REVIEW ARTICLE

Signs of the time: environmental input to the circadian clock

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Abstract

The circadian clock forms one of the most fascinating adaptations to life on earth. Organisms can not only anticipate the day/night cycle but can make use of an internal clock to measure daylength as an indicator of the changing of the seasons. The innate period of the clock is not exactly equal to 24 h, but is reset each day by environmental signals at dawn and dusk, most notably by changes in light and temperature. This ability to re-entrain also ensures that the clock is synchronized with the day/night cycle which in turn is crucial for anticipation of dawn and dusk. Recent advances in the field have identified the photoreceptors involved in resetting the clock in several systems. This has revealed surprising similarities, but also key differences in the circadian systems of plants, fungi, insects, and mammals. One recurring feature emerging from this research is that the photoreceptors themselves are under the control of the clock with transcript abundance being tightly regulated. Furthermore, elements of a feedback pathway whereby the clock modulates the activity of the light input pathway are now being identified.

Key words: Circadian clock, environmental input, photoreceptors, temperature.

Introduction

All organisms properly tested show a rhythm of metabolism, of physiological processes or even of behaviour in tune with the day/night cycle of the earth. In plants, the components of the photosynthetic machinery accumulate each day in anticipation of dawn; leaves or flowers often close up just before dusk to provide protection for more delicate tissues from the lower temperatures of night (Darwin, [1895] 1981; Enright, 1982). In insects, the adult flies eclose from their pupae in synchrony with dawn to provide the maximum chances of survival (Pittendrigh, 1954), in mammals such as ourselves our body has a rhythm of alertness and of temperature that causes us to be active during the day and to sleep at night (Moore-Ede et al., 1982). These phenomena are not purely responses to the external environment and will continue even in the absence of any external cues. A plant maintained in constant light will still show a cyclic production of its photosynthetic machinery (Millar and Kay, 1991), a human deep underground will continue to wake and sleep with an approximately 24 h rhythm as if still experiencing dawn and dusk (Luce, 1971; Sulzman, 1983). Such processes are controlled by an endogenous oscillator known as the circadian clock which continues to cycle, maintaining its own, approximately 24 h rhythm, as a result of the oscillation of the levels or activity of molecules within the cells of each organism. The molecules making up this core clock and their biochemical interactions have been well studied and in insects and mammals the components of this basic ‘clock mechanism’ are now known. These are discussed in more detail by Wager-Smith and Kay (2000). In plants, the clock mechanism remains more elusive, but several recent advances have begun to shed light on this (Carré and King, 2002).

The circadian clock does not run in isolation from the cycle of day and night. It must, itself, be set to the correct time. The clock must include a resetting mechanism by which it can first be synchronized with the day/night cycle so that the organism can correctly anticipate dawn and dusk. This clock resetting is a phenomenon very familiar to any travellers on long-haul flights. When a person travels through several time-zones, initially jet-lag is experienced.
whereby his/her circadian clock remains set to the timing of dawn and dusk in the place of departure though, gradually, over the course of a few days he/she finds his/her rhythm of sleep and wake has adjusted to the new timing of dawn and dusk.

The two most prevalent environmental cues which act as Zeitgebers (time givers) are the changes in light and temperature occurring at dawn and dusk and both are capable of resetting the clock (Edmunds, 1988; Roenneberg and Foster, 1997). The circadian clock can be thought of in terms of three major components: an 'input' pathway by which environmental cues act to synchronize the clock; the endogenous 'oscillator' itself; and the 'output' pathway whereby the rhythmic metabolic systems controlled by the clock are co-ordinated (Fig. 1).

This review focuses on the input to the clock. More details on clock output can be found in three recent papers by Harmer et al. (2000), McDonald et al. (2001) and Duffield et al. (2002), focusing on plants, insects and mammals, respectively. However, one caveat should be added to this separation in that it is now apparent that this traditional three component model of the circadian system is oversimplistic and it is becoming increasingly more difficult to consider any one of these components in isolation. For example, it has been clear for some time that the output pathway modulates (gates) the sensitivity of the input pathway (Fig. 1). Consequently, this review takes a fairly holistic approach in describing the input pathway.

**Light input to the circadian clock**

Light forms the dominant signal in resetting the clock and a close link between the circadian photoreceptors and the clock itself has been demonstrated for several systems (Devlin and Kay, 2001). In fact, the distinction between input, oscillator and output is becoming more and more blurred as more is discovered about the mechanism itself. In resetting, the clock exhibits a change in phase. That is, the hands of the circadian clock are moved forward or backwards. In terms of the molecular mechanism of the clock this would represent a change in the level or activity of a clock component to a level or activity that would normally be found at a different point in the cycle (Crosthwaite et al., 1995). The clock then continues as before. For most organisms the period of the circadian cycle is not quite 24 h and they show a slight resetting with each dawn and/or dusk. This plasticity allows an organism to adjust continually to changing daylength as the seasons of the year progress. The response of the clock to light is different at different times of day. The onset of light prior to the expected dawn will generally cause an advance in the phase of the rhythm whilst extension of the light period after the expected dusk will generally cause a delay in the phase of the rhythm. It is possible to produce a 'phase response curve' (PRC) illustrating this effect (Johnson, 1990). The effects of light pulses at different times of day on the phase of the rhythm are examined for organisms otherwise maintained in darkness. An example of a typical phase response curve is shown in Fig. 2. The curve shows a strong phase-advance response around subjective dawn.
and a strong phase-delay response around subjective dusk. The response to light is greatly reduced during the subjective day, as might be expected, when the organism will normally be ‘seeing’ light. For many organisms the PRC exhibits a ‘dead-zone’ during the subjective day where no response at all is seen to light (Johnson, 1990).

Entrainment of the circadian clock by such pulses of light is known as non-parametric entrainment as opposed to parametric entrainment to day/night cycles. Although the clock is sensitive to pulses of light for resetting, it is important that the clock should not be unduly influenced by aberrations in the environment. It must not, for example, be reset by a flash of lightning at night. In general, a prolonged pulse of irradiation is required to reset the clock (Nelson and Takahashi, 1991).

