**REVIEW
ARTICLE****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 ($Q_{10} \sim 1$) 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

Fig. 1. *Neurospora* conidiation rhythm viewed on race tubes. To monitor the phenotypic expression of the *Neurospora* clock, conidia are inoculated at one end of a race tube. Race tubes are hollow glass tubes about 40 cm long and 16 mm in diameter that are bent upwards at both ends to hold an agar medium. After growth for a day in constant light, the position of the growth front is marked and the culture is transferred to constant dark. The light-dark transfer synchronizes the cells in the culture and sets the clock running from subjective dusk. Following transfer, the growth front is marked every 24 h with the aid of a red light (which has no effect on the clock). Growth rate is constant and the positions of the readily visualized orange conidial bands (separated by undifferentiated surface mycelia) relative to the marked growth fronts allow determination of both period and phase of the rhythm.

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

Fig. 2. Domains of *N. crassa* FRQ. Structural domains of FRQ are indicated, as well as the alternative initiation codons used to produce the long (989 amino acids) and short (890 amino acids) forms of FRQ. ■, Nuclear localization signal (amino acids 195–200); ▨, helix–turn–helix domain (amino acids 169–215); ▤, conserved basic domains; ▩, conserved acidic domains; ▧, TG/SG repeats.

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* must oscillate as evidenced by the loss of circadian rhythms in strains expressing non-cycling *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 fulfils 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.*, 1994a; 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 (Morrow & 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 (Morrow *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) (Morrow *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 (μ ORFs). While deletion of the μ ORFs 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

Fig. 3. *Neurospora frq* feedback loop. The specifics of the feedback loop are described in the text. Circadian time (CT) is a formalism used to compare the properties of circadian rhythms from organisms or strains with different endogenous periods whereby the period is divided into 24 equal parts with each part defined as 1 circadian hour. By convention, CT0 represents subjective dawn and CT12 represents subjective dusk. This figure was adapted from Bell-Pedersen *et al.* (1996c).

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 (Goldbetter, 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.

Fig. 4. Working model for the *Neurospora* circadian clock. The three aspects of circadian timing, input of entraining stimuli, the oscillator and output from the oscillator, are presented. The feedback loop comprising the oscillator and involving both negative (FRQ) and positive (WC-2) activation of the clock component *frq* are shown. Time information leaves the oscillator (broken lines), perhaps via FRQ protein, to regulate the expression of the clock-controlled genes (ccgs). Light input to the oscillator initiates at an unknown photoreceptor and is relayed to the clock via WC-1 and WC-2.

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 mid-morning (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 repeated 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^L* 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 photo-responsive 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 (Shigeyoshi *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 effect 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*⁷ 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

Table 1. Summary of *Neurospora ccgs*Reprinted with permission from Bell-Pedersen *et al.* (1996c).

Gene	Average peak*	Identity†	Regulation‡		Reference
			Developmental	Light	
<i>ccg-1</i>	CT3	Unknown	+	+	Loros <i>et al.</i> (1989)
<i>eas</i> (<i>ccg-2</i>)	CT22	Hydrophobin	+	+	Loros <i>et al.</i> (1989); Bell-Pedersen <i>et al.</i> (1992); Lauter <i>et al.</i> (1992)
<i>ccg-4</i>	CT5	Unknown	+	+	Bell-Pedersen <i>et al.</i> (1996b)
<i>ccg-6</i>	CT19	Unknown	+	+	Bell-Pedersen <i>et al.</i> (1996b)
<i>ccg-7</i>	CT21	GAPDH	—	—	Bell-Pedersen <i>et al.</i> (1996b); Shinohara <i>et al.</i> (1998)
<i>ccg-8</i>	CT20	Unknown	—	—	Bell-Pedersen <i>et al.</i> (1996b)
<i>ccg-9</i>	CT19	Unknown	+	+	Bell-Pedersen <i>et al.</i> (1996b)
<i>cmt</i> (<i>ccg-12</i>)	CT18	CuMT	—	—	Bell-Pedersen <i>et al.</i> (1996b); Munger <i>et al.</i> (1987)
<i>al-3</i> §	CT10	GGPPS	+	+	Arpaia <i>et al.</i> (1995b)
<i>con-6</i>	ZT20	Unknown	+	+	Lauter & Yanofsky (1993)
<i>con-10</i>	ZT20	Unknown	+	+	Lauter & Yanofsky (1993)

* 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).

