm. With a better understanding of these biological rhythms, physicians and scientists can create new treatments and pharmaceutical drugs to prescribe illnesses related to the circadian rhythm.

2.1 Disease and Disease Progression Related to the Circadian Rhythm

The suprachiasmatic nucleus (SCN), found in the hypothalamus, is the circadian pacemaker for mammals. (Lu, et al., 2006). It is located in a region of the hypothalamus where destruction or pathological damage has been associated with interference of the circadian sleep-wake cycle. The SCN collects information through the eyes via the retina about the illumination of its surroundings. The information passes through a pathway called the retinohypothalamic tract inducing the SCN. The interpretation of the information is done day-by-day and passed to the pineal gland located on the epithalamus. If the SCN cells are removed and cultured, the cells maintain their own rhythm in the absence of external cues (Gillette, 1991).

2.2 Circadian Rhythm Sleep Disorder

Circadian Rhythm Sleep Disorder (CRSD) is a misalignment between an individual’s sleep patterns with the desired or social norm (Lu, et al., 2006). Individuals with CRSD often complain of difficulty falling asleep, maintaining sleep, and excessive sleepiness. There are three general criteria for CRSD according to the International Classification of Sleep Disorders. First, a constant or recurrent pattern of sleep disturbance that is thought to be directly due to either alteration in the circadian timing system or a misalignment between endogenous circadian
rhythms and external factors that affect the timing of sleep. Second, the sleep disturbance leads to insomnia, excessive sleepiness, or both. Third, the sleep disturbance is associated with impairment of function. CRSD can result in delayed sleep phase (DSP), advanced sleep phase (ASP), free-running type, and irregular sleep-wake cycles due to alterations in the endogenous circadian clock. Also, changes in the physical environment, such as jet lag and shift work disorder (SWD), can alter the circadian clock (Lu, et al., 2006).

In a study done by Lu, he examined patients with the above disorders and prescribed treatments for each case using chronology, light, and melatonin. DSP is characterized by an unusually long endogenous circadian period, hypersensitivity to evening light, and genetic mutation in circadian genes such as Per3, Aryalkylamine N-acetyltransferase, and Clock (Lu, et al., 2006). DSP patients have difficulties falling asleep, waking up, and excessive sleepiness which conflict with daytime function. Without social and/or work constraints, patients typically fall asleep between 2 to 6 AM and wake up between 10 AM and 1 PM. Sleep duration is truncated, leading to chronic sleep loss and impaired daily functions. In chronotherapy, progressive moves of the bedtime and rising time earlier by approximately 3 hours every 2 days are done until a final earlier bedtime schedule is achieved and maintained. Lu found chronotherapy to be useful. However, outside a clinical setting, he believes it is difficult to practice because of the strict conditions for social and professional restriction and the lengthy duration of treatment. In bright light therapy, bright light is administered at 2,000 to 2,500 lux for 1 to 2 hours between 6 and 8 AM in the morning. For patients with extreme DSP, light exposure can be delayed until later in the morning. For conformity of bright light therapy, exposure must be done at the correct time and for the correct duration. Also, the patient may need to restructure social and professional activities around the light regimen. Another effective treatment of DSP
patients involves administering melatonin. Melatonin is a hormone produced by pineal gland that aids in the regulation of the circadian rhythm (Altun, et al., 2007). In a study by Mundey, administrating 0.3 to 3 mg of melatonin to patient in the evening for 4 weeks has shown to be effective in treating DSP patients. Lu believes that more research needs to be done on the effectiveness of melatonin.

Patients that exhibit advanced sleep phase (ASP) have an advancement of the major sleep period featured by habitual and involuntary sleep onset and wake-up times that are several hours earlier relative to conventional and desired times (Lu, et al., 2006). ASP is common among middle-age and older adults. Possible causes for ASP are shortened endogenous circadian periods, increased retinal sensitivity to light in the morning, and genetic mutation of the Per2 or CK1δ gene(s) (Lu, et al., 2006). Common complaints of ASP sufferers are late afternoon or early evening sleepiness and the difficulty to maintain sleep during the early morning hours. Onset of sleep occurs between 6 and 9 PM and wake time is between 2 and 5 AM. Any attempts to conform to later sleep times leads to chronic partial sleep deprivation. Lu found that bright light exposure in the early evening was the most effective in delaying wake time, reducing wake time during sleep, and improving sleep efficiency.

In free-running type, the circadian rhythm is not stably entrained to the 24 hour day. Without a circadian rhythm entrained to a 24 hour day, the sleep period will drift later each day. Since light is the strongest synchronizing agent to the circadian clock, this type of CRSD is most common among blind people, who lack competent perception of light. Patients with free-running type will exhibit periods of insomnia, excessive sleepiness, or both, alternating with short asymptomatic periods (Lu, et al., 2006). Lu suggests the best treatment for free-running type is practicing sleep hygiene, structured social and physical activities, and—if needed—melatonin.
Irregular sleep-wake disorder is characterized by a lack of a clearly detectable sleep-wake circadian rhythm (Lu, et al., 2006). Sleep and wake periods are disbursed in three or more short episodes lasting 1 to 4 hours in a day with the longest sleep period usually from 2 to 6 AM. A possible cause is either a severance of the circadian clock, entrainment pathways, and/or a decreased exposure to environmental synchronizing agents. A treatment for irregular sleep-wake disorder proposed by Lu is increased social interactions, physical activity, and light exposure approximately 2,500 to 3,000 lux for 2 hours in the morning.

Jet lag is the result of a temporary misalignment between the endogenous circadian sleep/wake rhythm and the external physical environment due to a change in the time zone (Lu, et al., 2006). Jet lag affects travelers, especially the elderly. Individuals with jet lag temporarily experience sleep initiation and maintenance insomnia, daytime sleepiness, and decreased daytime performance. With increased time zone change, symptoms become more severe. For jet lag in westward flights, individuals should attempt to stay awake while light is out and not to sleep until nighttime at the destination. For individuals with jet lag traveling in an eastward flight, Lu suggests that one should stay awake, avoid bright light in the morning and exposing oneself to as much light as possible in the afternoon.

Shift work disorder (SWD) is characterized by an occurrence of insomnia, excessive sleepiness, and impaired performance during scheduled working hours amidst the usual sleep period. SWD is common among night and early morning shift workers. SWD patients have difficulty falling asleep or maintaining sleep, unrefreshing sleep, and sleepiness at work. Sleep is shortened by 1 to 4 hours in contrast to daytime workers (Lu, et al., 2006). A multimodal approach involving practicing bright light exposure during early shift hours, avoiding bright light by the use of dark glasses during the morning commute, and sleep hygiene such as avoiding
naps, exercising, using a bedtime ritual, etc. (Lu, et al., 2006) is used to align the circadian rhythm.

Lu explains that behavioral and environmental factors play an essential role in this disorder and treatment interventions need to address circadian physiology alongside behavioral and environmental influences. Circadian Rhythm Sleep Disorder (CRSD) is caused by changes in the circadian timing system or misalignment between endogenous rhythms and the external environment. However, the exact mechanism responsible for this disorder remains unknown.

2.3 Smith-Magenis Syndrome

A demonstration of a genetic disease that causes a sleep disorder is Smith-Magenis Syndrome (SMS). SMS is a clinically recognizable contiguous gene syndrome caused by an interstitial deletion of chromosome 17p11.2 (Leersnyder, et al., 2006). Deletion have ranged from <2 to >9 megabases and mutations in RAI1. Physical symptoms of SMS include mild dysmorphism, delayed development, abnormal behavior, severe sleep disturbances, and maladaptive daytime behavior due to unusual circadian rhythm. All SMS patients have some degree of mental retardation with IQ scores ranging 20 to 78. Dysmorphism include brachycephaly with midface hypoplasia and mouth shape characteristic with Cupid’s bow. Also, SMS patients exhibit speech delay with or without hearing loss, a hoarse deep voice, and short stature. Other variable features include renal abnormalities, seizures, cardiac defects, and scoliosis (Leersnyder, et al., 2006).

Sleep disturbance plays a major role for SMS patients and their families. SMS patients have a normal bedtime 8 to 9 PM regardless of age and gender. However, patients consistently wake up one to three times per night and fall back asleep within 30 minutes or more. While awake, patients exhibit hyperactivity, forcing parents or a caregiver to constantly look after the
patients and to devise artifices to keep them in the bedroom at night such as locking the door, removing furniture, etc. (Leersnyder, et al., 2006). With frequent sleep disturbance, parents or caregivers may become sleep deprived themselves. Most patients express morning tiredness and temper tantrums when tired; therefore, patients may nap 30 minutes or more during the day. Also, patients exhibit sleep attacks at the end of the day. The sleep disorder is due to a phase shift in melatonin in the circadian rhythm. Melatonin’s synthesis and release are stimulated by darkness and inhibited by light. Light entrainment is transmitted through the retino-hypothalamic tract (RHT) to reach the biological clock, SCN. The shift in melatonin shown in Figure 2 explains the tiredness in the morning and tantrums which are due to a rise in melatonin. Sleep attacks at the end of the day are due to the endogenous sleep onset of the patient that could be regarded as equivalent to a sleep phase advance (Leersnyder, et al., 2006).

There are a number of clock genes controlling the circadian rhythm. Subunit 3 of the COP9 signal transduction complex (COPS3) maps within the SMS critical region in 17p11.2 (Leersnyder, et al., 2006). COP3 signal transduction complex may not play a significant role with respect to melatonin phase shift in SMS but there may be an age-dependent penetrance or
variability for the expression of the phenotype (embryological changes). Zeitgebers transmit to
the SCN via the RHT for circadian rhythm of melatonin release. There may be an alteration of
the input/output-signaling pathway. However, the actual mechanism for the abnormal rhythm of
melatonin secretion in SMS is still unknown.

In a study by Leersnyder (Leersnyder, et al., 2006), he believed that behavioral problems
and night sleep insufficiency in SMS patients can be corrected by restoring melatonin circadian
rhythm. The circadian rhythm of melatonin is controlled by the sympathetic nervous system, and
β1-adrenergic reduces the production of melatonin. Leersnyder performed a trial of
administrating β1-adrenergic in the morning, finding a rapid decrease in plasma melatonin in all
SMS patients. Mean melatonin levels dropped from 68 to 8 pg/ml. With the treatment, naps and
sleep attacks disappeared and tantrums lessened from 1-2 each day to 1 or 2 per week. SMS
patients were better able to concentrate at school and were less hyperactive. Administering a
combination of morning β1-adrenergic and evening melatonin restored plasma circadian
melatonin rhythmicity, improved behavioral disturbances, and enhanced sleep in SMS patients.

