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Review

Detection and response of the *Neurospora crassa* circadian clock to light and temperature

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ABSTRACT

Circadian clocks are molecular timekeepers that provide organisms with a means to predict and prepare for environmental change. The filamentous fungus *Neurospora crassa* has provided an excellent model system in which the underlying molecular basis of circadian clocks has been elucidated. In Neurospora, and in other eukaryotes, circadian rhythmicity emerges from a network of positive and negative feedback regulation acting on clock genes and proteins. An essential attribute of the clock is that it can detect and respond to the daily cycle of light and dark and temperature change and integrate these environmental time cues to give an accurate depiction of the external day. In Neurospora many of the molecules that sense the daily changes in light and temperature are known. In this review we describe Neurospora's clock mechanism and how it is tuned to the real world by light and temperature.

1. Introduction

Circadian clocks are cellular timers whose endogenous periods approximate a solar day and whose ability to entrain, i.e. couple to and resonate with the 24-h solar day, enables organisms to anticipate daily changes in their environment (Dunlap *et al.*, 2004). Next to their innate cycle lengths and their ability for entrainment, another defining characteristic of circadian clocks is their ability to keep time in a range of ambient temperatures, a phenomenon known as temperature compensation. This property distinguishes circadian clocks from simple biochemical reactions or other non-circadian phenomena that are usually strongly temperature-dependent.

Because circadian clocks impose temporal order on a large number of cellular processes a breakdown of circadian organization has wide-ranging consequences. Defects in circadian clocks may result in reduced fitness due to lack of resonance with the rhythmic environment (Ouyang *et al.*, 1998; Dodd *et al.*, 2005), aberrant timing of development (Hicks *et al.*, 1996; Dowson-Day and Millar, 1999), aberrant cell cycle progression

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(Fu *et al.*, 2002; Borgs *et al.*, 2009), and disturbance of normal physiology and behaviour (Jones *et al.*, 1999; Toh *et al.*, 2001). The latter has serious consequences for human health and performance and may cause jetlag, sleep wake disorders, depression and early onset of cancer.

2. The Neurospora clock

The Neurospora circadian clock regulates diverse cellular processes but the most visually striking of these is the timing of asexual spore production (conidiation) (Pittendrigh *et al.*, 1959; Sargent and Briggs, 1967). However, the influence of the clock is not restricted to the timing of development. Rhythms in CO_2 production and lipid metabolism are also long known (Lakin-Thomas *et al.*, 1990) and more recently, microarray studies indicate that 10–15 % of Neurospora's genes are under clock control (Correa *et al.*, 2003; Nowrousian *et al.*, 2003). These rhythmically expressed genes encode ribosomal proteins, transcriptional regulators, enzymes involved in intermediary metabolism, cell-signalling, stress responses and mating (Vitalini *et al.*, 2006).

At the heart of the Neurospora circadian system is a negative feedback loop comprising the transcription factors WHITE COLLAR-1 (WC-1) and WC-2, the clock protein FREQUENCY (FRQ), FRQ-interacting RNA helicase (FRH), and the kinases and phosphatases that regulate their activity (Fig. 1) (Heintzen and Liu, 2007). WC-1 and WC-2 bind to the *frequency* (*frq*) promoter forming the White Collar Complex (WCC), and rhythmically activate *frq* transcription (Froehlich *et al.*, 2002; He *et al.*, 2002; Froehlich *et al.*, 2003). Once translated FRQ forms a homodimer that interacts with FRH (Cheng *et al.*, 2005) (forming the FRQ/FRH Complex (FFC)). The FFC interacts with CASEIN KINASE 1 (CK1) and CK2. In addition to their role as FRQ kinases, both CK1 and CK2 mediate FRQ-dependent phosphorylation of the WCC (He *et al.*, 2006).

Phosphorylated WCC is less able to bind to and activate transcription from the *frq* promoter and is translocated into the cytoplasm (Schafmeier *et al.*, 2006; Querfurth *et al.*, 2007). In the cytoplasm it may be dephosphorylated by PROTEIN PHOSPHATASE 2A (PP2A), allowing it to move back into the nucleus in an active state, or its phosphorylation may be further supported by cytosolic FRQ. Phosphorylation of the WCC in the nucleus and its dephosphorylation in the cytoplasm lead to a shuttling of inactive and active WCC between the nucleus and the cytoplasm on an ultradian timescale (Schafmeier *et al.*, 2008). Whereas transcriptionally active WCC is quickly degraded, phosphorylated WCC is more stable. Thus, as levels of FRQ increase through the afternoon and evening the WCC accumulates, but only late at night and early in the morning, as FRQ is degraded, is there sufficient active WCC to promote increased levels of *frq* mRNA (Fig. 2). Local changes in the structure and accessibility of the DNA also support rhythmic transcription of *frq* leading to the production of peak levels of *frq* RNA synthesis during the morning and consequently a peak in FRQ protein by mid-day. The chromodomain protein CLOCKSWITCH and at least one other as yet unidentified remodelling protein (Belden *et al.*, 2007) bring about these changes.