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; Loros, 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 *frq*/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

Fig. 5. Location of *eas* (*ccg-2*) promoter regulatory elements. The putative CAAT and TATAA transcriptional regulatory sequences are indicated. E, *EcoRI*; K, *KpnI*; N, *NdeI*; M, *MscI*; A, *AvaI*. ■, Activating clock element (ACE); □, negative developmental element (mycelial repressor); ▨, positive light element; ▩, light-responsive element; ▤, transcriptional activator. This figure was reprinted with permission from Bell-Pedersen *et al.* (1996a).

the biochemical and molecular level is the *eas* (*ccg-2*) gene. Nuclear run-on experiments demonstrated that *eas* (*ccg-2*) is transcriptionally regulated by the circadian clock (Loros & Dunlap, 1991), implicating the involvement of *cis*-acting regulatory elements mediating temporal control. Subsequent dissection of the *eas* (*ccg-2*) promoter localized a positive-acting clock element (ACE) to within a 45 bp promoter fragment, found to be distinct from other light and developmental elements governing its expression (Fig. 5) (Bell-Pedersen *et al.*, 1996a). Using an unregulated promoter/reporter system, it was shown that the ACE element is sufficient to confer high-amplitude rhythmicity on the reporter gene. The ACE sequences are currently being used to identify upstream regulatory factors responsible for cycling in attempts to trace the output pathway. In addition, the *eas* (*ccg-2*) light elements reside in regions that do not overlap the ACE (Bell-Pedersen *et al.*, 1996a), and *eas* (*ccg-2*) is photoresponsive in a clock-null strain (Arpaia *et al.*, 1993). These distinctions are particularly relevant because light provides a primary signal for resetting the phase of the circadian clock in *Neurospora*, indicating that the light response of *eas* (*ccg-2*) is a function of induced conidiation in response to light, rather than a function of the clock. Therefore, at least two independent pathways for *eas* (*ccg-2*) regulation appear to exist: one from the endogenous clock to generate the observed rhythmicity, and another through an asexual developmental pathway that involves input from environmental factors. The independent nature of light responsiveness and the clock's effect on gene expression is beginning to look like a common theme for clock- and light-regulated genes in phylogenetically diverse organisms including plants (Anderson *et al.*, 1997) and vertebrates (Zatz, 1994). These hierarchical levels of regulation probably reflect a need in the organism for flexibility in both the timing and amount of expression of these genes, depending on environmental conditions.

Concluding remarks

The past few years have seen significant advances in our understanding of the mechanisms of circadian rhythmicity, with the molecular genetic analysis of clocks continuing to provide a major contribution to the story.

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 kinases 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.

References

- Abastado, J. P., Miller, P. F., Jackson, B. M. & Hinnebusch, A. G. (1991). Suppression of ribosomal reinitiation at upstream open reading frames in amino acid-starved cells forms the basis for GCN4 translational control. *Mol Cell Biol* 11, 486–496.
- Albrecht, U., Sun, Z. S., Eichele, G. & Lee, C. C. (1997). A differential response of two putative mammalian circadian regulators, *mper1* and *mper2* to light. *Cell* 91, 1055–1064.
- Anderson, S. L. & Kay, S. A. (1997). Phototransduction and circadian clock pathways regulating gene transcription in higher plants. *Adv Genet* 35, 1–34.
- Anderson, S. L., Teakle, G. R., Martino-Catt, S. J. & Kay, S. A. (1994). Circadian clock- and phytochrome-regulated transcription is conferred by a 78 bp *cis*-acting domain of the *Arabidopsis* CAB2 promoter. *Plant J* 6, 457–470.
- Anderson, S. L., Somers, D. E., Millar, A. J., Hanson, K., Chory, J. & Kay, S. A. (1997). Attenuation of phytochrome A and B signaling pathways by the *Arabidopsis* circadian clock. *Plant Cell* 9, 1727–1743.
- Aronson, B. D., Johnson, K. A. & Dunlap, J. C. (1994a). The circadian clock locus *frequency*: a single ORF defines period length and temperature compensation. *Proc Natl Acad Sci USA* 91, 7683–7687.
- Aronson, B. D., Johnson, K. A., Loros, J. J. & Dunlap, J. C. (1994b). Negative feedback defining a circadian clock: autoregulation in the clock gene *frequency*. *Science* 263, 1578–1584.
- Arpaia, G., Loros, J. J., Dunlap, J. C., Morelli, G. & Macino, G. (1993). The interplay of light and the circadian clock: independent dual regulation of clock-controlled gene *ccg-2* (*eas*). *Plant Physiol* 102, 1299–1305.
- Arpaia, G., Loros, J. J., Dunlap, J. C., Morelli, G. & Macino, G. (1995a). Light induction of the clock-controlled gene *ccg-1* is not

