Keeping pace with *Neurospora* circadian rhythms

Deborah Bell-Pedersen

Tel: +1 409 847 9237. Fax: +1 409 845 2891. e-mail: dpedersen@bio.tamu.edu

Department of Biology, Texas A&M University, College Station, TX 77843-3258, USA

Keywords: *Neurospora crassa*, circadian rhythms, clock, light entrainment, clock-controlled genes (ccgs)

Overview

Daily (circadian) rhythms in biochemical, cellular and behavioural activities are observed in organisms as diverse as algae, fungi, fruit flies, mice and humans. A remarkable feature of these rhythms is that they are not simply a response to 24 h environmental cycles imposed by the Earth's rotation, but instead are generated internally by cell autonomous biological clocks. Circadian clocks allow organisms to predict and prepare for the changes that occur in the physical world and afford the ability to temporally coordinate and partition cellular activities to appropriate times of the day. Thus the circadian clock not only provides an interesting biological problem with which to study, but also grants a unique opportunity to investigate how a cellular phenomenon is adapted to the environment, ultimately resulting in specific behavioural patterns.

Recent advances in our understanding of the molecular and biochemical basis of circadian rhythmicity have come about through the use of genetically malleable model systems, one of which includes the filamentous fungus *Neurospora crassa*. Studies in *Neurospora* have identified several components required for circadian rhythmicity, including genes involved in input signalling pathways from the environment to the clock, the clock itself, and output from the clock. Progress in our understanding of the molecular genetic aspects of circadian rhythms in *Neurospora* and other organisms is occurring at impressive speed and is beginning to allow comparisons to be made between the different systems. Together, these data are revealing that the components involved in generating circadian rhythms have been conserved throughout evolution, despite findings that some of the specific details used by the clock to generate rhythms are found to differ. Thus it appears that the similar clock components may utilize somewhat distinct methods to achieve the same goal, namely auto-regulatory feedback required for self-sustained circadian rhythms in the organism.

Background

Just as people use a watch to guide them to do things in a timely fashion (such as preparing a review!), organisms use internal clocks to regulate biological activities so that they occur at appropriate times of the day (Pittendrigh, 1960). A marine dinoflagellate glows at night; a plant unfolds its leaves to capture the sun's energy; a fungal spore develops early in the morning for dissemination; an insect emerges from its pupal case in the moist early evening; a human sleeps: all of these events are intimately linked to the beat of endogenous clocks. In essence, the biological clock provides the innate capacity to sense environmental change (akin to a sundial) and to accurately measure the passage of time (like an hourglass). Together, these features allow maintenance of internal temporal order and the ability to anticipate dependable cyclic changes occurring in an organism's surroundings. This predictive prowess has likely provided an evolutionary advantage to organisms, thus contributing to the ubiquity of clocks in the biological world.

Similar to many areas of research in the life sciences today, the study of biological clocks (chronobiology) has progressed from the organism level, to anatomical dissection, to molecular analysis of genes. Early studies in the field, dating back to the early 1700s (deMairan, 1729), first focused on documenting and defining daily (circadian) rhythms (reviewed by Moore-Ede et al., 1982; Sweeney, 1987; Edmunds, 1988). The approximately 24 h rhythms in behaviour were shown to (1) persist under constant environmental conditions; (2) be reset in a time-dependent manner by environmental signals; and (3) have a rhythm with a period that remains unchanged over a broad physiologically relevant temperature range – termed temperature compensation. It is these properties that continue to provide the diagnostic characteristics of a circadian rhythm.

Among vertebrates, specialized circadian clock cells discretely localized in the brain and retina have been
identified and shown to establish temporal control over other parts of the body by generating circadian rhythms in synaptic activity and the release of humoral factors (Rusak & Bina, 1990; Cassone et al., 1993; van den Pol & Dudek, 1993; Turek, 1994; Strecker et al., 1995; Silver et al., 1996; Tosini & Menaker, 1996). Independent self-sustaining clocks have now been found in the suprachiasmatic nucleus (SCN), retina and non-mammalian pineal gland (Stephan & Zucker, 1972; Menaker, 1982; Zatz et al., 1988; Takahashi et al., 1989; Ralph et al., 1990; Underwood, 1990; Klein et al., 1991). Importantly, circadian rhythmicity is evident in primary cell cultures derived from each of these tissues (Robertson & Takahashi, 1988; Cahill & Besharse, 1993; Michel et al., 1993; Pierce et al., 1993; Welsh et al., 1995; Tosini & Menaker, 1996), establishing the cell-autonomous nature of the clock in multicellular organisms. These data, in combination with the demonstrations of circadian rhythms in unicellular eukaryotes and prokaryotes (Edmunds, 1988; Sweeney & Borgese, 1989; Kondo & Ishiura, 1994; Golden et al., 1997), indicate that circadian clocks can be appropriately studied at the level of the cell.