The photoreceptors involved in the perception of light leading to resetting of the circadian clock have been the subject of several recent advances in the field of circadian biology. Both plants and animals have a complex array of photoreceptors (Roenneberg and Foster, 1997; Whitelam and Devlin, 1998; Foster, 1998; Hall, 2000). The photoreceptors in plants are particularly well characterized. Light plays a key role in the development of a plant, controlling processes from germination, through seedling establishment, to determining the whole architecture of the plant (Kendrick and Kronenberg, 1994). Plants are exquisitely sensitive to small changes in the light environment in order to be able to adapt to take maximum advantage of the available light for photosynthesis. The first identification of the photoreceptors involved in resetting of the circadian clock came from research in the model plant, Arabidopsis thaliana where two families of photoreceptors, the red-absorbing phytochromes and the blue-absorbing cryptochromes combine to relay dawn and dusk signals to the endogenous oscillator (Somers et al., 1998a). In insects and mammals, the blue light photoreceptor, cryptochrome was also discovered and demonstrated to play a key role in the circadian clock. In insects,

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**Fig. 3.** Time-course showing the circadian rhythm of bioluminescence in a population of Arabidopsis thaliana seedlings expressing the firefly luciferase gene under the control of the Arabidopsis CAB2 promoter. The time-course follows the rhythm of transcription from the CAB2 promoter over 48 h. Two circular 10 cm Petri dishes of seedlings are represented for each time-point. Twenty-five images are shown, taken at 2 h intervals over the course of 2 d and should be followed as if reading a book, from left to right, one line at a time. The seedlings, germinated on growth medium were first entrained 12/12 h light/dark cycles for 6 d then transferred to constant light. The sequence commences during the morning of the first day after transfer to constant light. The CAB2 gene encodes part of the photosynthetic machinery and appropriately shows a peak of expression during the subjective day and a trough of expression during the subjective night. Images were taken using a NightOWL cooled CCD camera, Berthold Technologies, Cambridge, UK.
Cryptochrome is the main circadian photoreceptor although it has also been proposed to play a role in the oscillator mechanism in peripheral tissue (Emery et al., 1998, 2000; Stanewsky et al., 1998; Krishnan et al., 2001). In mammals, the main role of cryptochrome is as a component of the central clock mechanism itself (Kume et al., 1999; (van der Horst et al., 1999). The photoreceptors involved in the perception of light in resetting the clock remain elusive. It is known, however, that the eyes are essential for entrainment in mammals (Foster, 1998) yet several pieces of research have ruled out the involvement of the visual opsins (Freedman et al., 1999).

Circadian photoperception in plants

The earliest recorded observation of a circadian rhythm was made by Androsthenes (historian of Alexander the Great) around 400 BC. He observed that leaves of several tree species exhibit a horizontal position during the day and a more vertical position at night. However, it was in 1729 that the French astronomer, Jean Jacques d’Ortous de Mairan first demonstrated that this was the result of the action of an endogenous circadian clock. Mimosa, the sensitive plant, folds up its leaves at night and opens them again in the day. De Mairan showed that this rhythm, continued even when the plants were placed in deep shade demonstrating that bright sunlight was not required to trigger this response (de Mairan, 1729).

In plants, a large range of physiological processes are controlled by the circadian clock including rhythmic leaf movements (Engelmann et al., 1994; Millar et al., 1995a), photoperiodic induction of flowering (Devlin and Kay, 2000b) and stomatal opening (Somers et al., 1998b). However, much of the work studying the circadian clock in plants has involved the analysis of the rhythm of expression of the clock-controlled gene, light-harvesting chlorophyll a/b protein (better known as the chlorophyll a/b binding protein, CAB). CAB forms part of the photosynthetic machinery of the plant and, as would be predicted, the CAB transcript begins to accumulate prior to dawn, shows a peak of expression in the early part of the day then decreases again towards dusk (Millar and Kay, 1991). Millar et al. (1992) attached a firefly luciferase reporter gene (LUC) to the CAB promoter and transformed this CAB::LUC construct into plants. Using a highly sensitive photon-counting camera they were able to follow the rhythmic expression pattern of CAB in living seedlings by following the transient bioluminescence of the firefly luciferase produced as a result (Fig. 3).

This system allowed the isolation of several circadian clock mutants in A. thaliana (Millar et al., 1995a). One of these was the result of a mutation in the gene, TIMING OF CAB 1 (TOC1), that encodes a pseudo-response regulator involved in the central clock mechanism in A. thaliana (Strayer et al., 2000). The toc1 mutation affects all known observable rhythms in A. thaliana even in constant darkness and the TOC1 message, itself, displays circadian oscillation with a peak at subjective dusk, thus demonstrating its credentials as a central clock component (Somers et al., 1998a, b; Strayer et al., 2000). Other methods looking at the transcription factors responsible for regulation of the CAB gene identified two other central clock components, LATE ELONGATED HYPOCOTYL (LHY) and CIRCADIAN CLOCK ASSOCIATED 1 (CCA1), both MYB-type transcription factors (Wang and Tobin, 1998; Schaffer et al., 1998). Mutations in cca1 and lhy, likewise affect all known observable rhythms in A. thaliana and the CCA1 and LHY transcripts oscillate with a circadian rhythm showing a peak of expression at subjective dawn (Wang and Tobin, 1998; Schaffer et al., 1998). It was recently demonstrated that TOC1 is responsible for the positive regulation of CCA1 and LHY expression, whilst both LHY and CCA1 bind to the TOC1 promoter for the negative regulation of TOC1 expression (Alabadi et al., 2001). This loop (represented in the ‘Oscillator’ section of Fig. 4) is critical for clock function in A. thaliana and is proposed to form the fundamentals of the central circadian oscillator in A. thaliana.