- transduced through the circadian clock. *Mol Gen Genet* **247**, 157–163.
- Arpaia, G., Carattoli, A. & Macino, G. (1995b). Light and development regulate the expression of the *albino-3* gene in *Neurospora crassa*. *Dev Biol* **170**, 626–635.
- Ballario, P., Vittorioso, P., Magrelli, A., Talora, C., Cabibbo, A. & Macino, G. (1996). *White collar-1*, a central regulator of blue light responses in *Neurospora*, is a zinc finger protein. *EMBO J* **15**, 1650–1657.
- Bell-Pedersen, D., Dunlap, J. C. & Loros, J. J. (1992). The *Neurospora* circadian clock-controlled gene, *cgc-2*, is allelic to *eas* and encodes a fungal hydrophobin required for formation of the conidial rodlet layer. *Genes Dev* **6**, 2382–2394.
- Bell-Pedersen, D., Dunlap, J. C. & Loros, J. L. (1996a). Distinct cis-acting elements mediate clock, light and developmental regulation of the *Neurospora crassa eas* (*cgc-2*) gene. *Mol Cell Biol* **16**, 513–521.
- Bell-Pedersen, D., Shinohara, M., Loros, J. J. & Dunlap, J. (1996b). Clock-controlled genes isolated from *Neurospora crassa* are late night- to morning-specific. *Proc Natl Acad Sci USA* **93**, 13096–13101.
- Bell-Pedersen, D., Garceau, N. & Loros, J. J. (1996c). Circadian rhythms in fungi. *J Genet* **75**, 387–401.
- Borgstahl, G. E., Williams, D. R. & Getzoff, E. D. (1995). 1.4 Å structure of photoactive yellow protein, a cytosolic photoreceptor: unusual fold, active site, and chromophore. *Biochemistry* **34**, 6278–6287.
- Borjigin, J., Wang, M. M. & Snyder, S. H. (1995). Diurnal variation in mRNA encoding serotonin N-acetyltransferase in pineal gland. *Nature* **378**, 783–785.
- Cahill, G. M. & Besharse, J. C. (1993). Circadian clock functions localized in *Xenopus* retinal photoreceptors. *Neuron* **10**, 573–577.
- Carpenter, C. D., Kreps, J. A. & Simon, A. E. (1994). Genes encoding glycine-rich *Arabidopsis thaliana* proteins with RNA-binding motifs are influenced by cold treatment and an endogenous circadian rhythm. *Plant Physiol* **104**, 1015–1025.
- Carre, I. A. & Kay, S. A. (1995). Multiple DNA-protein complexes at a circadian-regulated promoter element. *Plant Cell* **7**, 2039–2051.
- Cassone, V. M., Warren, W. S., Brooks, D. S. & Lu, J. (1993). Melatonin, the pineal gland, and circadian rhythms. *J Biol Rhythms* **8** (suppl.), S73–81.
- Castiglione-Morelli, M. A., Guantieri, V., Villani, V., Kyriacou, C. P. & Tamburro, A. M. (1995). Conformational study of the Thr-Gly repeat in the *Drosophila* clock protein, PERIOD. *Proc R Soc Lond Ser B Biol Sci* **260**, 155–163.
- Chong, N. W., Cagampang, F. R., Coen, C. W., Campbell, I. C. & Powell, J. F. (1996). Rapid identification of novel genes expressed in a circadian manner in rat suprachiasmatic nuclei. *Neuroreport* **7**, 1199–1203.
- Comolli, J., Taylor, W. R. & Hastings, J. W. (1994). An inhibitor of protein phosphorylation stops the circadian oscillator and blocks light-induced phase shifting in *Gonyaulax polyedra*. *J Biol Rhythms* **9**, 13–26.
- Crosthwaite, S. K., Loros, J. J. & Dunlap, J. C. (1995). Light-induced resetting of a circadian clock is mediated by a rapid increase in *frequency* transcript. *Cell* **81**, 1003–1012.
- Crosthwaite, S. K., Dunlap, J. C. & Loros, J. J. (1997). *Neurospora wc-1* and *wc-2*: transcription, photoresponses, and the origins of circadian rhythmicity. *Science* **276**, 763–769.
- Curtin, K., Huang, J. & Rosbash, M. (1995). Temporally regulated entry of the *Drosophila period* protein contributes to the circadian clock. *Neuron* **14**, 365–372.
- Dharmananda, S. (1980). *Studies of the circadian clock of Neurospora crassa: light-induced phase shifting*. PhD thesis, University of California.
- Dunlap, J. C. (1993). Genetic analysis of circadian clocks. *Annu Rev Physiol* **55**, 683–728.
- Dunlap, J. C. (1996). Genetic and molecular analysis of circadian rhythms. *Annu Rev Genet* **30**, 579–601.
- Ederly, I., Zweibel, L., Dembinska, M. & Rosbash, M. (1994). Temporal phosphorylation of the *Drosophila period* protein. *Proc Natl Acad Sci USA* **91**, 2260–2264.
- Edmunds, L. N. (1988). *Cellular and Molecular Bases of Biological Clocks*. New York: Springer.
- Feldman, J. F. (1982). Genetic approaches to circadian clocks. *Annu Rev Plant Physiol* **33**, 583–608.
- Feldman, J. F. & Hoyle, M. N. (1973). Isolation of circadian clock mutants of *Neurospora crassa*. *Genetics* **75**, 605–613.
- Fonjallaz, P., Ossipow, V., Wanner, G. & Schibler, U. (1996). The two PAR leucine zipper proteins, TEF and DBP, display similar circadian and tissue-specific expression, but have different target promoter preferences. *EMBO J* **15**, 351–362.
- Francis, C. D. & Sargent, M. L. (1979). Effects of temperature perturbations on circadian conidiation in *Neurospora*. *Plant Physiol* **64**, 1000–1004.
- Fritz, B. H., Kasai, S. & Matsui, K. (1989). Free cellular riboflavin is involved in phase shifting the circadian clock of *Neurospora crassa* by light. *Plant Cell Physiol* **30**, 557–564.
- Fritz, B. H., Kasai, S. & Matsui, K. (1990). Blue light photoreception in *Neurospora* circadian rhythm: evidence for involvement of the flavin triplet state. *Photochem Photobiol* **51**, 607–610.
- Furuya, M. & Schafer, E. (1996). Photoperception and signaling of induction reactions by different phytochromes. *Trends Plant Sci* **1**, 301–307.
- Garceau, N. Y., Liu, Y., Loros, J. J. & Dunlap, J. C. (1997). Alternative initiation of translation and time-specific phosphorylation yield multiple forms of the essential clock protein FREQUENCY. *Cell* **89**, 469–476.
- Gekakis, N., Saez, L., Delahaye-Brown, A.-M., Myers, M. P., Sehgal, A., Young, M. W. & Weitz, C. J. (1995). Isolation of *timeless* by PER protein interactions: defective interaction between *timeless* protein and long-period mutant PER^L. *Science* **270**, 811–815.
- Goldbetter, A. (editor) (1996). *Biochemical Oscillations and Cellular Rhythms*. Cambridge: Cambridge University Press.
- Golden, S. S., Ishiura, M., Johnson, C. H. & Kondo, T. (1997). Cyanobacterial circadian rhythms. *Annu Rev Plant Physiol Plant Mol Biol* **48**, 327–354.
- Gooch, B. D., Wehseler, R. A. & Gross, C. G. (1994). Temperature effects on the resetting of the phase of the *Neurospora* circadian rhythm. *J Biol Rhythms* **9**, 83–94.
- Green, C. B. & Besharse, J. C. (1996). Identification of a novel vertebrate circadian clock-regulated gene encoding the protein nocturnin. *Proc Natl Acad Sci USA* **93**, 14884–14888.
- Hall, J. C. & Kyriacou, C. P. (1990). Genetics of biological rhythms in *Drosophila*. *Adv Insect Physiol* **22**, 221–298.
- Hardin, P. E., Hall, J. C. & Rosbash, M. (1990). Feedback of the *Drosophila period* gene product on circadian cycling of its messenger RNA levels. *Nature* **342**, 536–540.
- Hardin, P. E., Hall, J. C. & Rosbash, M. (1992). Circadian os-