Currently, molecular chronobiologists are focusing attention on genetically amenable model organisms to identify the genes whose activities constitute the workings of the biological clock (reviewed by Dunlap, 1996). Because clocks in diverse organisms share the same fundamental characteristics, it was anticipated that their mechanisms and possibly even their components have been conserved throughout evolution. While the details of the mechanism for generating rhythms vary between species, some of the components and the generalized scheme of a molecular feedback loop do appear conserved. This preservation, along with the ubiquity of clocks, ensures that advances in any system will have a broad impact on our understanding of how circadian clocks function in all organisms.

The fungus N. crassa is one model organism that has provided significant contributions to our knowledge of circadian biology (reviewed by Lakin-Thomas et al., 1990; Dunlap, 1993, 1996; Loros, 1995; Bell-Pedersen et al., 1996c). Neurospora not only has the advantage that powerful genetics and sophisticated molecular techniques are able to be performed on it, but it has a circadian rhythm of asexual spore development (conidiation) that is easily monitored on specialized tubes called race tubes (Fig. 1). The conidiation rhythm exhibits all of the key characteristics of a true circadian oscillation: the rhythm continues unabated in constant dark at 24°C with a period of 22 h (Pittendrigh et al., 1959); the period of the rhythm is temperature compensated (Q10 ~ 1) between 18 and 30°C (Nakashima & Feldman, 1980); and the rhythm can be entrained by various light–dark cycles (Sargent et al., 1966; Nakashima & Feldman, 1980; Dharmananda, 1980) and reset by temperature pulses (Francis & Sargent, 1979; Gooch et al., 1994; Rensing et al., 1995).

The aim of this review is to provide an overview of what we have learned about the mechanisms of circadian rhythmicity in Neurospora and to incorporate some of the notable similarities and distinctions between the Neurospora circadian system and that of other organisms.

The molecular aspects of Neurospora circadian rhythms

The oscillator

N. crassa entered the field of molecular chronobiology early with the discovery of the frq locus, which when mutated results in alteration of the circadian rhythm of conidiation (Feldman, 1982; Dunlap, 1996). Mutations in frq, generated by chemical or UV mutagenesis, were found to either lengthen, shorten or abolish the wild-type conidiation rhythm of 22 h, and in most isolates temperature compensation properties were affected (Feldman & Hoyle, 1973; Loros et al., 1986). Furthermore, the mutations caused no severe morphological defects in growth and development. These initial findings indicated that frq functions specifically in the organization and running of the Neurospora clock. From this point, considerable effort went into cloning the frq locus and this was accomplished via a chromosomal walk to the gene from nearby markers (McClung et al., 1989). In subsequent years, the importance of frq in circadian rhythmicity was verified using molecular biology techniques to demonstrate that frq and its corresponding protein, FRQ, are central components of the cellular oscillator (see below).

The criteria most often cited to establish that a gene encodes a component of a clock (Zatz, 1992; Aronson et al., 1994b) are: (1) mutations in the gene must cause a change in period or result in arrhythmicity for all output processes under control of that clock; (2) the activity or
amount of the component should oscillate with the same period as the overt rhythms; (3) prevention of the component's oscillation should result in arrhythmicity for all output pathways controlled by the clock; (4) environmental signals that shift the phase of the clock should similarly cause a rapid change in the levels or activity of the component; (5) induced alterations in activity of the component should act by a feedback mechanism to change the levels of the component. While these criteria provide the current framework for testing putative clock components, it should be emphasized that they may become clouded in systems containing multiple oscillators driving diverse output pathways and/or having more than one input.

For frq, it was shown that both frq mRNA and FRQ protein levels cycle with a 22 h period in wild-type strains grown in constant darkness, and the period of the oscillation is appropriately changed in both short- and long-period mutant strains (Aronson et al., 1994b). In support of a role for frq in the organization of the clock, the abundance of frq mRNA must oscillate as evidenced by the loss of circadian rhythms in strains expressing non-oscillating frq from an inducible promoter at an exogenous locus. In addition, driving frq levels from high to low independent of the time of day resets the clock to dusk (the initial low point in the frq mRNA cycle). Furthermore, a negative feedback loop is implied by the constant high levels of frq transcripts observed at all times of the day in strains that lack a functional FRQ protein, and by repression of the native frq locus in strains bearing an ectopic overexpressing version of the frq gene. These results demonstrate that FRQ resides within a molecular feedback loop and either directly or indirectly regulates the timing of its own synthesis. Consistent with these data, it was recently determined that nuclear localization of FRQ is required for frq molecular rhythms and overt circadian rhythmicity (Luo et al., 1998). Collectively, the results show that the frq gene fulfills the criteria established for a clock component.