In another study conducted by Leersnyder with control release of melatonin, his
administration of melatonin resulted in spikes of plasma melatonin with a slow decrease echoing
endogenous melatonin in the circadian rhythm. Mean sleep onset was delayed by 30 minutes,
sleep offset by 60 minutes, and the mean gain of sleep was 30 minutes. In most cases, sleep
awakening disappeared and wake-up time was delayed. Patients no longer woke up in the night
and more regular sleep stage organization and a rapid access to sleep stage 3-4. Deep and quiet
sleep was attained and day/night life was greatly improved. No adverse events, no side effects,
nor habituation was reported and children’s parents and caregivers were convinced for
continuation of treatment.
Leersnyder was able to model the first sleep and behavioral disorder in a genetic disease. He found that the best treatment was a combination of beta blockers and administrating melatonin. This combination restored the circadian rhythm of melatonin, suppressed inappropriate diurnal melatonin secretion, and improved sleep and behavioral disorders.

2.4 Disrupted Circadian Effects on Testosterone

The circadian rhythm can aid in our understanding of aging. William Bremner directed a study to investigate if levels of blood testosterone decreased as men aged (Bremner, et al., 1983). Decreased levels of testosterone accompany a host of aging problems in men such as loss of musculature, loss of body hair, and increase in osteoporosis which leads to frail bones, and a decrease in sexual desire and erectile function (Morales, et al., 2000). Previous studies yielded inconclusive results because studies carried out in the morning showed decrease levels of testosterone and studies carried out in the evening failed to show the decrease when comparing young and old men. Bremner believed, since a previous study showed normal young men to exhibit a circadian rhythm in serum testosterone with the highest level around 8 AM and lowest level in late afternoon or evening, that normal older men must have a circadian rhythm, however damped it may be.

Bremner studied 17 young men (age 23-28) and 12 old men (58-82). After a 24 hour adjustment period, hourly blood samples were taken through an indwelling peripheral venous cannula from 8 AM to 7 AM the next morning. The results showed young men demonstrated a clear circadian rhythm in serum testosterone levels. Maximal levels occurred at 8 AM and minimal levels occurred between 7 PM and 9 PM shown in Figure 3. In the circadian rhythm in older men, the amplitude was significantly lower and the mean testosterone levels were extremely lower in older men between 2 AM to 1 PM. Bremner’s study demonstrated a decrease
in testosterone levels in aging men. Also, the study found a loss in circadian rhythmicity with aging. Bremner raised the question, in 1983, of whether the loss is due to age related changes in gonadotropin production or to decreased testicular responsiveness to gonadotropins. Bremner’s study helped in the discernment of andropause, male menopause, which is the reduction of low testosterone and dehydroepiandrosterone (Vermeulen, 2000).

2.5 Circadian Rhythm’s Effect on Alzheimer’s Disease

Another age-related disease which alters the circadian rhythm in older patients is Alzheimer’s disease. Alzheimer’s disease is an incurable, degenerative, and terminal disease which affects 26.6 million people worldwide (Brookmeyer, et al., 2007). The true cause of Alzheimer’s disease is still unknown. Moreover, these patients exhibit cognitive impairment and dementia. Alzheimer’s disease affects the hypothalamus in different areas, including those for
neurotransmitters, neuromodulators, and neurohormonals. The disruption of the circadian rhythm in patients with Alzheimer’s disease can lead to decreased cognitivity, agitation during the day, and restlessness at night (Hoogendijk, et al., 1996). With nocturnal restlessness, the patient may disrupt the sleep of the caregiver; therefore, the patient is more likely to be institutionalized. Furthermore, poor lighting conditions and few outdoor activities in the nursing homes may exacerbate the circadian rhythm disturbance. Also, degenerative alterations in the retina and optic nerve affiliated with Alzheimer’s disease also decrease patients’ exposure to light, all of which lead to a desynchronization of the brain’s biological clock with the 24 hour environmental cues (Hoogendijk, et al., 1996).

In a study conducted by Hoogendijk, he analyzed treating patients with Alzheimer’s disease with exposure to bright light. Previously, treatment with hypnotic medication yielded limited therapeutic efficacy and decreased daytime alertness (Hoogendijk, et al., 1996). Circadian and circannual fluctuation, a yearly cycle, of vasopressin-expressing neurons decreases in the SCN with age; however, in Alzheimer’s disease patients it decreases substantially more. Therefore, Alzheimer’s disease patients spend more time napping compared to age-matched individuals. Hoogendijk assayed vasopressin-expressing neurons and found that young subjects exhibited low numbers of vasopressin-expressing neurons at night and high levels during the early morning, a phenomenon which is not seen in subjects over 50 years of age shown in Figure 4.

Moreover, vasopressin-expressing neurons are low in the summer and peak in the autumn for the young subjects. Hoogendijk correlated his findings to indicate that a dysfunction-pathologic structure relationship appeared to exist for sleep disturbances and that a decreased
number of vasopressin-expressing neurons in the SCN were present for older persons and Alzheimer’s disease patients.

![Figure 4: Number of vasopressin-expressing cells in health control subjects aged 0 to 100 years and patients with Alzheimer's Disease (AD) mean age of 79 years. Vertical lines denote standard error of the mean. Note the low values in the AD group and the 81-100 years-old group. These values were significantly different from the those for the groups aged 0-20 and 40-80. The overall effect of age on vasopressin-expressing cells was also significant. (Swaab, 1985) (Hoogendijk, et al., 1996)](image)

With the results found in Hoogendijk’s study, he found that using light to align sleep-wake distribution aided in improving the behavioral disorders, such as agitation and wandering, and sleep-wake disturbances with exposure to extra amount of light. Moreover, he believes it is advantageous to further research dysfunction-pathologic structure relationships for a better understanding of aging and Alzheimer’s disease.

2.6 Summary

These studies investigated diseases created by the malfunction of the circadian rhythm and the effect the circadian rhythm has on disease progression. For CRSD (Lu, et al., 2006), Lu found misalignment between the endogenous clock and the external environment can be treated with melatonin and exposure to light at specific time during the day. In a study by Leersnyder (Leersnyder, et al., 2006), he modeled the first sleep and behavioral disorder in a genetic disease
and treated this disorder with a combination of beta blockers and melatonin. Bremner found the amplitude of the circadian rhythm for testosterone significantly decreased as men aged (Bremner, et al., 1983). Hoogendijk found he was able to improve the behavioral disorders, such as agitation and sleep-wake disturbances with exposure to extra amount of light (Hoogendijk, et al., 1996). As more biological rhythms are analyzed in humans, research can investigate diseases and disease progression associated with the misalignment of the circadian rhythm.
CHAPTER 3 – LINK BETWEEN THE CIRCADIAN AND ULTRADIAN SYSTEMS

3.1 Introduction

As ultradian cycles are analyzed in yeast, research can develop a better understanding of biological rhythms and can provide insight into more complex organisms including humans. There are great similarities between circadian and ultradian systems. First, both systems have temperature compensation of periods which indicates a strong likelihood that the prime functions of both rhythms have temporal roles (Lloyd, et al., 2007). Practically all organisms live under conditions of temperature change that can occur quite quickly and at an accurate time either by man-made or biological events. Moreover, the organism must react to compensate for the fluctuation in temperature. Second, both systems exhibit a period sensitivity to Li⁺ (Lloyd, et al., 2005) (Lloyd, et al., 2007). Li⁺ is an inhibitor of glycogen synthase kinase 3 (GSK3) which regulates circadian rhythm in several organisms (Yin, et al., 2006). The inhibition to GSK3 lengthens the period of the cycle (Dokucu, et al., 2005). Also in ultradian rhythms, Li⁺ lengthens the period by inhibiting monoamine oxidase (Lloyd, et al., 2007). Third, alterations of periods have led to discovering the ability to change a system from a circadian time scale (24 hours) to an ultradian time scale (less than 24 hours). At very low temperatures or at high light intensities in rats, circadian patterns of drinking and motor activity were lost uncovering an ultradian rhythm (Klemfuss, 1992) (Rosenwasser, 1993). Mutation of genes central to the control of circadian rhythms leads to ultradian timekeeping. Mutations in per2 and cry1, in mice, reveal clear ultradian patterns (Lloyd, et al., 2007). In addition, ontogenesis is characterized by coherent patterns of gradually integrating ultradian and circadian physiology in rodents and humans.
Ultradian and circadian systems are not only similar in the structure of timing keeping and zeitgeber for alteration but are occasionally dependent on one another. Ultradian rhythms can be utilized to create circadian rhythms. There are many different mechanisms by which circadian rhythms maybe generated by using an ultradian clock: low-frequency generation in long feedback loops, deposition effect, frequency filtering, frequency beats, etc. (Lloyd, et al., 2007). In a feedback loop that acts from the terminating portion of a pathway to inhibit its initial steps, long period oscillations occur. In a multistep process, the period of the oscillation is longer than the half-life of the longest-lived intermediate (Gilbert, 1978). In deposition effect, the reversible exchange of an intermediate metabolite with a high amount of a reserve energy store can significantly increase the oscillation period. Complex behavior is illustrated by mathematical models of metabolic situation indicating the emergence of the low-frequency dynamics uses a spiral of ultradian cycles, after the system is re-initialized (Sel'kov, 1980). In frequency filtering, a low frequency rhythm is generated by a high frequency by a filter that counts the high frequency cycles (Levi, et al., 2005). In frequency beats, long period oscillations are created by two independent oscillators with different frequencies and common output complex waveforms (Chance, et al., 1967). In subharmonic resonance, physical systems may exhibit a low frequency collective mode of oscillation (Goodwin, 1963).

One area being investigated is the mutation/deletion of genes involved in the DNA damage response in budding yeast. The mutants have a deleted circadian checkpoint gene which is also shared as a cell cycle checkpoint such as TIM or Chk2 (Chen, et al., 2007). DNA damage may alter cell cycle progression and indirectly reset the biological clock through a putative reciprocal regulation between the two systems preventing the formation of a futile cycle. Shortened yeast metabolic cycle (YMC) period length from slow-growing cell cycle mutants of
yeast suggests the cell cycle can feed back to influence the metabolic cycle (Chen, et al., 2007). Though this reciprocal regulation of the metabolic and cell division cycles probably takes place in an indirect coupling mechanism that primarily controls growth, the potential for a direct mechanistic connection between cell division and metabolic cycle is currently being pursued.