- cillations in *period* gene mRNA levels are transcriptionally regulated. *Proc Natl Acad Sci USA* 89, 11711–11715.
- Harding, R. W. & Shropshire, W., Jr (1980). Photocontrol of carotenoid biosynthesis. *Annu Rev Plant Physiol* 31, 217–238.
- Heintzen, C., Melzer, S., Fisher, R., Kappeler, S., Apel, K. & Staiger, D. (1994). A light- and temperature-entrained circadian clock controls expression of transcripts encoding nuclear proteins with homology to RNA-binding proteins in meristematic tissue. *Plant J* 5, 799–813.
- Hicks, K. A., Millar, A. J., Carre, I. A., Somers, D. E., Straume, M., Meeks-Wagner, D. R. & Kay, S. A. (1996). Conditional circadian dysfunction of the *Arabidopsis* early flowering 3 mutant. *Science* 274, 790–792.
- Huang, Z. J., Edery, I. & Rosbash, M. (1993). PAS is a dimerization domain common to *Drosophila* Period and several transcription factors. *Nature* 364, 259–262.
- Huang, Z. J., Curtin, K. D. & Rosbash, M. (1995). PER protein interactions and temperature compensation of a circadian clock in *Drosophila*. *Science* 267, 1169–1172.
- Hunter-Ensor, M., Ousely, A. & Sehgal, A. (1996). Regulation of the *Drosophila* protein TIMELESS suggests a mechanism for resetting the circadian clock by light. *Cell* 84, 677–685.
- Ishida, N., Matsui, M., Nishimatsu, S., Murakami, K. & Mitsui, Y. (1994). Molecular cloning of a gene under control of the circadian clock and light in the rodent SCN. *Mol Brain Res* 26, 197–206.
- Johnson, C. H. (1994). Illuminating the clock: circadian photobiology. *Semin Cell Biol* 5, 355–362.
- King, D. P., Zhao, Y., Sangoram, A. M. & 9 other authors (1997). Positional cloning of the mouse circadian clock gene. *Cell* 89, 641–653.
- Klein, D. C., Moore, R. Y. & Reppert, S. M. (editors) (1991). *Suprachiasmatic Nucleus: The Mind's Clock*. New York: Oxford University Press.
- Klemm, E. & Ninnemann, H. (1979). Photoreceptors for circadian rhythms. *Photochem Photobiol Rev* 4, 207–265.
- Koh, M., Van Driessche, G., Samyn, B., Hoff, W. D., Meyer, T. E., Cusanovich, M. A. & Van Beeumen, J. J. (1996). Sequence evidence for strong conservation of the photoactive yellow proteins from the halophilic phototrophic bacteria *Chromatium salexigens* and *Rhodospirillum salexigens*. *Biochemistry* 35, 2526–2534.
- Kondo, T. & Ishiura, M. (1994). Circadian rhythms of cyanobacteria: monitoring the biological clocks of individual colonies by bioluminescence. *J Bacteriol* 176, 1881–1885.
- Kondo, T., Tsinoemas, N. F., Golden, S. S., Johnson, C. H., Kutsuna, S. & Ishiura, M. (1994). Circadian clock mutants of cyanobacteria. *Science* 266, 1233–1236.
- Lakin-Thomas, P. L., Cote, G. G. & Brody, S. (1990). Circadian rhythms in *Neurospora crassa*: biochemistry and genetics. *Crit Rev Microbiol* 17, 365–416.
- Lauter, F.-R. & Yanofsky, C. (1993). Day/night and circadian rhythm control of *con* gene expression in *Neurospora*. *Proc Natl Acad Sci USA* 90, 8249–8253.
- Lauter, F.-R., Russo, V. E. & Yanofsky, C. (1992). Developmental and light regulation of *eas*, the structural gene for the rodlet protein of *Neurospora*. *Genes Dev* 6, 2373–2381.
- Lavery, D. J. & Schibler, U. (1993). Circadian transcription of the cholesterol 7 α hydroxylase gene may involve the liver-enriched bZIP protein DBP. *Genes Dev* 7, 1871–1884.
- Lee, C., Parikh, V., Itsukaichi, T., Bae, K. & Edery, I. (1996). Resetting the *Drosophila* clock by photic regulation of PER and a PER-TIM complex. *Science* 271, 1740–1744.