The biochemical function of FRQ remains unknown; however, evidence is accumulating to suggest that the protein functions in transcriptional regulation. First, FRQ has several signature motifs that are consistent with it being a transcription factor, including a nuclear localization signal, a weak helix-turn-helix DNA-binding domain, and conserved acidic and basic regions (Lewis & Feldman, 1993; Aronson et al., 1994; Luo et al., 1998) (Fig. 2). Importantly, the sequence motifs compatible with FRQ being a transcription factor are found to be essentially conserved in FRQ homologues isolated from distantly related fungal species (Merrow & Dunlap, 1994; Lewis et al., 1996). Finally, nuclear localization of FRQ is required for clock function (Luo et al., 1998). Given the wealth of circumstantial evidence for FRQ acting as a transcription factor, one obvious challenge for the future is to directly demonstrate the DNA-binding ability of FRQ and in particular to test its competence in binding frq-specific sequences.

As might be expected, a complete cycle of the frq feedback loop takes 1 d to accomplish and at a rudimentary level this involves repression and derepression (activation) of the frq transcript (Merrow et al., 1997) (Fig. 3). At dawn, both frq mRNA and protein levels are low; however, the amount of frq transcript is beginning to rise (Garceau et al., 1997). About 4–5 h later frq mRNA reaches peak accumulation (just before noon) and two forms of the FRQ protein (Fig. 2 and see below) begin to amass. A 4–6 h delay in maximal FRQ protein levels relative to the peak in frq mRNA is observed wherein frq message levels begin to fall prior to FRQ protein reaching maximal accumulation. Thus, soon after FRQ protein is synthesized, it enters the nucleus (Luo et al., 1998) and rapidly acts (within 3 h) (Merrow et al., 1997) either directly or indirectly to repress the levels of frq mRNA. For the rest of the day, and into the early evening, FRQ remains at sufficient levels in the nucleus to keep frq turned off. FRQ is also progressively phosphorylated following synthesis, and as FRQ becomes heavily phosphorylated its levels begin to drop, suggesting a role for high-density phosphorylation in turnover (Garceau et al., 1997). Once the levels of FRQ fall below a critical mass, frq can no longer be efficiently repressed and can in turn be activated by positive factors encoded by wc-1 and wc-2 (Crosthwaite et al., 1997; see below) to restart the cycle.

Time delays imposed within the molecular feedback loop are required to achieve stable circadian rhythms of gene expression. For frq, post-transcriptional regulation probably contributes to the necessary time lags, including regulated translation of FRQ, phosphorylation and decay, but apparently does not depend on a delay in nuclear entry of FRQ (Luo et al., 1998). Currently, we only have clues to the underpinnings of these temporal features. First, frq mRNA contains a rather long 5' untranslated region (> 1 knt) with six short upstream open reading frames (uORFs). While deletion of the uORFs does not appear to eliminate overt rhythmicity or frq cycling (N. Y. Garceau & J. C. Dunlap, personal communication), it is possible that they participate in FRQ translational regulation under certain growth conditions (Garceau et al., 1997) similar to yeast GCN4 (Abastado et al., 1991). Furthermore, a role for phosphorylation in FRQ activity and/or decay is suggested and points to an involvement of protein kinases in clock function. Consistent with a role for phosphorylation in circadian clock activity is the finding that inhibitors of
protein phosphorylation were shown to stop the circadian oscillator and block light-induced phase shifting of the clock in *Gonyaulax polyedra* (Comolli *et al.*, 1994). Thus using saturating mutant screens for altered clock activity and/or the appropriate protein kinase inhibitors, we should in the future be able to identify the relevant kinases which act to modify FRQ.