The ultradian rhythm has been suggested for studies for many cell lines such as tumors (Chen, et al., 2007). The yeast metabolic cycle (YMC) provides two connections with cancer biology. First to gain a growth advantage in the environment, tumor cells deregulate the cell cycle at the cost of genome instability. This is reminiscent of the trade-off between growth and mutation accumulation observed in the YMC. Second to sustain accelerated cell expansion, tumor cells are more dependent on glycolysis than normal cells for energy illustrating a dependence on an ultradian rhythm. Therefore, it is feasible that more hypoxic and glycolytic redox environment of cancer cells could be part of the foundation for cell cycle deregulation (Chen, et al., 2007). Moreover, research of yeast can yield potential prescreening of drugs prior administration to humans based on the discernment of the YMC and the ultradian rhythm. If the characteristics and genes are properly located for the ultradian rhythm in yeast, predictions of drug interaction in rodents and humans tumor cell lines are quite possible. By having a prescreening process in yeast, the prescreening time can be reduced due to yeast’s ability to grow quickly (roughly 1-4 hours doubling time) and the cost of equipment and supplies for such experiments can be reduced returning faster results at a lower expense.

3.2 Ultradan Rhythm in Yeast

The glycolytic oscillation exhibited in the yeast *Saccharomyces cerevisiae* is one of the best biochemical oscillators known. First evidence of oscillatory behavior was reported by Duysens and Amesz in 1957 who studied the fluorescence of some glycolytic intermediates, such
as NADH, in yeast that undertook damped oscillations in the course of time (Goldbeter). In 1964, Chance was able to maintain glycolytic oscillations in yeast suspensions for relatively long periods of time. Later it was found if yeast cells were given continuous addition of glucose that NADH along with other elements of the glycolytic system and pH can exhibit sustained oscillations (Edmunds, 1988).

Yeast cells can undergo rapid glycolytic oscillations synchronization in stirred suspensions with synchronizing factors such as acetaldehyde, pyruvate, or external ethanol (Goldbeter). Sustained periodic behavior can be observed with a precise range of substrate injection rates. With control over the precise range of substrate injection rates, a model that illustrates amplitude and glycolytic oscillations can be derived for biological rhythms (Goldbeter). Since the glycolytic oscillations are well known in comparison to other rhythms, this mechanism permits for construction of realistic models which allow better discernment of the origin as well as the various properties of the periodic phenomenon.

Criteria were developed to assay chemical systems for oscillatory behavior based on reaction pathways. If X and Y are two substances, oscillations can occur if X activates its own production (with X being fixed), Y must tend to inactivate its own net production since increasing concentration increases the rate of removal of that chemical, and cross-coupling of opposite character so that increasing the concentration of X activates the net production of Y, and increasing Y inhibits the new production of X (or vice versa). The resulting oscillation will be a period independent of kinetic rate constants associated with these reactions (Edmunds, 1988).
3.3 Yeast Glycolysis

Determining the coordination of energy generation, metabolic reactions, transcriptional order, cell proliferation, and development is the central question of circadian and ultradian rhythms. A metabolic pathway that is regulated by the ultradian rhythm is yeast glycolysis. This metabolic pathway converts either glucose or fructose to ethanol and carbon dioxide. Periodic behavior originates at the step catalyzed by the enzyme phosphofructokinase (PFK) and is considered a positive feedback response (Edmunds, 1988). This is discerned by observing periodic behavior when glucose 6-phosphate or fructose 6-phosphate is taken as the glycolytic substrate. This observation allows the source of oscillation to be beyond the first two enzymes of the chain, hexokinase and phosphoglucose isomerase. However when PFK is bypassed by injecting fructose 1,6-biphosphate (FBP) or regulated by inhibitors (ATP) or activators (ADP or AMP), oscillations are greatly affected by the immediate appearance of phase shifts (Goldbeter).

The oscillations of NADH absorbance is self-sustained and has a free-running time of 2 to 70 minutes, an ultradian rhythm (Edmunds, 1988). Oscillations are also observed in muscle cells which also undergoes glycolysis. Here, PFK is inhibited by citrate ion which suppresses the oscillations in muscle cells. Glycolytic oscillations have been detected in tumor cells, insects, blowflies, heart cells, pancreatic β-cells, and many more (Goldbeter).

Under these complex time structures for energy generation, metabolic reactions, transcriptional order, cell proliferation, and development, organisms are required to synchronize operations of multiple processes in many time domains such as biochemical reactions which are accomplished in microseconds or even more swiftly (Lloyd, et al., 2007). Therefore, there is a demand to compartmentalize essential cellular and metabolic events occurring in synchrony with the metabolic cycle in simple eukaryotic cells (Tu, et al., 2005). To develop a deterministic
model, studies on continuous fed yeast cultures have described the temporal, genome-wide transcription and coordination of essential cellular and metabolic processes (Tu, et al., 2005). Oscillatory redox core in metabolism provide the ultradian time base for the YMC (Lloyd, et al., 2007). Here, the redox changes serve to be the zeitgeber for the clock and elicit phase shifts of the metabolic cycle (Chen, et al., 2007). The yeast cells under continuously fed culture become impulsively synchronized to a 40 minute rhythm and can be maintained for weeks or even months (Lloyd, et al., 2007). The system illustrates the time structures of living organisms because the processes and events occur inside the organism are amplified since the whole culture operates as a single cell. The most advantageous monitored output as shown in Figure 5 is the dissolved oxygen (O₂) by the organisms (Lloyd, et al., 2005). This supplies a phase relationship for many variables in the systems such as NAD(P)H, glutathione (GSH), ethanol, acetaldehyde, acetic acid, H₂S, amino acids, and other metabolites. Moreover, the heart of the ultradian clock in yeast is the redox cycle where nicotinamide nucleotides drive the reduction of soluble low molecular weight thiols or proteins and thereby protein disulfide bridges. Reoxidation then occurs by reaction with O₂ or reactive oxygen species (ROS) (Lloyd, et al., 2007). The main role of intracellular redox potentials is to balance reduced and oxidized forms of NAD(P)H/NAD(P)⁺ or 2GSH/GSSG which in turn harmonize energetic and biosynthetic functions.

Glycolysis and redox cycle, which are regulated by the ultradian rhythm, coordinate energy generation, metabolic reactions, transcriptional order, and cell proliferation transpiring in multiple time domains. This is accomplished by compartmentalizing essential cellular and metabolic events occurring simultaneously within the cell.
Figure 5: The oscillation of dissolved oxygen, redox state indicators and the signalling compounds, acetaldehyde and H$_2$S, in continuous cultures of S. cervisiae (a) NAD(P)H (b) GSH (c) acetaldehyde (d) extracellular H$_2$S (Lloyd, et al., 2005)

3.4 Gene Expression in Yeast

The ultradian clock in yeast provides the time base upon which all central processes are synchronized (Lloyd, et al., 2005). In a study by Lloyd, levels of mRNA were assayed to find genomic expression in yeast. Three superclusters of gene expression were found for 5329 transcript genes (Lloyd, et al., 2007). First, the oxidative (Ox) supercluster exhibits only 650 genes (Lloyd, et al., 2007) and contains mostly of genes encoding ribosomal proteins, translation initiation factors, amino acid biosynthetic enzymes, small nuclear RNAs, RNA processing enzymes, and proteins required for the uptake and metabolism of sulfur (Tu, et al., 2006). In the
next two superclusters, 4679 genes are expressed in the reductive phase. The reductive phase consists of reductive/building (R/B) and reductive/charging (R/C) (Lloyd, et al., 2007) (Tu, et al., 2006). The R/B supercluster peaks when cells begin the cease of oxygen consumption. This supercluster expresses primarily of nuclear-encoded mitochondrial genes and genes encoding histones, spindle pole components, and proteins essential for DNA replication and cell division (Tu, et al., 2005). The R/C supercluster encodes proteins involved in nonrespiratory modes of metabolism and protein degradation such as peroxisome, vacuole, proteasome, and cellular (Tu, et al., 2005). The periodic fluctuation in transcript abundance in the oscillation of the genes expressed in the YMC must be initiated by mRNA synthesis, turnover, or both (Tu, et al., 2005).

With the promoter region of genes expressed with matching temporal kinetics, it indicates that regulatory periodicity will be executed. 60% of all genes annotated to yeast transcription factors fluctuate in the YMC. Transcription factors that do not fluctuation may possess intrinsic metabolic sensors or be themselves coupled to signaling systems with the capability to sense redox or metabolic state (Tu, et al., 2005).

The cell division cycle (CDC) and the events of DNA replication and chromosome segregation are also governed by the ultradian clock. In favorable conditions, the organism initiates DNA replication at the transition between Ox and R/B phases in a 40 minute ultradian rhythm (Lloyd, et al., 2007). Cell division is confined only to the R/B and R/C phases. Neither DNA replication nor cell division is observed in the Ox phase for most cells (Tu, et al., 2005) (Chen, et al., 2007). Moreover, DNA replication and cell division are regulated temporally as a function by the YMC. An explanation of gating DNA replication to the reductive phase is the oxidative phase coincides with the G_1 phase when expressed genes are involved in protein synthesis and sulfur metabolism. Catabolism and liberation of stored sugar in late G_1 has been
proposed to contribute energy and materials to overcome a cycling accumulation limit to grant cell cycle entry (Chen, et al., 2007). The aforementioned cycle can be considered as a ‘limit-cycle’ oscillator model which a model of variable period length that depends on environmental conditions (Tu, et al., 2006). Also by the CDC confined to the reductive phase, the CDC evades the potential mutagenic redox environment of the oxidative respiratory phase (Tu, et al., 2005) by the output of ROS by mitochondrial respiration (Chen, et al., 2007). Observed by Lloyd at a growth rate determined by the continuous culture conditions that S-phase in the CDC is initiated by a group of yeast cells (~8% of total population). Gating of the S-phase by the coupling affects of the ultradian and YMC to the reductive phase is beneficial for protecting replication of DNA and genome integrity from oxidative damage (Schibler, et al., 2005) (Chen, et al., 2007) (Tu, et al., 2006). Also, aging of cells is partially due to an accumulation of reactive O$_2$ species-produced damage to cellular components, primarily lipids and proteins (Lloyd, et al., 2005) (Lloyd, et al., 2003).

3.5 Gene Selection

budding (Adams, et al., 2003) (Mitsui, et al., 1994) (Wang, et al., 2001) (Xu, et al., 2007) (Yaguchi, et al., 2000). BER1 is a less scrutinized yeast gene which is homologous to the *Arabidopsis* circadian rhythm gene SRR1 and is involved in microtubule stability (Fiechter, et al., 2008) and DNA damage repair (Lee, et al., 2007). To begin analysis of the ultradian rhythm in yeast, GTS1 and BER1 are the genes selected for this study.