The CAB::LUC reporter system was also used to analyse the light input pathway to the plant circadian clock. Millar et al. (1995b) demonstrated that the A. thaliana circadian

Fig. 4. A detailed plan of the circadian system in Arabidopsis thaliana. Critical to the maintenance of a circadian rhythm is a feedback loop made up of three proteins: TIMING OF CAB 1 (TOC1), CIRCADIAN CLOCK ASSOCIATED 1 (CCA1) AND LATE ELONGATED HYPOCOTYL (LHY). Levels of TOC1 oscillate with an approximately 24 h rhythm, peaking in the evening. Levels of CCA1 and LHY oscillate with an approximately 24 h rhythm, peaking in the morning. CCA1 and LHY proteins bind to an ‘evening’ element (AAAAATACT) in the TOC1 promoter negatively regulating transcription of the TOC1 gene. Conversely, TOC1 protein positively regulates transcription of the CCA1 and LHY genes. As levels of TOC1 protein rise, they promote transcription of LHY and CCA1. Then, as levels of CCA1 and LHY proteins subsequently rise, they inhibit transcription of TOC1, thereby negatively feedback back on their own transcription. Light input to the A. thaliana circadian clock is via phytochrome (PHY) and cryptochrome (CRY) photoreceptors, PHYB acts directly to promote transcription of CCA1 and LHY by associating with PHYTOCHROME INTERACTING FACTOR (PIF3) bound to a ‘G-box’ element (CACGTG) in the CCA1 and LHY gene promoters. Light signalling via PHYB and CRY1 involves direct association with a common circadian light input signal transduction component, ZELUPE (ZTL). Output from the clock potentially regulates these input pathways via two methods. Transcription of the PHYB, CRY1 and CRY2 genes is directly clock regulated, whilst sensitivity to light input signals is periodically regulated by the clock controlled gating factor EARLY FLOWERING 3 (ELF3). The CCA1 and LHY proteins are proposed to act in the output pathway regulating a range of clock controlled output genes. CCA1 and LHY positively regulate CHLOROPHYLL A B BINDING PROTEIN (CAB) and other ‘morning’ genes by binding to ‘morning’ promoter elements (AAAAATCT). CCA1 and LHY negatively regulate ‘evening’ genes by binding to ‘evening’ promoter elements (AAAAATATCT).
Input to the clock

INPUT

OSCILLATOR

OUTPUT
Photoreceptors involved in light input to the circadian clock in plants

In addition to chlorophyll, which carries out light harvesting for photosynthesis, plants possess several information gathering photoreceptors that allow them to regulate their development to take maximum advantage of their light environment. These fall into three families, the phytochromes, absorbing red and far red light and the cryptochromes and the phototropins, absorbing blue and UV wavelengths (Whitelam and Devlin, 1998; Christie and Briggs, 2001). The phytochrome family consists of five members in A. thaliana, phyA to phyE (Sharrock and Quail, 1989; Clack et al., 1994). All share the same basic structure, comprising a protein moiety of about 124 kDa and a covalently attached linear tetrapyrrole chromophore (Quail, 1991). Phytochromes exist in two photo-interconvertible forms, a red-absorbing, Pr form and a far-red-absorbing Pfr form (Quail, 1991). Phytochrome is synthesized in the inactive Pr form and upon absorption of a photon of light is converted to the active Pfr form that is responsible for regulating a range of physiological and developmental responses. PhyA is light labile and accumulates to high levels in etiolated seedlings (Quail, 1991). PhyA acts most prominently in germination and in seedling establishment and triggers a response to very low levels of light (Whitelam et al., 1993; Johnson et al., 1994). It is rapidly degraded in high fluences of red light and hence phyA is thought of as an ‘antenna’ detecting the small amount of light penetrating through the upper layer of soil indicating that a seedling is about to emerge into light (Yanovsky et al., 1995). PhyB–phyE are light stable and are generally involved in responses to higher fluences of red light (Hirschfeld et al., 1998; Whitelam et al., 1998). It is noticeable that the phytochromes also show a peak of absorption in the blue region of the spectrum and, consistent with this, phytochrome A has been implicated in some blue light responses (Whitelam et al., 1993). Sufficient phyA Pfr is formed in blue light to trigger a response.

The cryptochrome family in A. thaliana comprises two members, cry1 and cry2 (Devlin and Kay, 1999). Cryptochromes show a strong resemblance to the photolyases involved in the blue/UV light-dependent repair of DNA damage (Cashmore et al., 1999). The N-terminal section of each of the A. thaliana cryptochromes shares a strong homology with type II photolyase and, like the photolyases, cryptochromes bind two chromophores, a light-harvesting pterin and a catalytic flavin (Malhotra et al., 1995; Lin et al., 1995). Cry1 is light stable whilst cry2 is light labile and is degraded under high fluences of blue light (Lin et al., 1998). Cry2 has been demonstrated to be involved in seedling establishment in low fluence blue light whilst cry1 shows an involvement in such responses at both low and high fluences of blue (Lin et al., 1998).

Clocks ran faster in continuous light than in darkness. The response of an endogenous oscillator to continuous light is the sum of the phase advances and phase delays occurring throughout the circadian cycle. As light intensity increases, the magnitude of these phase shifts increases (Aschoff, 1979). The overall effect depends on the shape of the phase response curve. In diurnal organisms, such as A. thaliana, phase advances predominate over phase delays with the result that, in continuous light, increasing light intensity tends to lead to a shortening of the circadian period length. In nocturnal organisms, phase delays predominate over phase advances with the result that increasing light intensity tends to lead to a lengthening of period length. This phenomenon has become known as Aschoff’s rule (Aschoff, 1979). Millar et al. (1995b) showed that both red and blue light were capable of causing a shortening of the CAB::LUC rhythm in A. thaliana.
Somers et al. (1998a) demonstrated that both phytochromes and cryptochromes contribute to light input to the circadian clock in *A. thaliana*. By crossing the CAB::LUC transgene into null mutants for phyA, phyB, cry1 or cry2, roles for phyA and cry1 in the perception of blue light, and phyA and phyB in the perception of red light were demonstrated (Somers et al., 1998a). The assay made use of Aschoff’s rule whereby increasing fluence rate (light intensity) causes a decreasing period length in constant light (Aschoff, 1979). PhyA mutant seedlings showed a deficiency in the perception of low fluence rates of red and blue light consistent with the role of phyA as a low fluence photoreceptor in seedling establishment. PhyB mutant seedlings showed a deficiency in the perception of high fluence rate red light (Somers et al., 1998a). The responses of the phyA and phyB mutant seedlings nicely demonstrate the plasticity of recruitment of photoreceptors by the plant to allow meaningful detection of a range of fluence rates. Red light fluence rates above which the phyA response would saturate fall within the active range of phyB. At these fluence rates, phyA is degraded and phyB becomes the dominant red light photoreceptor. Studies with phyA phyB double mutants confirm these findings showing an additivity between the phyA and phyB monogenic phenotypes (Devlin and Kay, 2000a).