- Levine, J. D., Sauman, I., Imbalzano, M., Reppert, S. M. & Jackson, F. R. (1995). Period protein from the giant silkworm *Antheraea pernyi* functions as a circadian clock element in *Drosophila melanogaster*. *Neuron* 15, 147–157.
- Lewis, M. T. & Feldman, J. F. (1993). The putative *frq* clock protein of *Neurospora crassa* contains sequence elements that suggest a nuclear transcriptional regulatory role. *Protein Seq Data Anal* 5, 315–323.
- Lewis, M. T., Morgan, L. & Feldman, J. F. (1996). Analysis of frequency (*frq*) clock gene homologs: evidence for a helix-turn-helix transcription factor. *Mol Gen Genet* 253, 401–414.
- Linden, H. & Macino, G. (1997). *White collar-2*, a partner in blue-light signal transduction, controlling expression of light-regulated genes in *Neurospora crassa*. *EMBO J* 16, 98–109.
- Lindgren, K. M. (1994). *Characterization of ccg-1, a clock-controlled gene of Neurospora crassa*. PhD thesis, Dartmouth Medical School.
- Liu, Y., Tsinoemas, N. F., Johnson, C. H., Lebedeva, N. V., Golden, S., Ishiura, M. & Kondo, T. (1995). Circadian orchestration of gene expression in cyanobacteria. *Genes Dev* 9, 1469–1478.
- Liu, Y., Garceau, N. Y., Loros, J. J. & Dunlap, J. C. (1997). Thermally regulated translational control of FRQ mediates aspects of temperature responses in the *Neurospora* circadian clock. *Cell* 89, 477–486.
- Loros, J. (1995). The molecular basis of the *Neurospora* clock. *Semin Neurosci* 7, 3–13.
- Loros, J. J. & Dunlap, J. C. (1991). *Neurospora* clock-controlled genes are regulated at the level of transcription. *Mol Cell Biol* 11, 558–563.
- Loros, J. J., Richman, A. & Feldman, J. F. (1986). A recessive circadian clock mutant at the *frq* locus in *Neurospora crassa*. *Genetics* 114, 1095–1110.
- Loros, J. J., Denome, S. A. & Dunlap, J. C. (1989). Molecular cloning of genes under control of the circadian clock in *Neurospora*. *Science* 243, 385–388.
- Luo, C., Loros, J. J. & Dunlap, J. C. (1998). Nuclear localization is required for function of the essential clock protein FRQ. *EMBO J* 17, 1228–1235.
- McClung, C. R., Fox, B. A. & Dunlap, J. C. (1989). The *Neurospora* clock gene *frequency* shares a sequence element with the *Drosophila* clock gene *period*. *Nature* 339, 558–562.
- deMairan, J. J. (1729). Observation botanique. *Hist Acad R Sci (Paris)*, p. 35.
- Menaker, M. (1982). The search for principles of physiological organization in vertebrate circadian systems. In *Vertebrate Circadian Systems: Structure and Physiology*, pp. 1–12. Edited by J. Aschoff, S. Daan & G. A. Gross. Berlin: Springer.
- Morrow, M. & Dunlap, J. C. (1994). Intergeneric complementation of a circadian rhythmicity defect: phylogenetic conservation of the 989 amino acid open reading frame in the clock gene *frequency*. *EMBO J* 13, 2257–2266.
- Morrow, M., Garceau, N. Y. & Dunlap, J. C. (1997). Linearizing the circadian cycle to determine kinetic constants within the feedback loop. *Proc Natl Acad Sci USA* 94, 3877–3882.
- Michel, S., Geusz, M., Zaritsky, J. & Block, G. (1993). Circadian rhythm in membrane conductance expressed in isolated neurons. *Science* 259, 239–241.
- Millar, A. J., Carre, I. A., Strayer, C. A., Chua, N.-H. & Kay, S. A. (1995a). Circadian clock mutants in *Arabidopsis* identified by luciferase imaging. *Science* 267, 1161–1166.
- Millar, A. J., Straume, M., Chory, J., Chua, N.-H. & Kay, S. A.