GTS1 participates in many central cellular roles with *S. cerevisiae*. The protein product of the GTS1 gene, Gts1p, has a coding region for three Gly-Thr repeats (Adams, et al., 2003) (Mitsui, et al., 1994) shared by proteoglycans encompassing a polysaccharide attachment site (Mitsui, et al., 1994). The presences of these GT repeats in the clock-affecting genes from evolutionarily distant species, *Drosophila* and *Neurospora* species, implies that GT plays an important role in most clock-affecting proteins (Adams, et al., 2003) (Mitsui, et al., 1994). Gts1p embodies a zinc finger motif in the amino-terminal region and a glutamine (Q)-rich domain in the carboxy-terminal region (Iha, et al., 2001) therefore this protein was initially codified as a transcription factor (Yaguchi, et al., 2007). GTS1 exhibits partial homology with the circadian clock genes PER in *Drosophila* (Adams, et al., 2003) (Mitsui, et al., 1994) and FRQ in *Neurospora crassa* (Murray, et al., 2001). Therefore, the gene GTS1 was investigated to determine if it played a role in the ultradian rhythm in yeast. When GTS1 was deleted from a yeast strain, the mutant strains displayed either a complete loss of rhythmicity (Lloyd, et al., 2005) (Mitsui, et al., 1994) or shortening of the periodicity of oscillation in continuous cultures in which the cell cycle is self-synchronized or synchrony is induced (Adams, et al., 2003) (Murray, et al., 2001) (Saito, et al., 2002) (Wang, et al., 2001) (Xu, et al., 2007). These studies analyze the autonomously oscillating energy-metabolic pathway. The primary control of this pathway is by phosphofructokinase which transfers energy from glucose to NADH, acting as the
feed-forward activator, and then from NADH to ATP, acting as the feedback inhibitor. After consumption of the inhibitor ATP as fuel, glucose resumes the glycolytic pathway. The oscillations are detectable as a periodic change in the factors involved in energy metabolism such as: dissolved oxygen (DO) levels (Figure 6), CO₂ production, glucose and ethanol concentration, and amounts of stored carbohydrates (Saito, et al., 2002). DO oscillations arise from the periodic changes between respiration and respira-fermentation phases in which oxygen demands are relatively high and low, respectively. These ultradian oscillations occur spontaneously under aerobic conditions dependent on high cell density when the dilution rate through the vessel in a continuous flow reactor is proportion to the total volume of culture and the physical parameters such as temperature, aeration rate, and speed of agitation, are kept constant (Saito, et al., 2002) (Lloyd, et al., 2005) (Wang, et al., 2001) (Xu, et al., 2007). Other studies monitor DO from a continuous flow culture after being synchronized through a process of cell starvation over numerous days (Adams, et al., 2003) (Murray, et al., 2001).

Figure 6: Oscillation of dissolved oxygen. Continuous culture was started at time zero and continued at the dilution of 0.1 h⁻¹ with a synthetic medium containing 1% glucose at 30°C (Wang, et al., 2001).

Benomyl Resistant 1, BER1, belongs to an evolutionary conserved gene family of *Arabidopsis* with involvement in the circadian rhythm (Fiechter, et al., 2008). BER1 is homologous to SRR1, sensitivity to red light reduced, in *Arabidopsis* which is involved in red light response acting in the phytochrome B pathway. The cryptochromes and phytochromes in *Arabidopsis* play roles in photomorphogenesis and in the light input pathways that ultimately synchronizes the circadian clock with the external environment. Moreover, Srr1 mutant strains have shown defective PHYB-mediated signaling and abnormal expression of clock outputs. (Mas, 2005) (Stagier, et al., 2003). BER1 also has shown to be associated with microtubule stability (Fiechter, et al., 2008) and DNA damage repair (Lee, et al., 2007).

GTS1 and BER1 genes provide a locus for ultradian rhythm analysis in *S. cerevisiae*. Moreover, these genes are homologous to more complex eukaryotes such as *Drosophila*, *Neurospora*, and *Arabidopsis* providing insight into the circadian rhythm. By observing these
genes actions as an autonomous oscillating loop, generator of oscillations or regulator of the ultradian rhythm, the biological rhythm in yeast can be discerned and/or perturbed.

### 3.6 Synchronization of a Large Yeast Cell Population

Synchronization of a large yeast population is necessary when monitoring the ultradian rhythm. Developing an experiment for the analysis of a single *S. cerevisiae* cell for gene expression is arduous. Moreover, conventional batch and continuous yeast cultures are asynchronous with respect to individual rhythms because neighboring cells are in different stages of the cell cycle or ultradian rhythm making analysis difficult. Therefore, synchronization is paramount to discern a gene’s expression relating to the yeast metabolic cycle, cell cycle, or ultradian rhythm. At any instant most of the individual cells are assumed to divide in unison, synchronized yeast cultures can be utilized as an opportune instrument to unveil metabolic events (Henson, 2004) (Poulsen, et al., 2007) (Sheppard, et al., 1999), cell cycle modulated protein production (Walker, 1999), and ultradian rhythms (Klevecz, et al., 2004) (Llyod, et al., 2003) (Murray, et al., 2001) (Wang, et al., 2000) (Young, 2004) resembling behavior in a typical individual yeast cell.

Synchronization is achieved when the yeast culture reaches 80% or more in one of the cell cycle phases: G<sub>0</sub>/G<sub>1</sub>, S, or G<sub>2</sub>/M (Galli, et al., 1996) (Yeh, et al., 1995) the proportion of cells that have 1N or 2N DNA content using flow cytometry (Haase, et al., 1997), the percentage of cells with spindles, or by Northern blotting for known cell cycle regulated transcripts (Futcher, 1999). Synchronization can also be achieved when the yeast population exhibits a percent budded or percent unbudded above 90% (Day, et al., 2004). Evaluating percent budded discerns if the culture is in G<sub>0</sub>/G<sub>1</sub> or S plus G<sub>2</sub>/M. There are two methods to attain a synchronized yeast population: induction and centrifugal elutriation (Creanor, et al., 1994) (Day, et al., 2004)

3.6.1 Induction Methods

A method to achieve a synchronized yeast culture from a random yeast population is often a single event, chemical agents in sub-lethal forms or an environmental perturbation, which is used to synchronize a yeast culture by blocking cell cycle progression. The cells are subsequently liberated from the inhibition to restore essential growth conditions (Futcher, 1999) (Sheppard, et al., 1999) (Walker, 1999). The cells are then released back into a fresh medium, a method called block-and-release. A panel of chemical agents blocks cells at different stages of the cell cycle is as follows: alpha factor to arrest cells in G$_1$ phase for haploid yeast cells, hydroxyurea to arrest cells in S-phase, and nocodazole to arrest cells in G$_2$/M phase (Alvino, et al., 2007) (Creanor, et al., 1994) (Day, et al., 2004) (Futcher, 1999) (Walker, 1999). Types of environmental perturbations are heat-shock cycles and feeding-starving (Creanor, et al., 1994) (Futcher, 1999) (Saldanha, et al., 2004) (Walker, 1999). The induction method yields large
numbers of uniformly arrested cells without expensive equipment, making this method a common choice for biochemical and genetic studies (Day, et al., 2004) (Futcher, 1999). When one method fails to yield high synchrony, it is common to combine two different block-and-release cycles. Since the induction method blocks cell cycle progression and not cell growth and protein synthesis, the disadvantage to this method is its susceptibility to create artifacts, a distinct response from the cell cycle due to the blocking agent (Day, et al., 2004) (Futcher, 1999) (Shedden, et al., 2002). Moreover without re-enforcing events in the environment, synchrony will inescapably be lost due to the inherent variations between individual yeast cells in batch cultures (Sheppard, et al., 1999); therefore, a continuous flow reactor must be employed to sustain synchrony indefinitely (Lloyd, et al., 2005) (Llyod, et al., 2003) (Murray, et al., 2007) (Murray, et al., 2001). Although there are shortcomings of the induction method, this method has been instrumental in revealing a number of aspects of the cell cycle metabolism in yeast which would ordinarily been veiled in an asynchronous culture such as the ultradian rhythm of oxygen consumption (Lloyd, et al., 2005).

The aforementioned panel of chemical agents: alpha factor, hydroxyurea, and nocodazole are routinely employed for block-and-release to achieve high yeast culture synchrony for biochemical and genetic studies. The alpha factor is a 13 amino acid peptide which is created by mating type alpha cells and binds to a receptor found on mating type a (MATa) cells. By binding alpha factor, there is an inactivation of G1 cyclin/Cdc28 kinase complex in MATa cells which leads to G1 arrest with a 1N deoxyribonucleic acid (DNA) content (Creanor, et al., 1994) (Day, et al., 2004) (Futcher, 1999) (Walker, 1999). The yeast cells attempt to recover from the alpha factor arrest through various mechanisms. One important mechanism is the Bar1 protease which is secreted and degrades alpha factor (Futcher, 1999). There are two ways to combat this issue to
attain high synchrony: increase the amount of alpha factor, 100mg or more, or employ a MAT\textit{a bar1} mutant strain. By increasing the alpha factor amount, Bar1 protease will be overcome however synchrony will be transient. Utilizing a MAT\textit{a bar1} mutant strain, the amount of alpha factor will be 100-fold to 1000-fold less and the arrest is far more extended creating a flexible time for the yeast cells to be released (Futcher, 1999).

Hydroxyurea (HU) is a ribonucleotide reductase (RNR) inhibitor preventing the reduction of ribonucleotides to deoxyribonucleotides (deoxyribonucleotide triphosphates [dNTPs]) blocking DNA synthesis (Alvino, et al., 2007) (Day, et al., 2004) (Galli, et al., 1996) (Matmati, et al., 2009). Yeast cultures treated with high concentrations of HU (200mM) activate the S-phase checkpoint, cells arrest in early S-phase since accumulation of dNTPs are prevented, and cells engage the checkpoint to prohibit passage through the cell cycle into a catastrophic mitosis (Alvino, et al., 2007) (Galli, et al., 1996). In the presence of HU, the replication bubble expands with forks advancing at a synthesis rate of \(~50\text{bp per minute}\) which is significantly lower than unchallenged forks. When the HU inhibition is released in wild-type cells, transcription activated genes involved in replication and repair stabilize the idling replication complex to permit the resumption for progression (Alvino, et al., 2007). After several hours of HU exposure, wild-type cells can overcome the S-phase blockage to resume cell division (Matmati, et al., 2009).

analysis is employed to assay DNA content of individual yeast cells to determine the cell cycle phase.