Cry1 mutants showed a deficiency in response to low fluence rates of blue light, a wild-type response to intermediate fluence rates of blue light and a deficiency in response to high fluence rates of blue light. This clearly indicated a role for cry1 in blue light input to the clock. The cry2 monogenic mutant showed a wild-type response for blue light input to the clock (Somers et al., 1998a), but analysis of the cry1 cry2 double mutant showed a redundancy between cry1 and cry2 at intermediate fluence rates (Devlin and Kay, 2000a). This is consistent with the way in which cry1 and cry2 act in seedling establishment at these fluence rates. Both cry1 and cry2 act in seedling establishment in lower fluence rates of blue light, but cry2 is degraded at higher fluence rates leaving cry1 as the sole blue light photoreceptor for de-etiolation (Lin et al., 1998).

Significantly, for light input to the clock the cry1 mutant was also found to show a deficiency in the perception of low fluence rate red light in a similar manner to that seen in the phyA mutant (Devlin and Kay, 2000a). Given the fact that the absorption spectrum for the cryptochromes shows no peak in the red region of the spectrum, it must be concluded that cryptochrome is acting downstream of the photoreceptor phyA, presumably as a signal transduction component. Double mutant analysis of phyA cry1 in white light confirms that there is no additivity between these two mutations for low fluence rate light input to the clock (Devlin and Kay, 2000a). Both phyA and cry1 are capable of acting as photoreceptors in white light, yet cry1 is epistatic to phyA. This suggests that in low fluence rates of both red and blue light cry1 acts purely as a signal transduction component downstream of phyA. This role for cry1 appears unique to circadian photoperception. No evidence was found for a role for cry1 in seedling de-etiolation in low fluence rates of red light (Devlin and Kay, 2000a). A scheme for the action of the cryptochromes in blue light input to the clock is shown in Fig. 5a.

Evidence for the action of phyD and phyE in red light input to the clock was also demonstrated. In the regulation of physiological development in *A. thaliana* phyD and phyE show a conditional redundancy with phyB in that the roles of phyD and phyE only become apparent in the absence of phyB (Whitelam et al., 1998). This also appears to be the case for the action of phyD and phyE in light input to the clock. The phyD and phyE monogenic mutants show a wild-type response to red light, but when the phyA phyB phyD triple mutant was compared to the phyA phyB double mutant, the phyA phyB phyD triple mutant showed a relative deficiency in the perception of high fluence rate red light. Likewise the phyA phyB phyE triple mutant showed a deficiency in the perception of high fluence rate red light relative to the phyA phyB double mutant (Devlin and Kay, 2000a). The redundancy between phyD or phyE and phyB is, to some extent, understandable, given the fairly close similarity between the DNA sequences of phyB, phyD and phyE. In particular, phyB and phyD of *A. thaliana* are thought to have arisen as the result of a very recent gene duplication subsequent to the divergence of the Cruciferae and the Solanaceae (Mathews and Sharrock, 1997). Significantly the phyA phyB phyD triple mutant and the phyA phyB phyE triple mutant both still showed a significant shortening of period length as red light fluence rate increased, indicating, in each case, the action of the remaining phytochromes in red light input to the clock (Devlin and Kay, 2000a). Whether this represents the action of phyC awaits the creation of the phyA phyB phyD phyE quadruple mutant. One important point to note is that the photoreceptor mutants show no effect on the period length of the clock in darkness indicating that they are purely affecting the light input pathway rather than disrupting the clock mechanism itself (Devlin and Kay, 2000a). The roles of the phytochromes and the cryptochromes in light input to the circadian clock can be summarized as in Fig. 4b.

The third class of plant photoreceptors, the phototropins, are involved in the perception of blue wavelengths of light, leading to phototropism (Huala et al., 1998; Jarillo et al., 1998; Kagawa et al., 2001). Two phototropins are present in *A. thaliana*, nph1 (named after the mutant phenotype, non-phototropic hypocotyl) (Liscum and Briggs, 1995) and nph1 (nph1-like) (Kagawa et al., 2001). Phototropins consist of a protein moiety with two flavin chromophores held within PAS or LOV domains within the protein molecule (Christie et al., 1998). Analysis of the response of the nph1 mutant to a range of fluence rates of blue light showed no evidence for the involvement of nph1 in light
input to the clock (Harmer et al., 2000; supplemental data), however, it remains possible that nph1 could act redundantly with other photoreceptors.

Oscillating photoreceptor expression

In several systems, a close association between light input and the clock itself has been established with the result that the traditional distinction of input, oscillator and output is becoming blurred. Transcription of the *PHYB* gene and of the *CRY1* and *CRY2* genes in *A. thaliana* was recently shown to oscillate with a circadian rhythm in continuous light (Bognar et al., 1999; Harmer et al., 2000). *PHYB* shows a peak around subjective dawn whilst the *CRY*s show a peak in the later part of the subjective day (Harmer et al., 2000). The circadian photoreceptors, phyB, cry1 and cry2 are therefore part of both the input and output pathways from the clock and in this way the output pathway may feed back on the input pathway, adding an additional level of fine tuning to the clock resetting mechanism (Fig. 4). It is possible that the expression pattern of the circadian photoreceptors may, to some extent, contribute to the shape of the phase response curve giving increased sensitivity to light at the dawn and dusk transitions.

Such modulation of light responsiveness over the course of the circadian cycle has been termed gating (Millar and Kay, 1996). Gating is not only observed for clock resetting but also for several clock outputs which can also be directly regulated by light. Control of *CAB* gene expression is directly light responsive as well as being clock regulated. Following a period of darkness, *CAB* gene expression will show an acute spike in transcription in response to a light pulse. However, a strong circadian rhythm in this light responsiveness of *CAB* induction is observed, indicating that light-signalling to *CAB* is strongly gated by the clock (Millar and Kay, 1996). Very recently, the first candidate for a protein involved in this gating process was identified in *A. thaliana*. The *elf3* mutant shows an arrhythmic phenotype in constant white light (Hicks et al., 1996) but does show a circadian rhythm in darkness. McWatters et al. (2000) demonstrated that the *elf3* mutant fails to show any gating of the acute response of *CAB* in darkness. They concluded that the arrest of the oscillator in constant light is also a result of a loss of gating of light input, demonstrating that gating of phototransduction is an important part of the resetting response (McWatters et al., 2000) (Fig. 4).