A comparable 24 h transcription/translation-based feedback loop has been described for the fruit fly *Drosophila melanogaster* which is used to regulate circadian rhythms in pupal eclosion and adult locomotor activity (reviewed by Sehgal, 1995; Rosbash *et al.*, 1996). In *Drosophila*, transcription of the period (*per*) and timeless (*tim*) genes begins late in the day, and both genes peak in accumulation just after dusk (the opposite of *frq*) (Hardin *et al.*, 1990, 1992; Sehgal *et al.*, 1994, 1995). Like the delay in FRQ protein accumulation, both *PER* and TIM proteins display an approximate 6 h lag in peak levels relative to peak transcript levels (Zweibel *et al.*, 1991; Zeng *et al.*, 1994, 1996; Sehgal *et al.*, 1995; Hunter-Ensor *et al.*, 1996; Lee *et al.*, 1996). However, unlike the observed rapid entry of FRQ into the nucleus, nuclear entry of *PER* and TIM is temporally controlled by heterodimer formation (Curtin *et al.*, 1995). Both proteins possess a cytoplasmic localization signal and following synthesis the proteins first accumulate in the cytoplasm. Then around midnight as a result of *PER* and TIM joining, the cytoplasmic localization signals become masked (Saez & Young, 1996), rendering the *PER–TIM* complex proficient to enter the nucleus (Curtin *et al.*, 1995; Gekakis *et al.*, 1995; Zeng *et al.*, 1996). Once in the nucleus, TIM and PER act, possibly through other proteins, to shut down transcription of the genes which encode them. Again similar to FRQ, PER and TIM are subject to temporal phosphorylation, which may participate in turnover (Edery *et al.*, 1994; Zeng *et al.*, 1996). Protein levels start to fall just before dawn, and the cycle of transcription begins anew. Interestingly, in *PER-null* mutant strains, the levels of *per* mRNA remain low (about 50% of peak values) over the course of the day (Hardin *et al.*, 1990; Van Gelder & Krasnow, 1996), suggesting that the regulation of *per* is more complex than simple autoregulatory feedback inhibition. The maintenance of repressed *per* mRNA in the absence of *PER* protein cannot be explained by negative regulation of *per* by TIM, since in the absence of PER, TIM cannot enter the nucleus (Hunter-Ensor *et al.*, 1996; Myers *et al.*, 1996). Alternatively, the data suggest that PER may require interaction with both a negative (TIM) and positive element to accomplish the *per* feedback loop.

**Other clock components**

While the *frq* transcription/translation-feedback loop probably provides the core of the *Neurospora* clock, it is anticipated that other factors are involved in the loop. This was first suggested from genetic screens that uncovered mutations unlinked to *frq* that affect circadian rhythms in development, altering both period and in most cases the temperature compensation properties of the rhythm (Feldman, 1982; Dunlap, 1993, 1996). Also, theoretically, a negative feedback cycle would eventually begin to dampen and ultimately not support the long-lived rhythms typically observed (Goldbeter, 1996). Thus to maintain a robust self-sustained oscillation, a positive element must act within or on the feedback loop. Because *frq* transcription is fully activated in the absence of FRQ protein (unlike the PER story), this implies that the positive element(s) can function independently of FRQ.
Recently, two genes called white collar-1 (wc-1) and white collar-2 (wc-2), involved in all known light responses in Neurospora (Harding & Shropshire, 1980), were shown to be required for overt circadian rhythmicity and to positively activate frq expression (Crosthwaite et al., 1997). Mutations in wc-1 completely block light induction of frq, and mutations in wc-2 allow only a transient frq photoresponse. Surprisingly, lesions in either gene were found to impede accumulation of frq transcripts in the dark, thereby preventing sustained frq mRNA and protein cycling. Importantly, the short-lived light- and temperature-induced expression of frq observed in wc-2 mutant strains is not sufficient for circadian oscillations of frq, suggesting that wc-2 is necessary for circadian clock function and acts as a positive element within the clock feedback cycle (Fig. 4). Alternatively, the feedback loop is still intact in wc-1 strains: temporary activation from an inducible promoter results in a full cycle of rhythmic FRQ expression. Based on these data, wc-2 is suggested to encode a positive component of the oscillator and wc-1 has been classified as a positive-acting clock-associated gene providing a necessary link between the light photoreceptor and a component of the oscillator. Therefore, it is likely that WC-2 acts at subjective dawn within each feedback cycle to turn on frq transcription, at the time when FRQ protein has fallen below a critical level needed to repress frq transcription. Future examination of the regulation of the wc genes, particularly with regards to their potential to cycle in activity or abundance, and their expression levels in a FRQ-null strain should help to clarify their role in the organization of the Neurospora clock.