Other induction methods are heat shock cycles. Heat shock cycling is a very simple technique to synchronize large cultures by introducing repetitive heat shocks to a yeast culture (Creanor, et al., 1994). In this method, yeast cells are grown overnight at 32°C then transferred to a water bath at 41°C for 30 minutes. After, the culture is placed in a shaker for 130 minutes at 32°C, roughly one generation. This process is repeated five to six times to reach synchrony (Creanor, et al., 1994). Another application of heat shock cycling is to restrict cdc mutant strains of Sch. Pombe and S. Cerevisiae at a high temperature, approximately 35°C, then shifting the cultures back to a lax temperature of 25°C inducing synchrony. These temperature-sensitive mutants cause provisional, uniform, and readily reversible arrest of the cell cycle in mitosis provided induction conditions do not result in cell death (Creanor, et al., 1994). Cells in log-phase culture shifted to the restrictive temperature will cease cell division at the same point in M-phase. Moreover after the yeast culture is released to a lax temperature, synchrony tends to decay rapidly after the first cell cycle. This is due to the continual growth during the restrictive temperature while cells are not dividing. Consequently, the yeast cells are twice as large as normal yeast cells which have the effect of shortening subsequent cycle times as the cells attempt to get back to normal size causing asynchrony (Creanor, et al., 1994). The relation of heat shocked synchronized cells to normal cells cycle may have variation particularly due to the environmental shock at the start of each cell cycle (Mitchison, et al., 1965).

Feed-starve cycles are another method to achieve synchrony. In this method, yeast cell cultures consume all nutrients causing the starved yeast cells to cease all growth and entering a nonproliferating state referred to as stationary phase or G₀ (Creanor, et al., 1994) (Werner-
Washburne, et al., 1993). These unbudded yeast cells allow maintenance of viability for extended periods without added nutrients retaining the ability to resume growth readily when proper nutrients are provided (Werner-Washburne, et al., 1993). During this process, smaller cells which are difficult to synchronize are separated through centrifugal sedimentation and resuspended in starvation medium (Creanor, et al., 1994) (Mitchison, et al., 1965). With multiple feed-starve cycles treatments, yeast cells are inoculated into fresh medium to induce synchrony (Creanor, et al., 1994) (Mitchison, et al., 1965) (Walker, 1999). Multiple cycles which ranges from twelve to seventeen days are usually required to achieve synchrony. A budding profile plot analyzing percent budded and unbudded cells can be generated to determine the degree of synchrony (Creanor, et al., 1994). The largest disadvantage of the feed-starve method is the extend time needed and the multiple handling operations necessary.

3.6.2 Centrifugal Elutriation

Centrifugal elutriation separates cells on the basis of their size, mass, shape, and sedimentation rate (Day, et al., 2004) (Walker, 1999). Using centrifugal force, yeast cells sediment in the presence of a counter flow of medium (Figure 7). Cells are inoculated into the funnel shaped chamber of the spinning rotor which is preset at a flow rate (Walker, 1999). In this technique, large budded or irregularly shaped cells sediment towards the outer edge of the elutriation chamber while smaller cells are less affected by centrifugal forces. Moreover due to the high surface area of smaller cells, small cells tend to be pushed toward the top of the elutriation chamber by the counter flowing medium (Day, et al., 2004). These layers containing the smaller cells can be elutriated out of the chamber by increasing the counter flow rate and harvested (Day, et al., 2004) (Walker, 1999). Centrifugal elutriation is regarded as the preferred method for synchronizing cell population because this technique attains the purest synchronized
population of dividing cells without extraneous environmental stresses (Mitchison, et al., 1965). There are two main advantages of this technique. First because selection is based on cell size, centrifugal elutriation does not perturb the normal coordination between cell growth and cell division generating a synchronous culture in the most physiologically relevant manner. Second, this technique can be applied to any yeast strain making it universally applicable (Day, et al., 2004). The major disadvantage of centrifugal elutriation is the need for large and expensive centrifugation equipment. Furthermore, the manipulation and centrifugation of yeast cells may induce stress responses. Therefore, this technique should be compared to results of at least one other synchronizing technique (Day, et al., 2004).

**Figure 7: Process of Centrifugal Elutriation**

(A) Small and large yeast cells begin to enter the elutriation chamber. (B) Centrifugal elutriation creates a size gradient with smaller cells towards the top and larger cells towards the bottom of the elutriation chamber. (C) Increasing the counter flow elutriation chamber (Day, et al., 2004).

### 3.6.3 Summary

When monitoring the ultradian rhythm through gene expression, synchronization of a large yeast population is essential. For this study, a block-and-release synchronization technique was utilized. Hydroxyurea and nocodazole were used to synchronize cultures in S-phase and G2/M phase of the cell cycle. Also to achieve a higher level of synchrony, hydroxyurea and nocodazole were used in subsequent treatments. The block-and-release technique provided a large synchronous culture without subjecting the culture to extreme environmental pressure such
as high temperatures as in heat shock or low pH as in cell starvation. Moreover, in developing a tool for more complex eukaryotes such as human, these extreme environmental conditions are in far contrast to natural conditions of moderate pH and temperature. Due to expense constraints, centrifugal elutriation was not employed; however, it provides a high level of synchrony with very few artifacts.
CHAPTER 4 – MATERIALS AND METHODS

4.1 Introduction to Materials and Methods

Yeast cells are arguably the most studied organisms and are recognized as the model system for simple eukaryotes. Yeast cells are advantageous to examine because of their rapid growth, ease of transfer to different media, well-defined genetic system, affordability, and highly adaptable DNA transformation system (Sherman, 2002). Moreover, the first fully sequenced genome of a eukaryote was the yeast cell *Saccharomyces cerevisae*. Many human diseases can be related to yeast; therefore yeast has become a model eukaryotic system for diverse biological studies (Sherman, 2002). To analyze the ultradian rhythm in yeast, three steps were executed: a novel yeast strain created, synchronized, and analyzed using flow cytometry for batch and continuous flow systems.

4.2 Yeast Medium

Yeast cells were cultured in complete liquid media, on the surface of complete solid agar plates, or histidine drop out solid agar plates. Generally, YPD is a rich medium (2% glucose, 2% bactopeptone, and 1% yeast extract) in which all necessary nutrients are provided for complete liquid and solid agar (2% agar) medium. In minimal media, a particular metabolite is omitted in this case histidine (Treco, et al.). This is often employed to assess uptake of DNA post transformation in yeast cells.

4.3 Creation and Verification of Deleted Ultradian Genes

For this study, we report the creation and characterization of a yeast-based promoter-reporter construct to detect the ultradian rhythm in yeast. To accomplish this task, two novel
yeast strains were synthesized: ICY100 for GTS1 analysis and ICY101 for BER1 analysis. These yeast strains were derived from the S. cerevisiae strain BY 4743 (MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 / MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 purchased from Thermo Scientific) using a PCR based gene deletion method (Longtine, et al., 1998). Plasmid pFA6a-GFP(S65T)-HIS3MX6 was isolated from E. coli DHα by alkaline lysis with SDS Minipreparation (Sambrook, et al., 2001). Successful plasmid extraction was verified by running the isolated plasmids through an agarose gel. Forward and reverse primers were designed to generate a knockout cassette composing of a fusion encoding histidine resistance flanked by 45 bp regions homologous to the 45 bp region upstream and downstream from either the GTS1 ORF or BER1 ORF shown in Table 1. Agarose Gel Electrophoresis was employed to corroborate the construction of the cassette.

Table 1: PCR primers used to construct knockout cassette

<table>
<thead>
<tr>
<th>Primer</th>
<th>Purpose</th>
<th>Primer Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>GTS1 F</td>
<td>GTS1 Forward Deletion Primer</td>
<td>5’ TCT GTC AAT CAC AGA TTA TAG TTT ATT TAT ACT TAG</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TCA AAA ATG CGG ATC CCC GGG TTA ATT AA 3’</td>
</tr>
<tr>
<td>GTS1 R</td>
<td>GTS1 Reverse Deletion Primer</td>
<td>5’ ATG ATA TGT AAT GCG GAG CAG CGG CAA TGT ATC TTA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TTT TTA GAA TTC GAG CTC GTT TAA AC 3’</td>
</tr>
<tr>
<td>BER1 F</td>
<td>BER1 Forward Deletion Primer</td>
<td>5’ ACC TGA TAC CGA ACT TAA TGA CCT TGA CCG GTC TTC</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TGC TGT ATG CGG ATC CCC GGG TTA ATT AA 3’</td>
</tr>
<tr>
<td>BER1 R</td>
<td>BER1 Reverse Deletion Primer</td>
<td>5’ TAC AGT TTA GTT ATA AAG TAC TAT GTA GTG AAT GAT</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ATG TTA GAA TTC GAG CTC GTT TAA AC 3’</td>
</tr>
</tbody>
</table>

ICY 100 and ICY 101 cells were transformed using a lithium acetate protocol (Gietz, et al., 1992). By utilizing primers approximately 500 bp upstream from the target gene start site and a primer within the histidine resistance coding region, transformants were selected on histidine deficient medium. After, the new strains were corroborated by using PCR verification (Table 2)
and agarose gel electrophoresis. Successfully transformed yeast cells were harvested and stored at -80°C in YPD broth + 50% glycerol.