- (1995b). The regulation of circadian period by phototransduction pathways in *Arabidopsis*. *Science* 267, 1163–1166.
- Mittag, M. (1996). Conserved circadian elements in phylogenetically diverse algae. *Proc Natl Acad Sci USA* 93, 14401–14404.
- Mittag, M., Eckerskorn, C., Strupat, K. & Hastings, W. J. (1997). Differential translational initiation of *lbp* mRNA is caused by a 5' upstream open reading frame. *FEBS Lett* 411, 245–250.
- Molina, C. A., Foulkes, N. S., Lalli, E. & Sassone Corsi, P. (1993). Inducibility and negative autoregulation of CREM: an alternative promoter directs the expression of ICER, an early response repressor. *Cell* 75, 875–886.
- Moore-Ede, M., Sulzman, F. M. & Fuller, C. A. (1982). *The Clocks That Time Us*. Cambridge, MA: Harvard University Press.
- Munger, K., Germann, U. A. & Lerch, K. (1987). Isolation and regulation of expression of the *Neurospora crassa* copper metallothionein gene. *Exper Suppl* 52, 393–399.
- Myers, M., Wager-Smith, K., Rothenfluh-Hilfiker, A. & Young, M. (1996). Light-induced degradation of TIMELESS and entrainment of the *Drosophila* circadian clock. *Science* 271, 1736–1740.
- Nakashima, H. (1982). Phase shifting of the circadian clock by diethylstilbestrol and related compounds in *Neurospora crassa*. *Plant Physiol* 70, 982–986.
- Nakashima, H. & Feldman, J. F. (1980). Temperature-sensitivity of light-induced phase shifting of the circadian clock of *Neurospora*. *Photochem Photobiol* 32, 247–251.
- Paietta, J. & Sargent, M. L. (1981). Photoreception in *Neurospora crassa*: correlation of reduced light sensitivity with flavin deficiency. *Proc Natl Acad Sci USA* 78, 5573–5577.
- Pierce, M. E., Sheshberadaran, H., Zhang, Z., Fox, L. E., Applebury, M. L. & Takahashi, J. S. (1993). Circadian regulation of iodopsin gene expression in embryonic photoreceptors in retinal cell culture. *Neuron* 10, 579–586.
- Pilgrim, M. L. & McClung, C. R. (1993). Differential involvement of the circadian clock in the expression of genes required for ribulose-1,5-bisphosphate carboxylase/oxygenase synthesis, assembly, and activation in *Arabidopsis thaliana*. *Plant Physiol* 103, 553–564.
- Pilgrim, M. L., Caspar, T., Quail, P. H. & McClung, C. R. (1993). Circadian and light-regulated expression of nitrate reductase in *Arabidopsis*. *Plant Mol Biol* 23, 349–364.
- Pittendrigh, C. S. (1960). Circadian rhythms and the circadian organization of living things. *Cold Spring Harbor Symp Quant Biol* 25, 159–184.
- Pittendrigh, C. S., Bruce, B. G., Rosensweig, N. S. & Rubin, M. L. (1959). Growth patterns in *Neurospora*. *Nature* 184, 169–170.
- van den Pol, A. N. & Dudek, F. E. (1993). Cellular communication in the circadian clock, the suprachiasmatic nucleus. *Neuroscience* 56, 793–811.
- Ralph, M. & Menaker, M. (1988). A mutation of the circadian system in golden hamsters. *Science* 241, 1225–1227.
- Ralph, M., Foster, R. G., Davis, F. C. & Menaker, M. (1990). Transplanted suprachiasmatic nucleus determines circadian period. *Science* 247, 975–978.
- Rensing, L., Kallies, A., Gebauer, G. & Mohsenzadeh, S. (1995). The effects of temperature change on the circadian clock of *Neurospora*. *Ciba Found Symp* 183, 26–41.
- Reppert, S. M., Tsai, T., Roca, A. L. & Sauman, I. (1994). Cloning of a structural and functional homolog of the circadian clock gene period from the giant silkworm *Antheraea pernyi*. *Neuron* 13, 1167–1176.