WC-1 and WC-2 bear sequence similarity to the GATA family of transcription factors found in fungi and vertebrates and both have been shown to bind to promoters of blue-light-regulated genes in Neurospora (Ballario et al., 1996; Linden & Macino, 1997). In addition, WC-1 and WC-2 contain sequences with similarity to a motif called PAS. The PAS domain was first identified within the Drosophila PER protein [Drosophila PER, mammalian ARNT (a dimerization partner of the dioxin receptor) and SIM (the product of the single-minded gene)] (Huang et al., 1993) and shown to be involved in dimerization of PER to TIM (Gekakis et al., 1995). Notably, the PAS motif, which generally consists of paired repeat sequences and is often coupled to a bHLH domain, is found in proteins involved in both the circadian clock and in photoreception in bacteria and plants (Borgstahl et al., 1995; Koh et al., 1996). This suggests a possible evolutionary link between the clock's ability to anticipate daily light-dark cycles and light-responsive proteins in prokaryotes. The presence of PAS in clock-associated genes from diverse organisms has also fuelled speculation that biological clocks may have arisen from a common ancestor rather than being reinvented over and over again. This argument is further supported by the identification of a PAS domain in a putative clock component from mouse encoded by the clock gene (Vitaterna et al., 1994; King et al., 1997). Mutations in clock result in activity rhythms with a long period in heterozygotes and eventual arrhythmicity in homozygous mutants in constant darkness. More recently, a mammalian per homologue (mper1) was cloned using oligodeoxynucleotides directed against the per PAS domain (Tei et al., 1997). Consistent with a role in the clock, mper1 was found to oscillate in both the SCN and retina with peak accumulation occurring in the midmorning (Tei et al., 1997; Sun et al., 1997). A second mammalian homologue, designated mper2, was also identified and shown to cycle in abundance in the SCN and retina (Shearman et al., 1997). These findings are particularly relevant (and exciting!) because they provide strong evidence for the widespread conservation of clock components.

Putative clock components have also been identified in the cyanobacteria (kai) (Kondo et al., 1994), plants (toc) (Millar et al., 1995a) and hamsters (tau) (Ralph & Menaker, 1988). In all cases, mutations of these genes result in altered rhythms (period changes or arrhythmicity). It is noteworthy that most rhythm-affecting mutations, including those found in mouse, plants, Drosophila and Neurospora, are semidominant (Hall & Kyriacou, 1990; Dunlap, 1993; Vitaterna et al., 1994; Millar et al., 1995a), suggesting that the products of these genes act in multi-subunit complexes or encode regulatory proteins. Only time will tell if a similar transcription/translation-based feedback loop comprises the clock in these other species. However, a different feedback mechanism is speculated to exist in prokaryotes since they lack a nuclear envelope, elimin-
ating the possibility of time delays imposed by nuclear migration and uncoupled transcription/translation. In addition, a potentially distinct mechanism for rhythmicity was recently uncovered in the nocturnal silkworm *Antheraea pernyi* (Sauman & Reppert, 1996). A per gene homologue was cloned from the silkworm and found to fully complement *Drosophila* PER-null mutants (Reppert et al., 1994; Levine et al., 1995). Similar to *Drosophila*, per mRNA and protein levels cycle in the eye of *A. pernyi* with a 4–6 h delay between the two rhythms with concomitant PER nuclear entry. However, surprisingly, PER was only detected in the cytoplasm of brain cells with no evidence for temporal transport into the nucleus. Despite this, expression of per mRNA and protein is rhythmic in the brain, but lacks the temporal delay in peak protein levels. Here, per regulation is suggested to involve post-transcriptional control through an antisense transcript which is restricted to per-expressing brain cells. Taken together, these results imply that the same clock component can use distinct modes of regulation in different cell types to achieve the negative feedback essential for rhythmicity. Curiously, an *frq* antisense transcript has also been detected; however, the role of this transcript, if any, in the clock is unclear (S. K. Crosthwaite, K. A. Johnson & J. C. Dunlap, personal communication).

**Temperature regulation of the clock**

One fascinating property of clocks is that they function normally within a wide range of physiological temperatures. How these temperature limits are set has remained elusive. Studies in *Neurospora* have revealed that this phenomenon may be related to translational regulation of FRQ, since the growth temperature of the cultures determines how much of two possible forms of FRQ are produced (Liu et al., 1997). The different FRQ forms arise from alternative initiation of translation and include a long form of 989 amino acids initiating at the first ATG (ATG#1) and a shorter form of 890 amino acids initiating at a third ATG (ATG#3) (Fig. 2). Either type can suffice for clock activity at some, but not all, temperatures, and elimination of either form reduces the temperature range permissive for rhythmicity. Specifically, at high temperatures (approaching 30°C) the total level of FRQ rises and translational initiation at the first ATG is favoured, whereas at lower temperatures (around 18°C) translational initiation at ATG#3 is favoured. The increased overall levels of FRQ observed at high temperature indicate that quantities sufficient for clock activity at low temperature are not adequate at higher temperatures. In addition, at low temperature, equivalent amounts of the long form of FRQ protein will not suffice for rhythmicity and vice versa, implying that the two forms differ qualitatively as well as quantitatively. Together these data indicate that the temperature limits permissive for rhythmicity are influenced by overall FRQ levels (too little or too much FRQ stops the clock) and that activity at temperature extremes is dictated by the different forms.