Candidates for factors involved in the light input pathway downstream of the photoreceptors themselves have also been identified in plants. The *zeitlupe* (*ztl*) mutant of *A. thaliana* was identified as showing a long period length for the rhythm of *CAB::LUC* expression in constant light (Somers et al., 2000). It also shows a long period length for expression of other circadian clock regulated genes and for the rhythm of cotyledon movement (Somers et al., 2000). The *ztl* phenotype is strongly dependent on fluence rate, displaying a greater period lengthening effect at lower fluence rates (Somers et al., 2000). This suggests that *ztl* specifically disrupts light input to the clock. *ZTL* forms part of a small family of three closely related proteins along with *FKF1* and *LKP2* (Nelson et al., 2000; Kiyosue and Wada, 2000). FKF1 also affects circadian regulated gene expression and mutations in both *ZTL* and *FKF1* both cause late flowering (Somers et al., 2000; Nelson et al., 2000). Recently *ZTL* was demonstrated to bind to both phyB and cry1 using yeast two-hybrid and *in vitro* binding studies, further suggesting a close association with the light input pathway to the circadian clock (Jarillo et al., 2001) (Fig. 4).

Another candidate signal transduction component downstream of phytochrome in light input to the clock is PHYTOchrome INTERACTING FACTOR 3 (*PIF3*). *PIF3* was identified in a yeast two-hybrid screen for phytochrome interacting factors (Ni et al., 1998). The *PIF3* gene encodes a bHLH-type transcription factor that binds to G-box sequences present in the promoters of the genes encoding the critical clock components, *LHY* and *CCA1* (Martinez-Garcia et al., 2000). When a phytochrome molecule is converted to the Pfr form it moves from the cytoplasm to the nucleus and there binds to the promoter-bound *PIF3* transcription factor (Kircher et al., 1999; Ni et al., 1999). *CCA1* and *LHY* gene expression is light-regulated and *PIF3* has been shown to be essential for normal light regulation (Ni et al., 1999). This has led to the conclusion that binding of phytochrome to *PIF3* acts as the switch to trigger an increase in expression of the *CCA1* and *LHY* genes. Such a system would allow a pulse of light to reset the clock by triggering a change in *CCA1* and *LHY* message levels (Fig. 4).

Photoreceptors involved in light input to the circadian clock in insects

Following the discovery of cryptochromes in plants (Ahmad and Cashmore, 1993), molecules showing strong similarity to photolyases, but which failed to show any photolyase function, were identified in animals (Sancar, 2000). Because of this similarity to the plant cryptochromes, these molecules were also termed cryptochromes. As with the plant cryptochromes, the N-terminus of the animal cryptochromes shows strong homology to the photolyases whilst the C-terminal forms a unique extension (Cashmore et al., 1999; Devlin and Kay, 1999; Sancar, 2000). However, the animal cryptochromes show a stronger homology with the 6-4 photolyases than with the type II photolyases (Cashmore, 2000). However, the animal cryptochromes show a stronger homology with the 6-4 photolyases than with the type II photolyases (Cashmore et al., 1999). It is thought that the animal cryptochromes arose subsequent to the plant cryptochromes by divergence from the 6-4 photolyases. The phylogeny of the plant crypto-
Fig. 6. The action of cryptochrome in light input to the circadian clock in the fruit fly, Drosophila melanogaster. The circadian clock consists of a negative feedback loop whereby the TIMELESS (TIM) and PERIOD (PER) proteins mediate a rhythmic suppression of their own transcription via repression of a transcriptional activation complex made up of the CLOCK (CLK) and CYCLE (CYC). In response to light CRYPTOCHROME (CRY) binds to TIM negating the action of the TIM–PER dimers and resetting the clock to a point at which tim and per transcription is high. The D. melanogaster cry gene shows a circadian rhythm of expression with a peak in the later part of the day. It is proposed that this oscillation may modulate light resetting of the clock.

Cryptochromes, however, suggests that they arose much earlier. In fact, they are predicted to have arisen prior to the divergence of plants and animals (Cashmore et al., 1999) suggesting that plant-like, type II photolyase-derived cryptochrome has been subsequently lost in animals. None-the-less, the same blue-light-sensing mechanism seems to have been adapted once again to provide a circadian photoreceptor in animals, particularly in insects where cryptochrome forms the primary circadian photoreceptor (Emery et al., 2000; Devlin and Kay, 2001). The core clock components have been well characterized in the insect, Drosophila melanogaster, where a transcriptional feedback loop involving four key molecules generates sustained oscillation at the molecular level with a relatively tight circadian period (Allada et al., 1998; Rutila et al., 1998; Darlington et al., 1998). Transcription of the genes, period (per) and timeless (tim) leads to a rise in PER and TIM protein levels in the afternoon and early evening. PER–TIM dimers then enter the nucleus and repress their own transcription by inhibiting the activity of a positively acting transcriptional activation complex consisting of the proteins CLOCK (CLK) and CYCLE (CYC). Consequently, levels of PER and TIM protein fall again as these proteins are degraded until they reach a level at which they no longer inhibit their own transcription and the cycle begins again (Allada et al., 1998; Rutila et al., 1998; Darlington et al., 1998) (Fig. 6).

A second, interlocked negative feedback loop causes CLK to cycle in antiphase with PER and TIM. In this, CLK feeds back as a repressor of its own transcription whilst PER and TIM act as derepressors (Glossop et al., 1999).