Table 2: Primers constructed to verify transformation

<table>
<thead>
<tr>
<th>Primer</th>
<th>Purpose</th>
<th>Primer Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>GTS1 V</td>
<td>GTS1 Verification</td>
<td>5’ AAC ATA CCT TTA TGT CGG TTG C 3’</td>
</tr>
<tr>
<td>BER1 V</td>
<td>BER1 Verification</td>
<td>5’ TTT GTA ACG ACT GCT AAA TAA CAA A 3’</td>
</tr>
<tr>
<td>GFP V</td>
<td>GFP Verification</td>
<td>5’ AAA GGG CAG ATT GTG TGG AC 3’</td>
</tr>
</tbody>
</table>

4.4 Synchronization of Yeast Cell Populations

For the yeast cells to act in unison, a block-and-release method was employed in which cells were blocked by a chemical agent as described by Day (Day, et al., 2004) into S or G2/M phase of the cell cycle. Using this method allowed for a synchronized cell cycle and ultradian rhythm when cells were released from the chemical blocking agent. To create a synchronous ICY 100 culture, two chemical agents for synchrony hydroxyurea (HU) and nocodazole (NOCO) were tested. To determine the level of synchronization, samples were prepared for flow cytometry to discern percent 1N and 2N DNA or percent budded to unbudded yeast cells using a protocol developed by Creanor (Creanor, et al., 1994). Calibur flow cytometer (BD Biosciences, San Jose, CA) at the School of Veterinary Medicine, LSU configured for propidium iodide fluorescence measurements. Forward and side scatter measurements were made using linear amplification, and fluorescence measurements were made with logarithmic amplification for linear amplification for DNA replication. A total for 15,000 cells per sample were analyzed on a Macintosh G5 computer (Apple Computer, Cupertino, CA) running Cellquest graphics software (BD Biosciences, San Jose, CA). Single color fluorescence analyses in the form of histograms were used to examine DNA cell cycle components.
First, cultures were inoculated with high concentrations of HU (200mM) activating the S-phase checkpoint. HU is a ribonucleotide reductase which inhibits the reduction of ribonucleotides to dexocyribonucleotide preventing the accumulation of dNTPs and therefore arrests cells in early S-phase (Alvino, et al., 2007) (Galli, et al., 1996). Cultures were grown overnight in YPD to early log phase. Early log phase was determined by a growth curve generated by optical density readings at 600nm from a DU Series 700 Spectrophotometer (Bechman Coulter, Fullerton, CA). Then, the culture was diluted using YPD to a cell concentration between 0.2-0.5 x 10^7 cells/mL. Cell concentrations were determined by a hemacytometer. HU was added to a final concentration of 200mM. 1 mL samples were taken at 2 hours and 3 hours post treatment of HU in triplicates. These points were analyzed with flow cytometry. When the percentage of cells in S-phase exceed 80%, the culture was determined synchronized.

Second, cultures were inoculated with 15 μg/mL nocodazole (NOCO) which reversibly binds to tubulin and inhibits microtubule polymerization preventing nuclear migration and nuclear division in yeast (Day, et al., 2004) (Jacobs, et al., 1982) (McKinney, et al., 1993). NOCO arrests cells in G₂/M with large buds with DNA content of 2N (Day, et al., 2004) (Futcher, 1999). This mitotic arrest effects growth and morphology of yeast and the development of multicellular forms with mother and daughter cells with the same size (Jacobs, et al., 1982). Cultures were grown overnight in YPD plus 1% dimethyl sulfoxide (DMSO) to early log phase. Then, the culture was diluted using YPD plus 1% DMSO to a cell concentration between 0.2-0.5 x 10^7 cells/mL. After, the culture was inoculated with 15 μg/mL of NOCO. 1 mL points were taken at 2 hours and 3 hours post inoculation of NOCO in triplicates. These points were analyzed
with flow cytometry to evaluate percent budded and unbudded. When the percentage of budded cells exceed 90%, the culture was determined synchronized.

Third, cultures were treated with HU and NOCO in sequence. First, the culture was synchronized with the method described before with HU for 3 hours. Second, the culture was washed of any HU with warm phosphate buffer saline (PBS) twice and synchronized with the method described for NOCO for 2 hours. 1 mL points were taken at the end of the sequence in triplicates and analyzed using flow cytometry to discern budded and unbudded cells. When the percentage of budded cells exceed 90%, the culture was determined synchronized.

4.5 Flow Cytometry Experiments of Asynchronous and Synchronous Populations

Flow cytometry experiments were performed on asynchronous and synchronous ICY100 populations to evaluate the GFP expression. Fluorescence intensity was determined by comparison with BY 4743 cells. All samples were tested on a FACS Calibur flow cytometer (BD Biosciences, San Jose, CA) at the School of Veterinary Medicine, LSU configured for GFP and Propidium Iodide fluorescence measurements. Forward and side scatter measurements were made using linear amplification, and fluorescence measurements were made with logarithmic amplification for GFP expression and linear amplification for DNA replication. A total for 15,000 cells per sample were analyzed on a Macintosh G5 computer (Apple Computer, Cupertino, CA) running Cellquest graphics software (BD Biosciences, San Jose, CA). Single color fluorescence analyses in the form of histograms were used to examine GFP expression.

For asynchronous cultures, experiments were conducted in batch and continuous flow. The continuous flow reactor employed was the BIOFLO 3000 bench-top fermentor (New Brunswick Scientific Co., Inc., Edison, NJ). In a batch system, asynchronous BY 4743 and triplicates of ICY 100 were grown to log phase then diluted to roughly 0.2 OD at 600nm using
YPD. 1 mL samples were taken every 30 minutes for 6 hours. Samples were washed using phosphate buffer saline (PBS) twice. A wash consisted of centrifuging the sample at 3000 rpm for 5 minutes then decanting supernatant and resuspending cells in PBS. After the second wash, samples were resuspended in PBS and placed on ice in a refrigerator at 4°C overnight. Samples were then analyzed using flow cytometry to detect GFP expression.

In a continuous flow system, ICY 100 was supplied a continuous feed of warm YPD at 1.8 mL/min, a dilution rate of 0.0036 min⁻¹, at standard conditions. 1 mL samples were taken every 10 minutes. The culture was grown to log phase inserted into a chemostat and raised to a final volume 500 mL using warm YPD starting with an OD of approximately 0.2.

GFP expression of synchronous cultures (batch and continuous) was measured for ICY 100. Prior to each experiment, synchronization was assessed via flow cytometry. As mentioned in the literature, synchronization of a yeast population within batch systems can last typically two generation (Day, et al., 2004). Therefore, a continuous flow experiment within a chemostat was also performed with the goal of maintaining synchronization beyond two generations. Samples from each experiment were analyzed for GFP expression by flow cytometry. In batch, BY 4743 and IC 100 in triplicates were grown overnight to log phase. IC 100 was then synchronized using HU and NOCO then transported to warm YPD as previously described in Section 4.4. After, 1 mL samples were collected from each culture to discern level of synchronization prior to experimentation evaluating budded and unbudded cells. While cultures grew in a shaker at 110 rpm and a temperature of 30°C, 1 mL samples were collected every 10 minutes for 4 hours. Samples were washed using PBS twice, placed on ice, and incubated at 4°C overnight. Each point was analyzed with flow cytometry assessing GFP expression. In a chemostat, BY 4743 and ICY 100 synchronized using HU and NOCO as previously described in
4.4 and placed in warm YPD. The final volume of the synchronized cultures was 160 mL. The culture were inserted into the chemostat and raised to a final volume 500 mL using warm YPD. 1 mL samples were collected from each culture to discern level of synchronization prior to experimentation. After, warm YPD flowed in and cells plus medium flowed out at a constant rate of 3.5 mL/min with dilute rate of 0.007 min⁻¹. Cultures were grown in a chemostat at 110 rpm and a temperature of 30°C. 1 mL points were collected every 10 minutes for 200 minutes. Samples were washed using PBS twice, placed on ice, and incubated at 4°C overnight. Each point was analyzed with flow cytometry assessing GFP expression.
CHAPTER 5 – RESULTS AND DISCUSSION

The ultradian rhythm was characterized by monitoring the oxygen consumption of polyploid yeast cells (Adams, et al., 2003) (Chen, et al., 2007) (Klevecz, et al., 2004) (Murray, et al., 2007) (Llyod, et al., 2003) (Saito, et al., 2002) (Wang, et al., 2000) (Xu, et al., 2007). To investigate changes in the ultradian rhythm, Murray (Murray, et al., 2001) selected and deleted genes in yeast then grew the new strain in synchrony within a continuous flow reactor to evaluate the effect on oxygen consumption. However, these approaches required lengthy synchronizations lasting weeks and an acidic medium (pH 3.4). In this research, discerned genes regulating the ultradian rhythm in diploid *Saccharomyces cerevisiae* were monitored by a promoter-reporter construct utilizing green fluorescent protein (GFP) by transforming yeast cells with a knockout cassette by homologous recombination. The transformed yeast strains were grown in standard medium and synchronized using a block-and-release method which drastically shortened the time to synchronize to 6 hours. By monitoring GFP with flow cytometry in asynchronous and synchronous cultures, this reporter gene characterized the gene’s expression level allowing frequent monitoring of the ultradian rhythm.

5.1 Transformed Yeast Strain ICY 100

The novel yeast strain, ICY 100, was generated by transforming the diploid strain, BY4743, with the GTS1P-GFP promoter-reporter construct (Figure 8). This construct knocked out one of the two GTS1 genes and was verified using PCR (Figure 9). A growth curve (Figure 10) of ICY 100 where the end of lag-phase approximately at an optical density (OD) of 0.2. Log-phase appeared after 0.2 OD and concluded approximately at 1.7 OD, lasting almost 500
minutes. After the culture exceeded an OD value of 1.7, the yeast cells began the stationary phase.

Figure 8: Verification of GTS1 Knockout Cassette Run Through an Agarose Gel – Wells: (1) 1kb Ladder, (2)-(6) GTS1 Knockout Cassette at ~2.5kb, (7) ACT1 Positive Control at ~1kb, (8)-(10) Isolated Plasmid

Figure 9: Verification Knockout Cassette Transformation Run Through an Agarose Gel for GTS1- Wells (1) 1kb Ladder, (2)-(11) Verification Strain ~1kb, (12) ACT1 Positive Control ~1kb, (13)-(14) Isolated Yeast DNA
Figure 10: Growth Curve of ICY 100. ICY 100 was grown overnight. Samples were collected starting (t=0) at an optical density of 0.01 and continued until stationary phase was reached.

A flow cytometry experiment was executed utilizing BY 4743 and triplicates of ICY 100 in a batch culture. Samples were taken every 30 minutes for 6 hours and analyzed using flow cytometry to detect GFP expression. Data collected from the GFP expression of IC 100 were normalized to the control sample BY 4743 GFP expression shown in Figure 11. The graph illustrates a 2 to 2.55 fold increase in GFP discerning that the transformed cassette embodying GFP had successfully been inserted. However evaluating the GFP expression over 6 hours, there is no distinguishable biological rhythm or oscillation that can be construed as an ultradian rhythm.
Figure 11: ICY 100 Triplicates of GFP Expression Collected by Flow Cytometry Normalized to GFP Expression of BY 4743 with Standard Deviation. Samples were taken every 30 minutes for 6 hours.