Relatively few studies have addressed temperature compensation at the molecular level in other systems. In *Drosophila*, PER is physically flexible and this pliancy is dependent on a TG repeat region of the protein (Castiglione-Morelli et al., 1995) (a TG repeat is also present in FRQ; McClung et al., 1989). Flexibility increases (1) as the temperature is raised and (2) in isolates with longer repeat lengths (which have co-evolved with flanking regions of the protein). It is likely that PER elasticity is required for TIM interactions since mutation of the PER PAS domain (such as that observed in the *per*1 mutation; Huang et al., 1995) results in defective temperature compensation. Mutations of PER which affect flexibility, as well as temperatures outside of the physiological range, may restrict movement and alter the clock by limiting TIM/PER heterodimerization. In addition, in some *Drosophila* species the clock protein TIM utilizes alternative methionines for initiation of translation similarly to FRQ (Rosata et al., 1997). Although the function of the alternative TIM forms has not been determined, it may be that, similar to FRQ, the alternative forms extend the temperature range permissive for rhythmicity.

**Input from the environment to the oscillator**

The circadian clock can be reset by various environmental signals, with light being the most conserved and predominant clock-entraining stimulus. In *Neurospora*, a flavin-mediated response involving an as yet unidentified blue-light photoreceptor is observed in all known light-regulated events (Klemm & Ninnemann, 1979; Paietta & Sargent, 1981), including light resetting of the circadian clock (Dharmananda, 1980; Nakashima, 1982; Fritz et al., 1989, 1990). While various light-input pathways exist in different organisms, all models for rhythmic entrainment propose that light acts to rapidly alter the activity of a central clock component (Pittendrigh, 1960). Consistent with this prediction, the levels of *frq* mRNA were shown to increase within 5 min after a brief light pulse, independent of FRQ negative feedback inhibition (Crosthwaite et al., 1995), with induction requiring the products of the *wc* genes (Crosthwaite et al., 1997). In addition, a direct correlation was observed between the light-induced levels of *frq* transcript and the magnitude of phase shifts in the conidiation rhythm resulting from the same light treatment. These data provide molecular clarification of how a single light pulse can produce two different responses, either a phase advance or a phase delay of a rhythm, depending on the time of day the light signal is perceived. Specifically in *Neurospora*, a light pulse given in the late night when *frq* mRNA levels are low or rising rapidly causes mRNA levels to reach the midday point, resulting in an advance of the cycle. Alternatively, a light pulse administered in the early evening when *frq* levels are falling slows transcript decline and delays the next cycle. Finally, a light pulse during midday, when *frq* is already at peak levels, does not cause an appreciable change in the phase of the conidiation rhythm. One obvious goal for the future is to identify the photoreceptor molecule(s) responsible for light signalling to the clock.
In *Drosophila*, TIM (but apparently not PER) provides a link between light and the clock; however, unlike the induction of *frq* transcription by light, light triggers the degradation of TIM protein (Hunter-Ensor et al., 1996; Lee et al., 1996; Myers et al., 1996; Zeng et al., 1996). Hence the clock in *Drosophila* can only be photoresponsive when TIM is present. A light pulse in the early evening, when TIM and PER proteins are joining but have not yet moved into the nucleus, causes a delay in the feedback loop until the levels of TIM can be replenished. Conversely, in the late evening, when both *tim* and *per* mRNAs are at a low point in the cycle and PER/TIM heterodimers are in the nucleus, destruction of TIM by a light pulse results in increased transcription of both *per* and *tim* transcripts, advancing the cycle.

While a lot is known about the physiological effects of light on circadian clocks and of the components involved in light harvesting and signalling in photosynthetic organisms (Johnson, 1994; Millar et al., 1995b; Furuya & Schafer, 1996; Hicks et al., 1996; Anderson & Kay, 1997) and animals (reviewed by Takahashi, 1993; Turek, 1994), the molecular mechanisms controlling light resetting of the circadian pacemaker were until recently unknown. In mammals, light is ineffective in causing a phase change during the subjective day. Furthermore, experiments aimed at disrupting the clock with transcriptional or translation inhibitors indicate a requirement for macromolecular synthesis during the early morning for normal progression through the circadian cycle, similar to *Neurospora* (reviewed by Takahashi, 1993). Taking this one giant leap further, and presuming that the ability to perceive light is the same throughout the day, these observations would be compatible with an excitatory effect of light on a day-active central clock component, similar to *Neurospora* *frq*. This prediction turns out to be true – both *mper1* and *mper2* expression is rapidly induced in the SCN following a brief light pulse (Shigeysahi et al., 1997; Albrecht et al., 1997; Shearman et al., 1997). Therefore, while *mper1* and *mper2* bear strong sequence similarity to *Drosophila per*, their expression in the dark and in response to light is most like *Neurospora frq*. These data strongly reinforce the notion that the *Neurospora* clock is functionally similar to the mammalian clock.