- Robertson, L. M. & Takahashi, J. S. (1988). Circadian clock in cell culture. II. *In vitro* photic entrainment of melatonin oscillation from dissociated chick pineal cells. *J Neurosci* 8, 12–21.
- Rosata, E., Trevisan, A., Sandrelli, F., Zordan, M., Kyriacou, C. & Costa, R. (1997). Conceptual translation of *timeless* reveals alternative initiating methionines. *Nucleic Acids Res* 25, 455–457.
- Rosbash, M., Allada, R., Dembinska, M. & 7 other authors (1996). A *Drosophila* circadian clock. *Cold Spring Harbor Symp Quant Biol* 61, 265–278.
- Rouyer, F., Rachidi, M., Pikielny, C. & Rosbash, M. (1997). A new gene encoding a putative transcription factor regulated by the *Drosophila* circadian clock. *EMBO J* 16, 3944–3954.
- Rusak, B. & Bina, K. G. (1990). Neurotransmitters in the mammalian circadian system. *Annu Rev Neurosci* 13, 387–401.
- Saez, L. & Young, M. W. (1996). Regulation of nuclear entry of *Drosophila* clock proteins PERIOD and TIMELESS. *Neuron* 17, 911–920.
- Sargent, M. L., Briggs, W. R. & Woodward, D. O. (1966). Circadian nature of a rhythm expressed by an invertaseless strain of *Neurospora crassa*. *Plant Physiol* 41, 1343–1349.
- Sauman, I. & Reppert, S. M. (1996). Circadian clock neurons in the silkworm *Antheraea pernyi*: novel mechanisms of Period protein regulation. *Neuron* 17, 889–900.
- Sehgal, A. (1995). Genetic dissection of the circadian clock: a *timeless* story. *Semin Neurosci* 7, 27–35.
- Sehgal, A., Price, H., Man, B. & Young, M. (1994). Loss of circadian behavioral rhythms and *per* oscillations in the *Drosophila* mutant *timeless*. *Science* 263, 1603–1606.
- Sehgal, A., Rothenfluh-Hilfiker, A., Hunter-Ensor, M., Chen, Y., Myers, M. & Young, M. W. (1995). Circadian oscillations and autoregulation of *timeless* RNA. *Science* 270, 808–810.
- Shearman, L. P., Zylka, M. J., Weaver, D. R., Kolakowski, L. F., Jr & Reppert, S. M. (1997). Two *period* homologs: circadian expression and photic regulation in the suprachiasmatic nuclei. *Neuron* 19, 1261–1269.
- Shigeyoshi, Y., Taguchi, K., Yamamoto, S. & 8 other authors (1997). Light-induced resetting of a mammalian circadian clock is associated with rapid induction of the *mPer1* transcript. *Cell* 91, 1043–1053.
- Shinohara, M. L., Loros, J. L. & Dunlap, J. C. (1998). Glyceraldehyde-3-phosphate dehydrogenase is regulated on a daily basis by the circadian clock. *J Biol Chem* 273, 446–450.
- Silver, R., LeSauter, J., Tresco, P. A. & Lehman, M. N. (1996). A diffusible coupling signal from the transplanted suprachiasmatic nucleus controlling circadian locomotor rhythms. *Nature* 382, 810–813.
- Stehle, J. H., Foulkes, N. S., Molina, C. A., Simoneaux, V., Pevet, P. & Sassone-Corsi, P. (1993). Adrenergic signals direct rhythmic expression of transcriptional repressor CREM in the pineal gland. *Nature* 365, 314–319.
- Stephan, F. K. & Zucker, I. (1972). Circadian rhythms in drinking behavior and locomotor activity of rats are eliminated by hypothalamic lesions. *Proc Natl Acad Sci USA* 69, 1583–1586.
- Strecker, G. J., Bouskila, Y. & Dudek, F. E. (1995). Neurotransmission and electrophysiological mechanisms in the suprachiasmatic nucleus. *Neurosciences* 7, 43–51.
- Sun, Z. S., Albrecht, U., Zhuchenko, O., Bailey, J., Eichele, G. & Lee, C. C. (1997). *RIGUI*, a putative mammalian ortholog of the *Drosophila period* gene. *Cell* 90, 1003–1011.
- Sweeney, B. M. (1987). *Rhythmic Phenomena in Plants*, 2nd edn. San Diego: Academic Press.
- Sweeney, B. M. & Borge, M. B. (1989). A circadian rhythm in