In D. melanogaster it was known that visual photoreceptors alone were not responsible for light input to the clock. The norpA<sup>pl</sup> mutation causes the compound eyes and ocelli to be completely unresponsive to light, although monogenic norpA<sup>pl</sup> mutant flies show normal entrainment (Wheeler et al., 1993; Yang et al., 1998). The discovery of cryptochrome provided a strong candidate for the circadian photoreceptor. A D. melanogaster mutant lacking cryptochrome, named cry<sup>baby</sup> (cry<sup>b</sup>) has been most informative in revealing the role of cryptochrome in the D. melanogaster clock. In the cry<sup>b</sup> mutant, rhythms of PER and TIM expression in the body of the fly fail to entrain to light/dark cycles (Stanewsky et al., 1998). Curiously, cry<sup>b</sup> mutant flies were still observed to display behavioural locomotor rhythms. It was subsequently discovered that the rhythm of per and tim expression within lateral neuron cells remains entrainable by light suggesting that photoreceptors other than D. melanogaster cryptochrome (dCRY) are able to entrain these rhythms. When the norpA<sup>pl</sup> mutation was combined with the cry<sup>b</sup> mutation, norpA<sup>pl</sup> cry<sup>b</sup> double mutant flies now failed to show normal entrainment of behavioural rhythms (Stanewsky et al., 1998) suggesting that behavioural entrainment in D. melanogaster is mediated by a combination of signals from cryptochrome and from the visual photopereception pathway.

Phase shifting is also compromised in the cry<sup>b</sup> mutant flies. In response to a pulse of light that will induce a phase shift in behavioural rhythms in wild-type flies, the cry<sup>b</sup> mutant fails to show any phase shift (Stanewsky et al., 1998). Responses of overexpressors of dcry, however, vary. Emery et al. (1998) observed an enhanced response to light pulses for phase shifting in overexpressors of dcry. Ishikawa et al. (1999) observed a decreased response. An assay based on Aschoff’s rule (Aschoff, 1979) has also been used definitively to demonstrate the role of cryptochrome as a circadian photoreceptor. In D. melanogaster, period length in constant light increases with increasing fluence rate to the extent that, at very high intensities of light, wild-type flies become arrhythmic (Aschoff, 1979; Konopka et al., 1989). Mutant cry<sup>b</sup> flies do not respond to this increasing fluence rate and continue to display strong rhythmicity even in intense constant illumination. This indicates that circadian photoperception is completely impaired in the absence of cryptochrome (Emery et al., 2000). A wild-type phenotype can be
restored in the cryb mutant flies by expression of wild-type dCRY in lateral neuron cells. dCRY is thus demonstrated to be the only circadian photoreceptor that impinges directly on the clock in D. melanogaster (Emery et al., 2000). This leaves a question as to how the visual photoreceptors can entrain the clock in a cryb mutant. It was proposed by Emery et al. (2000) that visual stimuli may act to drive a rhythm of physical activity in light/dark cycles and that this, in turn, somehow feeds back to entrain the behavioural rhythm that can subsequently be observed in cry flies under constant conditions.

The mode of action of dCRY in resetting the clock at the molecular level was identified by Ceriani et al. (1999). Using a yeast two-hybrid assay, dCRY was shown to interact directly with TIM. This interaction was observed in the light, but not in darkness, pointing to a light-dependent interaction of dCRY with the PER-TIM dimer (Ceriani et al., 1999). Furthermore, in a D. melanogaster cell culture system, dCRY was found to negate the action of the PER-TIM dimer in feeding back on per and tim transcription in light but not in darkness, effectively resetting the clock to a point at which per and tim transcription are high (Ceriani et al., 1999).

The D. melanogaster cryptochrome dcr shows a circadian rhythm of expression with a peak in the later part of the day. As is proposed to be the case for the plant photoreceptors, this oscillation may modulate light resetting of the clock and affect the shape of the phase response curve (Ishikawa et al., 1999) (Fig. 6).

In a recent study by Krishnan et al. (2001), a further role for dCRY in the D. melanogaster clock was proposed. In addition to the role of dCRY as the circadian photoreceptor in D. melanogaster brain, Krishnan et al. (2001) showed that dCRY may be part of the oscillator mechanism itself in peripheral tissue. A circadian rhythm of olfactory responses can be observed in the antennae of D. melanogaster. In wild-type flies maintained in constant darkness, this rhythm could be entrained by temperature cycles, but such temperature cycles were unable to entrain the rhythm in cry flies. This suggests either that dCRY is essential for temperature entrainment in D. melanogaster or that it forms part of the oscillator mechanism in the antenna. As there is no precedent of dCRY having a thermoreceptive role, the latter seems more likely. This result stresses the care that needs to be exercised when denoting a particular gene/protein as a photoreceptor or clock component.

Photoreceptors involved in light input to the circadian clock in mammals

In mammals, cryptochrome plays an integral role in the central clock mechanism. Two cryptochromes, CRY1 and CRY2 exist in mammals (Devlin and Kay, 1999), though it is uncertain whether they play a role within light input to the clock. In mice, the clock mechanism is made up of a similar transcriptional feedback loop to that discovered in insects, though CRY replaces TIM within the mammalian system and there are three per genes present (Kume et al., 1999; Field et al., 2000; Reppert and Weaver, 2001). Transcription of the mCry and mPer genes begins in the late afternoon and levels of the proteins in the cytoplasm rise steadily. mCRY–mCRY dimers or mCRY–mPER dimers then form, enabling their entry into the nucleus where these dimers inhibit the action of their own transcriptional activators, CLK and CYC, and thus exert a negative feedback on their own transcription so that levels of cry and per subsequently fall again. It appears that inhibition of the CLK–CYC complex is effected largely by the CRY proteins, thus the role of CRY has become central to the clock mechanism itself (Reppert and Weaver, 2001). Consistent with this, mCry1+/− mCry2+/− mice are arrhythmic in constant light (van der Horst et al., 1999). Again a second, interlocking feedback loop exists. In this case mPER2 acts as a promoter of CYC transcription, whilst mCRY plays a role in stabilizing mPER2 (Shearman et al., 2000).