A chemostat was also employed sampling data point every 10 minutes as shown in Figure 12. The culture was grown to log phase inserted into the chemostat and raised to a final volume 500 mL using warm YPD, 30°C. GFP expression was normalized to the GFP expression at time zero. As time proceeded past 5 hours, GFP expression steadily decreased while slightly fluctuating. These fluctuations could not be considered an ultradian rhythm because the fluctuation was only slight and the trend of expression exhibited an overall decrease as time passed.
Figure 12: Chemostat Analysis of Green Fluorescent in IC Y100. Samples were analyzed by flow cytometry. GFP expression is normalized to the initial expression. Samples were taken every 10 minutes for 5 hours.

For the asynchronous batch and continuous flow systems, there were no distinct ultradian rhythms. Since the yeast cells were not growing and dividing in unison, neighboring yeast cells were in dissimilar states of the ultradian rhythm. Therefore, the aforementioned asynchronous yeast cultures create an average GFP expression output with no significant fluctuation.

5.2 Transformed Yeast Strain IC Y101

The novel yeast strain, IC Y101, was created by transforming the diploid strain, BY4743, with the GFP(S65T)-HIS3MX6 cassette (Figure 13). This construct knocked out one of the two BER1 genes via homologous recombination generating a BER1P-GFP promoter-reporter construct and was verified by PCR (Figure 14). A growth curve (Figure 15) of IC 101 illustrates a lag-phase approximately ends at an optical density (OD) of 0.5. Log-phase appears after 0.5 OD and concludes approximately 1.4 OD lasting roughly 3 hours. After the culture has reached an OD value above 1.4, the yeast cells begin stationary phase.
Figure 13: Verification of BER1 Knockout Cassette Run Through an Agarose Gel - Wells: (1) 1kb Ladder, (2)-(6) BER1 Knockout Cassette ~2.5kb, (7)-(8) ACT1 Positive Control ~1kb

Figure 14: Verification of Knockout Cassette Transformation Run Through an Agarose Gel for BER1 - Wells (1) 1kb Ladder, (2)-(5) Verification Strain ~1kb, (6) ACT1 Positive Control
Figure 15: Grow Curve of ICY 101 by analyzing optical density over time.

A flow cytometry experiment was performed with ICY 101 grown in a batch culture. Samples were taken every 30 minutes for 6 hours. Samples were then analyzed using flow cytometry to detect GFP expression. Triplicates of ICY 101 GFP expression data were normalized to the sample expressing the highest GFP expression as shown in Figure 16. Illustrated in Figure 17 is the average of the triplicate samples of ICY 101.

After analysis of this data, transformed ICY 101 was not expressing GFP levels higher than the control, BY4743. Moreover, the GFP expression could not be discerned as a biological rhythm since expression lacked any fluctuation. Therefore, ICY 101 was not tested in synchronous cultures in batch and continuous flow.
Figure 16: Batch ICY 101 Green Fluorescent Expression Normalized to the Sample Expressing the Highest Green Fluorescence.

Figure 17: In batch, the average of green fluorescent expression in the triplicate samples of ICY 101.
5.4 Synchronization of a Largest Yeast Population Using Block-and-Release

To synchronize this culture, cells were blocked into an S or G₂/M phase of the cell cycle by a chemical agent and released via warm PBS wash. This technique, known as block-and-release, is described by Day (Day, et al., 2004). To create a synchronous ICY 100 culture, two chemical agents for synchrony hydroxyurea (HU) and nocodazole (NOCO) were employed. To determine the level of synchronization, samples were prepared for flow cytometry to discern percent 1N and 2N DNA using a protocol developed by Creanor (Creanor, et al., 1994).

ICY 100 cultures were grown overnight and treated with 200mM of HU activating the S-phase checkpoint. 1 mL points were taken at 2 hours and 3 hours post treatment of HU in triplicates. These points were analyzed with flow cytometry shown in Figure 18.

![Fluorescence from propidium iodide which allows discernment of 1N and 2N cells. Markers set relative to control: M1-G₀/G₁, M2-G₂/M, M3-S.](image)

Figure 18: ICY 100 with HU 200mM concentration. ICY 100 was grown overnight in YPD to early log phase. Then the culture was diluted using YPD to a cell concentration between 0.2-0.5 x 10⁷ cells/mL. HU was added to a final concentration of 200mM. 1 mL points were taken at 2 hours and 3 hours post treatment of HU in triplicates. Points were collected and analyzed using flow cytometry. Left: cell cycle of ICY 100 after 2 hours after exposure. Right: cell cycle of ICY 100 after 3 hours after exposure. Y-Axis: Cell Count. X-Axis: Fluorescence from propidium iodide which allows discernment of 1N and 2N cells. Markers set relative to control: M1-G₀/G₁, M2-G₂/M, M3-S.
From the data collected from flow cytometry shown in Table 3, the ICY 100 cultures exposed to HU lasting 2 and 3 hours yielded similar amounts of G0/G1, S, and G2/M. However, the level of synchronization did not exceed 80% for any of the phases and therefore failing to reach synchronization. Moreover, the flow cytometry analysis suggests that HU blocked ICY 100 in G0/G1 far more than S phase which is in contrast to the literature. Subsequent time points were not attained due to suggestions in the literature describing the yeast cells’ ability to overcome HU over prolong times (Matmati, et al., 2009). With the data obtained, ICY 100 with the use of the chemical agent HU nudges the cells closer to some sort of synchrony.

Table 3: HU 200mM ICY 100 Culture Cell Cycle Percentages over Time with Standard Deviation.

<table>
<thead>
<tr>
<th>Exposure Time (hrs)</th>
<th>G0/G1 (%)</th>
<th>S (%)</th>
<th>G2/M (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>79.66 ± 1.72</td>
<td>9.37 ± 0.52</td>
<td>9.36 ± 1.08</td>
</tr>
<tr>
<td>3</td>
<td>73.50 ± 10.19</td>
<td>13.33 ± 7.19</td>
<td>11.33 ± 2.54</td>
</tr>
</tbody>
</table>

In a separate experiment, ICY 100 cultures were treated with nocodazole (NOCO) which reversibly binds to tubulin and inhibits microtubule polymerization preventing nuclear migration and nuclear division in yeast (Day, et al., 2004) (Jacobs, et al., 1982) (McKinney, et al., 1993). NOCO holds cells in G2/M and cells arrest with large buds with DNA content of 2N (Day, et al., 2004) (Futcher, 1999). The cultures were inoculated with 15 μg/mL of NOCO. 1 mL samples were taken at 2 hours and 3 hours post inoculation of NOCO in triplicates and analyzed with flow cytometry to evaluate cells budded and unbudded as shown in Figure 19. When evaluating synchronization by NOCO, Day (Day, et al., 2004) explained the culture must exceed a budding percentage above 90% to be considered synchronized. From the data collected by the flow cytometer shown in Table 4, the ICY 100 cultures exposed to NOCO for 2 hours generated the most budded cells and the least of amount of unbudded cells. However, the level of synchronization did not exceed 90% for budded cells and therefore failed to reach synchrony.
With the data obtained, the ICY 100 cultures treated with the chemical agent NOCO came closer to achieving synchrony.

Figure 19: ICY 100 with 15 μg/mL of NOCO. ICY 100 was grown overnight in YPD to early log phase. Then the culture was diluted using YPD to a cell concentration between 0.2-0.5 x 10^7 cells/mL. NOCO was added to a final concentration of 200mM. 1 mL points were taken at 2 hours and 3 hours post treatment of NOCO in triplicates. Points were collected and analyzed using flow cytometry. Left: cell cycle of ICY 100 after 2 hours after exposure. Right: cell cycle of ICY 100 after 3 hours after exposure. Y-Axis: Cell Count. X-Axis: Fluorescence from propidium iodide which allows discernment of 1N and 2N cells. Markers set relative to control: M1-G0/G1, M2-G2/M, M3-S.

Table 4: NOCO 15 μg/mL ICY 100 Culture Percentages of Budded and Unbudded Yeast Cells Over Time with Standard Deviation.

<table>
<thead>
<tr>
<th>Exposure Time (hrs)</th>
<th>Budded Cells</th>
<th>Unbudded Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>89.20 ± 1.85</td>
<td>8.82 ± 1.25</td>
</tr>
<tr>
<td>3</td>
<td>84.79 ± 0.32</td>
<td>13.16 ± 0.42</td>
</tr>
</tbody>
</table>

With each chemical agent providing a level of synchrony close to full synchrony, an experiment was created to use HU and NOCO in sequence. First, ICY 100 was synchronized with the method described before with HU for 3 hours because of relative higher level of S phase. Second, the culture was washed of any HU by warm PBS (30°C) twice and synchronized with the method described for NOCO. 1 mL samples were taken at the end of the sequence in triplicates and analyzed using flow cytometry to discern budded and unbudded cells as shown in Figure 20.
Figure 20: ICY 100 with HU 200mM followed by 15 μg/mL of NOCO. Points were collected and analyzed using flow cytometry. Y-Axis: Cell Count. X-Axis: Fluorescence from propidium iodide which allows discernment of 1N and 2N cells. Markers set relative to control: M1-G0/G1, M2-G2/M, M3-S.

After the analysis of budded and unbudded cells through flow cytometry data, the percentage of cells in each phase was as follows: 95.06 ± 0.36 budded cells and 4.56 ± 0.26 unbudded cells. Therefore using this method, ICY 100 was able to attain synchrony in G2/M phase.

5.5 Analyzing GFP from Synchronized ICY 100 in Batch

With synchronized ICY 100, experiments were executed to discern GFP expression in a batch system. Since the yeast cells were growing and dividing in unison, neighboring yeast cells were in similar stages of the ultradian rhythm. As mentioned in the literature, synchronization of a yeast population within batch systems can last to a maximum of two generation. To discern longevity of synchrony of ICY 100 in a batch system, ICY 100 was grown overnight in YPD to early log phase then synchronized by the method previously mentioned. 1 mL samples were taken in triplicates and analyzed using flow cytometry. Shown in Table 5 is the data collected from the flow cytometer. The synchronization of ICY 100 drops by 10% in the first hour.
however stabilizes to 73.69% of budded cells after 4 hours as seen in Figure 21 and 22 the unbudded cells maintain 25.13% of the total population.

Table 5: Synchronized ICY 100 with HU and NOCO sequence and resuspended in warm YPD. In batch, points were collected as shown and prepared for flow cytometry to assess percentage of cells budded and unbudded with standard deviation. The points with a † are the average of only two points.