**Output**

We have already seen that *frq* is coupled to the input pathway through at least the *wc* genes; however, to date no candidate clock component has been connected to an output pathway. Therefore, while organisms have a clock to temporally control a wide range of activities, very little is known about how this regulation takes place or of the clock proteins responsible for signalling time information to the rest of the cell. A commonly used strategy for studying output pathways first involves the identification of genes that are rhythmically expressed but unlike the clock genes when inactivated have no affect on oscillator function. Once isolated, the genes can be used to identify the upstream regulatory factors involved in rhythmic expression, proceeding in a step-wise fashion until an oscillator component is reached.

Although the first systematic screen for clock-controlled genes (ccgs) was carried out in *Neurospora* (Loros et al., 1989), rhythmically expressed genes have now been identified and are being studied in a wide range of organisms, including the cyanobacteria (Liu et al., 1995; Golden et al., 1997), plants (Pilgrim et al., 1993; Pilgrim & McClung, 1993; Anderson et al., 1994; Carpenter et al., 1994; Heintzen et al., 1994; Carre & Kay, 1995; Anderson & Kay, 1997), *Drosophila* (Van Gelder et al., 1995; Van Gelder & Krasnow, 1996; Rouyer et al., 1997) and vertebrates (Lavery & Schibler, 1993; Molina et al., 1993; Pierce et al., 1993; Stehle et al., 1993; Ishida et al., 1994; Borjigin et al., 1995; Chong et al., 1996; Green & Besharse, 1996; Fonjallaz et al., 1996). In many cases, it is still not known if the rhythmically expressed genes participate in the clock mechanism (similar to *frq*, *per* and *tim*) or are true output genes. Furthermore, the clock apparently exerts its effect on the regulation of these genes at the transcriptional level; however, precedence also exists for clock regulation occurring post-transcriptionally. For example, in *G. polyedra*, the luciferin-binding protein LBP is regulated by the circadian clock at the translational level (Mittag et al., 1997) and this regulation may involve a conserved 3' UTR binding protein (CCTR) which is elevated during the day and low at night (Mittag et al., 1997; Mittag, 1996). Also worth noting is that in the cyanobacterium *Synechococcus* it was discovered that transcription of virtually all genes is circadianly regulated (Liu et al., 1995). Classification of the genes was possible based on common phase and amplitude, and subsequent studies showed that rhythmicity of one class could be attributed to the activity of a particular sigma factor (Tsinoremas et al., 1996).

Currently, 11 ccgs have been identified in *Neurospora* and are summarized in Table 1. Verification of clock regulation for most of the genes was achieved by demonstrating that the period of the mRNA abundance rhythm equals the period of the strain examined. For example, in the long-period *frq* allele, with an endogenous period of 29 h, the period of the peak in mRNA accumulation approached 29 h and eventually cycled 180° out of phase with the wild-type strain (Loros et al., 1989; Bell-Pedersen et al., 1992, 1996b). In all cases tested, the clock was shown to function normally in strains containing inactivated copies of the ccgs, demonstrating that they are part of an output pathway and are not involved in oscillator function (Bell-Pedersen et al., 1992; Lindgren, 1994; M. L. Shinohara, J. J. Loros & J. C. Dunlap, personal communication).

All of the *Neurospora* ccgs peak in transcript accumulation in the late night to early morning; however, they differ in overall expression levels, amplitude and absolute peak of accumulation (Bell-Pedersen et al., 1996b). This time of peak abundance coincides with the time of conidiation, initially suggesting that some or all of these genes might be involved in the asexual spore
developmental pathway. While many of the genes are induced during conidiation and by light treatments, suggesting a direct role in asexual spore development (Arpaia et al., 1993, 1995a; Lauter et al., 1992; Lindgren, 1994; Bell-Pedersen et al., 1996b), not all of the ccgs are induced during conidiation (including ccg-7, -8 and -12). These findings indicate that clock-regulated output pathways distinct from conidiation exist in Neurospora and may include the previously reported circadian rhythms in the sexual developmental cycle (Lakin-Thomas et al., 1990) or might reflect unknown output pathways. In either case, these data suggest that different regulatory pathways exist in Neurospora to control diverse outputs from the clock. In addition, comparisons of transcript accumulation of the ccgs in both wild-type and FRQ-null mutant strains revealed differential regulation, whereby the genes are found to be activated, repressed or unchanged in cells that lack FRQ (Bell-Pedersen, unpublished); however, it is not known if FRQ interacts directly with these genes.