Some evidence points to a role for cry in light input to the mammalian clock, though this is not definitive. It is difficult to prove a role for CRY in light input to the mammalian clock based on studies of the mCry1+/− mCry2+/− double mutant mice as the clock itself no longer runs in these mutants (van der Horst et al., 1999). Evidence for altered response to phase-shifting pulses of light has been demonstrated for the mCry2−/− monogenic mutants. mCry2−/− mutant mice show an enhanced response to a phase-shifting light pulse during the subjective night (Thresher et al., 1998). One of the early events associated with phase resetting in response to a light pulse is a rapid induction of mPer1. mCry1+/− and mCry2−/− monogenic mutants display aberrant mPer1 induction (Vitaterna et al., 1999). Studies of light-induced mPer1 transcription in mCry1+/− mCry2+/− double mutant mice also suggest a role for mCRY as a circadian photoreceptor. mCry1+/− mCry2−/− double mutant mice fail to show mPer1 induction in response to a light pulse given during the night. However, if mCry1+/− mCry2+/− mutants are left in darkness for 52 h prior to a light pulse, then light-induction of mPer1 is observed, suggesting the action of another, possibly light-labile, photoreceptor (Okamura et al., 1999). In addition, mPer2 is still induced in response to a light pulse in mCry1+/− mCry2−/− mutants and, whilst induction of mPer2 has not been shown to lead to clock resetting, this also indicates the involvement of photoreceptors other than cryptochrome acting upon the clock system (Vitaterna et al., 1999).

mCRYs do play a role as photoreceptors for behavioural modification in mice. As well as showing a circadian regulation of locomotor activity, whereby they are active during the subjective night and inactive during the
subjective day, wild-type mice show a simple responsive behaviour to light/dark cycles whereby they remain inactive whenever the light is on. The cryptochromes have been shown to act redundantly with the classical retinal photoreceptors in the perception of light in the control of this response (Selby et al., 2000). This result indicates that they can act as photoreceptors and that they do play a dual role, both as part of the clock mechanism and as photoreceptors in their own right. Whether they contribute to circadian photoperception will be more difficult to ascertain.

It is certain, however, that the mammalian circadian photoreceptor is located in the eye as enucleation results in loss of circadian entrainment (Foster, 1998). Experiments with rodless and coneless rats, however, have demonstrated that neither rods nor cones are essential for clock resetting (Freedman et al., 1999). mCRY is found in the retinal ganglion layer of the eye, thus cryptochrome is not ruled out as the ocular photoreceptor (Miyamoto and Sancar, 1998). However, it is probable that the action of a novel mammalian photoreceptor located in the eye is being witnessed. This is also suggested by the action spectrum for mammalian clock resetting which shows a peak of action at ~500 nm, corresponding better to an opsin (Provencio and Foster, 1995; Yoshimura and Ebihara, 1996) than to cryptochrome (Sancar, 2000). Novel opsins have, indeed, recently been found in *Xenopus* and in salmon (Soni et al., 1998; Provencio et al., 1998b).

**Differences in circadian photoperception between plants and animals**

It is notable that the *A. thaliana cry1 cry2* double mutant is still rhythmic, clearly indicating that, in *A. thaliana*, the cryptochromes are not essential for the running of the clock as they are in mammals but are purely involved in light input (Devlin and Kay, 2000a). The clock itself seems to have evolved independently in plants and in animals and, although much of the clock `mechanism' appears conserved between insects and mammals (Williams and Sehgal, 2001), these components have not been found in plants. It is, therefore, no surprise that the circadian photoreceptors also arose independently in plants and animals, but it remains an interesting observation that, in both cases, molecules derived from the blue-light sensing photolyases are closely involved with the clock.

Another distinct feature of plant and animal clocks is that, whilst in animals there is a strong central co-ordination of circadian oscillations throughout the organism mediated via the brain, in plants there appears to be no such co-ordination. Within mammals a region of the hypothalamus known as the suprachiasmatic nucleus (SCN) acts as a central clock regulating rhythms throughout the rest of the body (Yamazaki et al., 2000). The SCN shows a direct synaptic connection to the eyes, thought to be the route of clock resetting light signals (Provencio et al., 1998a). Whilst rhythms can be maintained in isolated mammalian tissues, clock resetting by light has not been demonstrated in these peripheral tissues and appears to require central control via signals from the SCN (Sakamoto et al., 1998; Yamazaki et al., 2000). Within flies the central control appears to be exhibited by the lateral neurones (Stanewsky et al., 1998). Rhythmic expression of the clock genes within the lateral neurones alone has been demonstrated to be sufficient to maintain locomotor rhythms of the whole fly (Stanewsky et al., 1998). Isolated body parts of *D. melanogaster* can also maintain rhythmic clock gene expression and, furthermore, can be entrained by light signals indicating that, although the clock is controlled centrally in flies, clock resetting throughout the organism is not absolutely dependent on signals from the lateral neurones (Plautz et al., 1997). In plants, it has been demonstrated the intact cotyledons on the same plant can be stably entrained to different light/dark cycles independently and will continue to cycle out-of-phase when transferred back to constant conditions (Thain et al., 2000). This indicates that not only is each part of the plant capable of independent clock resetting but that there is no systemic co-ordination of the clock throughout the plant as a whole. All co-ordination of rhythms throughout the plant is therefore dependent on each part of the plant detecting the same environmental signals.

**Photoreceptors involved in light input to the circadian clock in fungi**

Yet another independently evolved clock mechanism is found in the fungus, *Neurospora crassa*. *N. crassa* formed a model organism in which many of the principles of an oscillator based on a transcriptional feedback loop were established (Dunlap, 1999). The *frequency* (*frq*) gene of *N. crassa* forms part of a self-sustaining oscillator whereby the FRQ protein feeds back on *frq* transcription (Aronson et al., 1994). In *N. crassa* two proteins, WHITECOLLAR 1 and 2 (WC-1 and WC-2), that are required for all known photoresponses, have been shown to be essential for light input to the clock (Harding and Melles, 1983; Russo, 1988; Sommer et al., 1989; Lauter and Russo, 1991; Arpaia et al., 1993). WC-1 and WC-2 act as positive regulators that maintain robust FRQ cycling (Crosthwaiete et al., 1997). WC-1 and WC-2 form a transactivating complex known as the whitecollar complex (WCC) which activates *frq* transcription (Ballario et al., 1998; Talora et al., 1999; Denault et al., 2001). As FRQ protein subsequently rises, the FRQ protein interacts directly with this complex to negate its action on the *frq* promoter so that FRQ protein is no longer produced and the levels of FRQ subsequently fall again (Denault et al., 2001). WC-1 is responsible for the light induction of the *frq* transcript that results in a
phase shift in the rhythm (Crosthwaite et al., 1997). Interestingly, the WC-1 is also rhythmically expressed under the control of FRQ protein (Denault et al., 2001). As with the oscillating light input components in A. thaliana and D. melanogaster this may form a mechanism of gating of the light responsiveness of the clock to appropriate times of day. Very recently a further putative gating component was identified in N. crassa. The protein, VIVID (VVD) regulates the light response in N. crassa (Heintzen et al., 2001). VVD forms an independent autoregulatory transcriptional feedback loop. Transcription of vvd is regulated by the WCC and VVD protein is rapidly induced by light. The VVD protein then acts to negate the WCC, effectively reducing the light responsiveness of N. crassa. Consequently, vvd mutants of N. crassa show severely dampened gating and an alteration in the phase response curve (Heintzen et al., 2001) (Fig. 7).