<table>
<thead>
<tr>
<th>Point</th>
<th>Time (mins)</th>
<th>Budded Cells</th>
<th>Unbudded Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>95.06 ± 0.36</td>
<td>4.57 ± 0.26</td>
</tr>
<tr>
<td>2</td>
<td>60</td>
<td>85.36 ± 0.84</td>
<td>13.83 ± 0.98</td>
</tr>
<tr>
<td>3</td>
<td>120</td>
<td>78.42 ± 1.44</td>
<td>20.47 ± 1.64</td>
</tr>
<tr>
<td>4</td>
<td>150</td>
<td>75.99 ± 0.61†</td>
<td>22.84 ± 0.34†</td>
</tr>
<tr>
<td>5</td>
<td>180</td>
<td>72.57 ± 0.91†</td>
<td>25.93 ± 1.25†</td>
</tr>
<tr>
<td>6</td>
<td>210</td>
<td>75.11 ± 0.97</td>
<td>23.33 ± 0.40</td>
</tr>
<tr>
<td>7</td>
<td>240</td>
<td>73.69 ± 1.53</td>
<td>25.13 ± 1.33</td>
</tr>
</tbody>
</table>

Figure 21: Synchronized culture of ICY 100 in batch. Points were collected as shown and analyzed using flow cytometry to assess percentage of cells budded and unbudded. The red asterisk points are the average of two points.
Then in batch, BY 4743 and ICY 100 in triplicates were grown overnight to log phase then synchronized using HU and NOCO as previously described in 5.2.2. After synchronization was completed, cultures were transported into warm YPD (30°C). 1 mL samples were collected from each culture to discern level of synchronization prior to experimentation. BY 4743 exhibited 91.82% budded prior to experimentation. Budding percentages for ICY 100 were: A-91.80%, B-92.27%, and C-93.61% budded. 1 mL samples were collected every 10 minutes for 4 hours. Each point was analyzed with flow cytometry assessing GFP expression. The data collected from the triplicates of ICY 100 were averaged and normalized to the control, BY 4743. (Figure 23).
In batch, BY 4743 and the triplicates of ICY 100 began with high levels of synchronization. When normalized to the control BY 4743 in batch, ICY 100 exhibited a 2.46 to 3.33 fold increase in GFP expression, an increase greater than asynchronous cultures. After analysis of GFP expression, there is an oscillation between the start time and 150 minutes with two periods. The first period begins with a GFP expression of 2.72 then increases to 2.96 after 30 minutes then decreases to 2.62 at 70 minutes. The second period begins with a GFP expression of 2.88 then increases to 3.09 at 100 minutes then decreases to 2.76 at 140 minutes. Therefore from the data, there is a 70 minute period of oscillation for the gene GST1. This period fits the description by Lloyd and Murray (Lloyd, et al., 2007) that a temperature-compensated clock period in yeast is about an hour (30-90 minutes). After 170 minutes, the data illustrates a loss of fluctuation which behaves in same manner as the aforementioned oscillations. Since the block-and-release synchronization method can only embrace synchrony for one to two generations, it was discerned that the loss of fluctuation was due to the loss of synchronization. With the doubling time roughly 2 hours, synchronization would have been present for 240 minutes if
synchronization was kept for two generation which is not illustrated by the GFP expression output. This corroborates the results earlier regarding the synchronization’s duration. ICY 100 for this experiment is holding synchrony for one generation which is $111.7 \pm 2.64$ minutes. The succeeding generation begins to lose synchrony beginning roughly at 150 minutes. Therefore after the budding percentage decrease below 75%, the culture can be assumed no longer in synchrony.

5.6 Analyzing GFP from Synchronized ICY 100 in Continuous Flow

A continuous flow experiment within a chemostat was also performed in hopes that synchronization would be held for an extended period of time. The longevity of ICY 100 synchrony was analyzed in a chemostat system. 1 mL samples were taken before insertion into the chemostat and after 5 minutes within the chemostat. Subsequent points were taken in 30 minute intervals. After samples were analyzed using flow cytometry to discern percent budded and unbudded cells. The percentages of the combined synchronized ICY 100 culture in each phase prior to this chemostat experiment were as follows: budded cells of 94.3% and unbudded cells of 4.59%. Samples from each experiment were analyzed for GFP expression by flow cytometry. Shown in Table 6 are the data collected from flow cytometry. The synchronization of IC 100 was maintained above 85% of budded cells over the 4 hour period within the chemostat. Unbudded cells phase increases to 11.53% of the total culture. Over an extended period of time, the percentage of unbudded cells would likely increase while percentage budded cells would decrease below synchronization standards based on the trend data as shown in Figure 24.
Table 6: Synchronized ICY 100 culture within a chemostat at a constant dilution rate of. Points taken as followed and analyzed using flow cytometry to assess the percent of cells within each phase of the cell cycle.

<table>
<thead>
<tr>
<th>Point</th>
<th>Time (mins)</th>
<th>Budded Cells</th>
<th>Unbudded Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5</td>
<td>92.98</td>
<td>5.66</td>
</tr>
<tr>
<td>2</td>
<td>30</td>
<td>92.59</td>
<td>5.88</td>
</tr>
<tr>
<td>3</td>
<td>60</td>
<td>92.03</td>
<td>6.40</td>
</tr>
<tr>
<td>4</td>
<td>90</td>
<td>90.43</td>
<td>7.19</td>
</tr>
<tr>
<td>5</td>
<td>120</td>
<td>90.88</td>
<td>5.51</td>
</tr>
<tr>
<td>6</td>
<td>150</td>
<td>89.35</td>
<td>6.72</td>
</tr>
<tr>
<td>7</td>
<td>180</td>
<td>88.36</td>
<td>8.92</td>
</tr>
<tr>
<td>8</td>
<td>210</td>
<td>89.69</td>
<td>8.60</td>
</tr>
<tr>
<td>9</td>
<td>240</td>
<td>87.39</td>
<td>11.53</td>
</tr>
</tbody>
</table>

Figure 24: Synchronized ICY 100 culture within a chemostat at a constant dilution rate of. The first point was taken 5 mins after cell insertion. Subsequent points were taken every 30 mins for 4 hours and analyzed using flow cytometry to assess the percent of cells within each phase of the cell cycle.

Then in a chemostat, BY 4743 and ICY 100 were grown overnight to log phase then synchronized in batch using HU and NOCO as previously described in Section 5.4. After synchronization was completed, cultures were transported into warm YPD. The final volume of the batch cultures was 160 mL. The synchronized batch cultures were inserted into the chemostat
and raised to a final volume 500 mL using warm YPD. BY 4743 was synchronized prior to experimentation to 93.76% budded. ICY 100 culture was synchronized prior to experimentation to 88.35% budded. 1 mL samples were collected every 10 minutes for 200 minutes. Each point was analyzed with flow cytometry assessing GFP expression. The data collected from ICY 100 was averaged and normalized to the control, BY 4743 (Figure 25).

Figure 25: Synchronized ICY 100 in a chemostat. Samples collected for flow cytometry and normalized to the control BY 4743.

When synchronized ICY 100 was placed in a chemostat, no significant fluctuation could be discerned similar to the ultradian rhythm. ICY 100 begins with a 2.21 fold increase of GFP expression from the control, BY 4743. This GFP expression fluctuates slightly until 140 minutes at which point the GFP expression increases to a 2.52 fold increase. This increase remains roughly 2.50 until 200 minutes. From this data and the data collected regarding the duration of synchrony in the chemostat, the majority of cells remain in budded phase while still growing and dividing. This lengthening of budded cells from the batch system is due to the chemostat’s fundamental ability to generate a steady state. This ability allows the culture to remain budded
longer. However with the cells remaining in a budded stage longer in the chemostat, this culture did not engender a synchronous culture generating an ultradian oscillation.
CHAPTER 6 – CONCLUSION AND FUTURE WORKS

In this study, the ultradian rhythms characterized in diploid *Saccharomyces cerevisiae* by using a GTS1P-GFP promoter-reporter construct. The transformed yeast strain was grown in standard medium and synchronized. By monitoring GFP with flow cytometry in synchronous cultures, the promoter-reporter construct, GTS1-GFP, monitored the ultradian rhythm gene’s expression facilitating a better understanding of the ultradian rhythm.

For the GTS1-GFP construct in ICY 100, cultures were grown in asynchronous and synchronous batch and continuous flow systems. For asynchronous cultures in batch and continuous flow no biological rhythm could be retrieved because neighboring yeast cells were in dissimilar stages of the ultradian rhythm. Therefore, the asynchronous yeast cultures caused an average GFP expression output with an indistinctive oscillation. For synchronous cultures in batch, a biological rhythm with two distinct oscillations was discerned in a synchronized batch culture. Even though synchrony dissipated after the first generation, the construct was able to expose an oscillation of the GST1 with a 70 minute period which have not been observed previously. Although GTS1 aids in the retention of the ultradian rhythm for oxygen consumption (Murray, et al., 2001), this study shows GTS1 follows a longer period. Moreover, this suggests that not all ultradian rhythms exhibited in yeast are 40 minutes. Further research is needed to identify the periods for other genes associated with the ultradian rhythm. A continuous flow reactor was employed to prolong the synchronous culture past the first generation. However with a prolonged budded cell percentage, the ICY 100 culture did not exhibit any biological rhythm which was caused by the inability of the cells in a continuous culture to readily the cell cycle after synchronization.
The strategy of creating a promoter-reporter construct to discern the characteristics of the ultradian rhythm in diploid *S. cerevisiae* can be improved. First, the block-and-release method for synchronizing large yeast population is too unstable and unable to maintain synchrony. Populations had to be carefully washed with PBS otherwise yeast populations were unable to reach the threshold of synchrony. When synchronized yeast populations were placed in batch, synchrony was unable to be maintained past the first generation. To allow for a more stable and maintainable synchrony, yeast cell starvation in a continuous culture would be the most efficient as described by Lloyd and Murray (Lloyd, et al., 2007) (Lloyd, et al., 2003). However, the medium utilized in cell starvation was acidic, pH 3.4. GFP is unstable in such acidic conditions. Therefore with yeast cell synchronized with cell starvation, a new promoter-reporter construct must be developed with a reporter gene capable of surviving in such harsh conditions. GFP was utilized here for its common use as a reporter gene in various species, expression in non-homologous species, and ease of detection with UV light. Another method, however expensive, is the use of a centrifugal elutriation machine which generates a more efficient synchronization (Day, et al., 2004). In this method, the creation of artifacts is prevented and if utilized with a continuous flow reactor should yield a synchronous culture, indefinitely. By applying these suggestions, the yeast strain would be able to maintain synchrony indefinitely while allowing the reporter gene to characterize the expression of GTS1.
REFERENCES


Yeast Extract Phosphate (YEP) [Book Section] // Yeast Extract Glucose Agar.


VITA

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