Based on sequence analyses and comparisons of the sequences to known genes, the functions of some of the ccgs were identified. The eas (ccg-2) gene was found to encode a characteristic fungal hydrophobin; a component of the hydrophobic rodlet layer of conidia important for water repellency and spore dissemination (Bell-Pedersen et al., 1992; Lauter et al., 1992). The ccg-12 gene was found to be identical to the previously cloned cmt gene of Neurospora (Munger et al., 1987) encoding copper metallothionein (Bell-Pedersen et al., 1996b), ccg-7 encodes glyceraldehyde-3-phosphate dehydrogenase (Shinohara et al., 1998) and al-3 encodes geranylgeranyl pyrophosphate synthase, essential for the biosynthesis of carotenoids (Arpaia et al., 1995b). The function of the other genes remains unclear. The ccg-1 gene encodes a small polypeptide of 71 amino acids which bears no distinguishing features and is not similar to other proteins in the databases (Lindgren, 1994; Lorus, 1995). A ccg-1-inactivated strain produced no discernible phenotype under a variety of growth conditions analysed, which is particularly surprising since ccg-1 mRNA represents a significant portion of total Neurospora mRNA (up to 8% of a morning-specific cDNA library) (Bell-Pedersen et al., 1996b). Recent inactivation of ccg-6 and ccg-9 revealed a slow growth phenotype and reduced circadian expression of the developmental rhythm (M. L. Shinohara, J. J. Loros & J. C. Dunlap, personal communication). These results initially suggested a possible role for the genes in the clock mechanism itself. However, in both ccg-9- and ccg-6-null strains the fra/FRQ rhythm was normal, indicating that the clock still functions properly in these strains. Interestingly, rhythmicity of some of the other ccgs appears to be affected in a ccg-9-null strain (M. L. Shinohara, J. J. Loros & J. C. Dunlap, personal communication), suggesting that CCG-9 may function in signalling time-of-day-specific regulation.

The most highly characterized Neurospora ccg at both

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**Table 1. Summary of Neurospora ccgs**

Reprinted with permission from Bell-Pedersen et al. (1996c).

<table>
<thead>
<tr>
<th>Gene</th>
<th>Average peak*</th>
<th>Identity†</th>
<th>Regulation‡</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Development</td>
<td>Light</td>
</tr>
<tr>
<td>ccg-1</td>
<td>CT3</td>
<td>Unknown</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>eas</td>
<td>CT22</td>
<td>Hydrophobin</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>ccg-4</td>
<td>CT5</td>
<td>Unknown</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>ccg-6</td>
<td>CT19</td>
<td>Unknown</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>ccg-7</td>
<td>CT21</td>
<td>GAPDH</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>ccg-8</td>
<td>CT20</td>
<td>Unknown</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>ccg-9</td>
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<td>+</td>
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<tr>
<td>cmt</td>
<td>CT18</td>
<td>CuMT</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>al-3S</td>
<td>CT10</td>
<td>GGPPS</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>con-6</td>
<td>ZT20</td>
<td>Unknown</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>con-10</td>
<td>ZT20</td>
<td>Unknown</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

* The peak in message accumulation varies slightly in different experiments (Bell-Pedersen et al., 1996b), and with the exception of al-3, con-6 and con-10, was determined from Northern blots of the same rhythmic RNA probed with the indicated ccgs. The peak in al-3 message accumulation was estimated by us from Northern blots presented in Arpaia et al. (1995b), whereas con-6 and con-10 were shown to peak about 20 h after a light pulse representing zeitgeber time (ZT) 20 (Lauter & Yanofsky, 1993). CT, Circadian time.

† GAPDH, glyceraldehyde-3-phosphate dehydrogenase; CuMT, copper metallothionein; GGPPS, geranylgeranyl pyrophosphate synthase.

‡ Developmental and light regulation of the ccgs. +, Increased transcription following developmental induction and light treatment; -, no effect.

§ Only the longer al-3c transcript has been demonstrated to be rhythmic (Arpaia et al., 1995b).
Several new potential clock genes have been identified through mutation and are currently being studied to determine their potential role in generating rhythms, and eventually these studies will allow more specific comparisons to be made between clock systems in diverse organisms. *Neurospora* continues to provide a prototype for analysis of all three major areas of clock research, including the genes and gene products that make up the oscillator, and components involved in input signalling to and output signalling from the clock. A great number of important yet addressable questions are continuously arising from these studies and include such things as: (1) how does FRQ modulate gene expression; (2) what are the light elements involved in the clock mechanism; (3) what is the biochemical basis for temperature compensation; (4) what is the identity of the photoreceptors for light input into the clock; and (5) what are the components of the clock that signal time information to the output genes? If the rate of clock analysis in *Neurospora* continues at the same pace, we can expect that it will not be long before we have the answers to these and other questions.

**Acknowledgements**

I would like to thank Jay Dunlap for his helpful comments on this review and members of the laboratories of Jennifer Loros and Jay Dunlap for sharing unpublished data.

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