Temperature input to the clock

The response of the circadian clock to temperature shows something of a paradox. The clock exhibits a temperature compensation whereby, under constant conditions, the period length of the rhythm is almost unaffected by the external temperature. The phenomenon of temperature compensation was first observed by Pittendrigh (1954) studying the eclosion rhythm of Drosophila pseudoobscura pupae. He found that this rhythm maintains the same period length over a wide range of temperature. Similarly, the rhythms studied in plants show very low sensitivity to temperature (Salisbury et al., 1968; Somers et al., 1998b). For most biochemical reactions the rate will increase 2–3-fold for a 10 °C increase in temperature ($Q_{10}=2–3$). $CAB::LUC$ rhythms in A. thaliana show a slight shortening of period length in response to increasing temperature with a $Q_{10}$ of 1.0–1.1 (Somers et al., 1998b).

However, despite the temperature compensation which is built into the endogenous clock, a sudden temperature change is able to act as a Zeitgeber and entrain the clock to a new phase. Clocks can, in fact, be entrained to a temperature rhythm such as that which occurs with the day/night cycle in the natural environment (Bünning, 1973; Somers et al., 1998b; Merrow et al., 1999), though much less is known about the temperature input to the clock in entrainment.

Temperature entrainment has been used experimentally to examine the position of mutations within the clock system. The $frq$ null mutant of N. crassa shows an arrhythmic phenotype in constant conditions. The $frq$ null fails to entrain to light/dark cycles, but will stably entrain to temperature cycles, suggesting that $frq$ is a critical clock component essential for light-mediated entrainment of circadian rhythms in N. crassa (Merrow et al., 1999). It appears that another $frq$-independent oscillator is responsible for the rhythms observed in $frq$ mutants in response to temperature entrainment (Roenneberg and Merrow, 2000).

Such a $frq$-independent oscillator had also previously been proposed on the basis that the $frq$ null mutant will eventually display a (very long period) rhythm after an extended period in constant conditions (Loros and Feldman, 1986). Curiously, wc-1 and wc-2 mutants cannot be entrained by either light or temperature indicating a wider involvement in the clock (Crosthwaite et al., 1997).

Similarly temperature entrainment was used to demonstrate that the elf3 mutant of A. thaliana specifically affects light input to the oscillator. As discussed earlier, the elf3 mutant has lost gating light signalling causing the action of the oscillator to be masked in continuous light (McWatters et al., 2000). Temperature entrainment can restore rhythmicity in the elf3 mutant demonstrating that the mutation specifically affects the light input pathway to the clock (McWatters et al., 2000).

Temperature cycles were used to demonstrate that the toc1 mutation in A. thaliana lies within the central oscillator as opposed to being in an input pathway (Somers et al., 1998b). The toc1 mutant displays a short period length for a number of rhythmic outputs even in darkness indicating that it does not lie in the light input pathway (Somers et al., 1998b). toc1 was shown not to
affect temperature compensation and following temperature entrainment the tocl mutant also showed a short period length. Furthermore, in cycles of alternating high and low temperature, the peak of CAB::LUC expression in the tocl mutant led that of the wild type (Somers et al., 1998b). A feature of mutants affecting the period length of the clock is that the timing of the peak of the rhythm relative to the entraining signal will differ from that seen in the wild type. In a short period mutant the peak will lead that of the wild type and in a long period mutant the peak will lag behind that of the wild type. Thus the tocl mutant does not affect the process of temperature entrainment of the clock and so does not lie within the temperature input pathway. tocl is, therefore, proposed to affect the oscillator mechanism itself (Somers et al., 1998b).

Conclusions

A common feature of circadian systems in all species examined is the close association between light input and the oscillator itself. Several systems display an oscillation in expression of the circadian photoreceptors themselves. Phytochrome B in A. thaliana shows a circadian rhythm of transcription with a peak in the late part of the night/early morning (Bognar et al., 1999; Harmer et al., 2000). The A. thaliana cryptochromes oscillate with a peak in the late part of the day (Harmer et al., 2000). D. melanogaster cryptochrome also shows a circadian oscillation with a peak in the late part of the day (Ishikawa et al., 1999). Interestingly, whilst it is uncertain whether the mammalian cryptochromes are circadian photoreceptors their expression also oscillates under circadian control. mCry1 oscillates with a circadian rhythm in the SCN, also showing a peak in the late part of the day, whilst both mCry1 and mCry2 oscillate with a circadian rhythm in peripheral skeletal tissue (Kume et al., 1999).

Clearly the output from the clock feeds back to regulate input. As well as direct regulation of photoreceptor levels a gating or modulation of responsiveness to signals from the photoreceptors is an integral part of many circadian systems. This can regulate the degree of clock resetting in response to the signals from the light environment at different times of day (Roenneberg and Foster, 1997). This feature of these clocks means that they will respond most strongly to environmental conditions at crucial times of day, namely dawn and/or dusk when the acquisition of information about the light environment is most important.

Recently, elements involved in gating responses have begun to be characterized (McWatters et al., 2000; Heintzen et al., 2001). The elucidation of such regulatory gating or modulating mechanisms will be important for the full understanding of the central oscillator itself. It may even be hypothesized that a feedback loop modulating the activity of a photoreceptor could have been at the very origin of circadian clocks.

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