cell division in a prokaryote, the cyanobacterium *Synechococcus* WH7803. *J Phycol* 25, 183–186.

Takahashi, J. S. (1993). Circadian-clock regulation of gene expression. *Curr Opin Genet Dev* 3, 301–309.

Takahashi, J. S., Murakami, N., Nikaido, S. S., Pratt, B. L. & Robertson, L. M. (1989). The avian pineal, a vertebrate model system of the circadian oscillator: cellular regulation of circadian rhythms by light, second messengers, and macromolecular synthesis. *Recent Prog Horm Res* 45, 279–352.

Tei, H., Okamura, H., Shigeyoshi, Y., Fukuhara, C., Ozawa, R., Hirose, M. & Sakaki, Y. (1997). Circadian oscillation of a mammalian homologue of the *Drosophila period* gene. *Nature* 389, 512–516.

Tosini, G. & Menaker, M. (1996). Circadian rhythms in cultured mammalian retina. *Science* 272, 419–421.

Tsinoremas, N. F., Ishiura, M., Kondo, T., Andersson, C. R., Tanaka, K., Takahashi, H., Johnson, C. H. & Golden, S. S. (1996). A sigma factor that modifies the circadian expression of a subset of genes in cyanobacteria. *EMBO J* 15, 2488–2495.

Turek, F. W. (1994). Circadian rhythms. *Recent Prog Horm Res* 49, 43–90.

Underwood, H. (1990). The pineal and melatonin: regulators of circadian function in lower vertebrates. *Experientia* 46, 120–128.

Van Gelder, R. N. & Krasnow, M. A. (1996). A novel circadianly expressed *Drosophila melanogaster* gene dependent on the *period* gene for its rhythmic expression. *EMBO J* 15, 1625–1631.

Van Gelder, R., Bae, H., Palazzolo, M. & Krasnow, M. A. (1995). Extent and character of circadian gene expression in *Drosophila*

melanogaster: identification of 20 oscillating mRNAs in the fly head. *Curr Biol* 5, 1424–1436.

Vitaterna, M. H., King, D. P., Chang, A.-M. & 7 other authors (1994). Mutagenesis and mapping of a mouse gene, Clock, essential for circadian behavior. *Science* 264, 719–725.

Welsh, D. K., Logothetis, D. E., Meister, M. & Reppert, S. M. (1995). Individual neurons dissociated from rat suprachiasmatic nucleus express independently phased circadian firing rhythms. *Neuron* 14, 697–706.

Zatz, M. (1992). Perturbing the pacemaker in the chick pineal. *Discov Neurosci* 8, 67–73.

Zatz, M. (1994). Photoendocrine transduction in cultured chick pineal cells. IV. What do vitamin A depletion and retinaldehyde addition do to the effects of light on the melatonin rhythm? *J Neurochem* 62, 2001–2011.

Zatz, M., Mullen, D. A. & Moskal, J. R. (1988). Photoendocrine transduction in cultured chick pineal cells: effects of light, dark, and potassium on the melatonin rhythm. *Brain Res* 438, 199–215.

Zeng, H., Hardin, P. E. & Rosbash, M. (1994). Constitutive overexpression of the *Drosophila period* protein inhibits *period* mRNA cycling. *EMBO J* 13, 3590–3598.

Zeng, H., Qian, Z., Myers, M. & Rosbash, M. (1996). A light-entrainment mechanism for the *Drosophila* circadian clock. *Nature* 380, 129–135.

Zweibel, L. J., Pe, H., Liu, X., Hall, J. C. & Rosbash, M. (1991). A post-transcriptional mechanism contributes to circadian cycling of a *per-B-galactosidase* fusion protein. *Proc Natl Acad Sci USA* 88, 3882–3886.