FRQ is also phosphorylated and its phosphorylation state is an important determinant of both phase and period length. Due to progressive phosphorylation of FRQ by CK1 and CK2, towards the end of the day it becomes hyperphosphorylated. Hyperphosphorylated FRQ is targeted for ubiquitination and degradation by the proteasome, releasing repression of the WCC (He and Liu, 2005). Mutation analysis reveals that phosphorylation sites located in the N-terminal half of FRQ result in period lengthening, whilst mutation of sites towards the C-terminus results in period shortening (Baker *et al.*, 2009; Tang *et al.*, 2009). Moreover, the pattern of phosphorylation changes

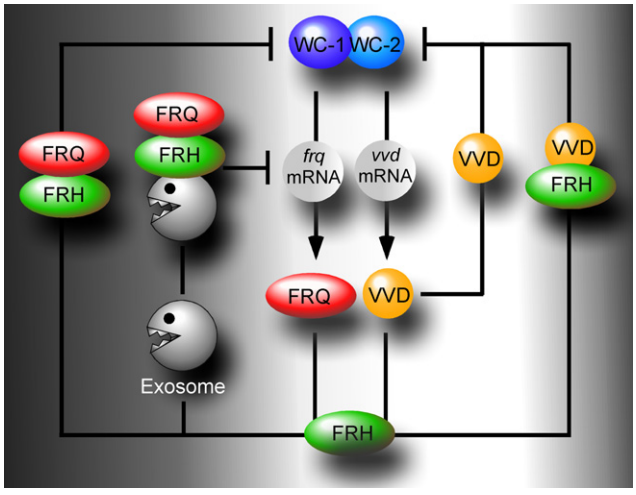

Fig. 1 – Negative feedback at the heart of the *Neurospora crassa* circadian clockwork. During the night (dark grey) the FRQ–FRH Complex (FFC) rhythmically represses the activity of the White Collar Complex (WCC) to generate molecular circadian oscillations. In a post-transcriptional feedback loop the FFC is recruited to the exosome and rapidly degrades *frq* transcript. In addition a VVD–FRH complex represses WCC activity to bring about rapid resetting of the Neurospora clock at dusk (light grey). Not shown are positive feedback loops that reinforce this feedback cycle but are not essential for rhythmicity.

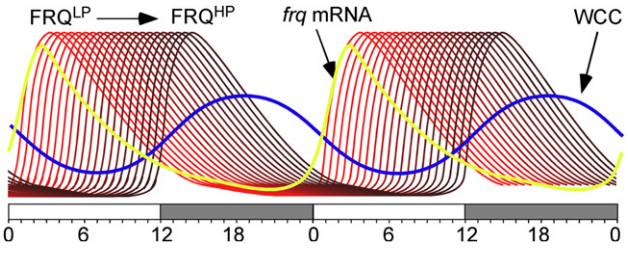

Fig. 2 – Rhythms in central clock gene expression in Neurospora: schematic representation of *frq* transcript (yellow), FRQ protein (bright red to dark red) and WCC (blue) expression/activity during two circadian cycles. Subjective day (open box) and subjective night (shaded boxes). FRQ matures from a newly synthesized and hypophosphorylated nuclear repressor of WCC (FRQ^{LP}; bright red lines) to a highly phosphorylated form (FRQ^{HP}; dark red lines) that supports cytoplasmic accumulation of the WCC. The progressive phosphorylation of FRQ creates a wave of FRQ phosphorylation profiles that cover a wide range of phases within the circadian day.

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depending on the time of day such that late in the day the C-terminal is maximally phosphorylated. In this state FRQ is stable, however with time it is destabilized due to phosphorylation of the N-terminus, leading eventually to its degradation. The activity of the protein phosphatases PPA1, PPA2 and PP4 also impacts on the negative feedback loop regulating the phosphorylation state of FRQ and the WC proteins (Heintzen and Liu, 2007).

An additional negative feedback loop involves the products of the *vivid* (*vvd*) gene. This feedback loop plays roles in Neurospora's response to light (Heintzen *et al.*, 2001; Schwerdtfeger and Linden, 2003; Elvin *et al.*, 2005). Recent data suggest that VVD inhibits the WCC by interacting with the WCC and FRH to mute light-induced transcription and facilitate rapid down-regulation of light responses at dusk (Chen *et al.*, 2010; Hunt *et al.*, 2010; Malzahn *et al.*, 2010). With regards to the clock, VVD helps to establish clock phase by muting clock resetting at dawn and promoting it, *via* the inhibition of *frq* transcription, at dusk (Elvin *et al.*, 2005) (see below for more detail).

The central negative feedback loop is supported by a positive feedback circuit that ensures the timely accumulation of newly synthesized WCC. Though the levels of *wc-1* and *wc-2* RNA do not cycle, their transcription is promoted by FRQ and FRQ-dependent phosphorylation of the WCC increases its stability and accumulation in the cytoplasm (Fig. 2). This ensures that when FRQ degrades there is already a new pool of WCC waiting to activate the next round of transcription (Lee *et al.*, 2000; Schafmeier *et al.*, 2008). Cycling levels of *frq* RNA are additionally enhanced through the action of FRH. FRH is a cofactor of the *Neurospora* exosome and the complex of FRQ and FRH binds to *frq* transcript targeting it for degradation. Thus, when FRQ levels are low *frq* RNA is more stable and *vice versa* (Guo *et al.*, 2009).

3. Light resetting and clock entrainment

By definition, circadian clocks have periods that deviate from the 24-h solar day. Therefore, to stay in synchrony with the natural world clocks must couple to environmental rhythms, such that a stable phase (time of day) relationship between the clock and the environment is established. The coupling process is called entrainment (Johnson *et al.*, 2003). The ability to entrain is key to the function of circadian clocks because in this way organisms stay in tune with the environment and can adapt to environmental transitions long before they happen. This is in contrast to non-circadian processes in which the environment periodically forces a response pattern onto an inherently arrhythmic process. The most powerful environmental cues that entrain circadian clocks to the solar day are light and temperature. Both factors change with metronomic predictability over the course of the day and throughout the year in all but the most extreme latitudes.

One can study the ability of these cues to reset (and entrain) a circadian clock by giving light or temperature pulses at different times of the day and measuring the effect these pulses have on the phase of the rhythm (Fig. 3). A plot that displays the response against the time when pulses are given is known as a phase response curve (PRC, Fig. 3C) (Hastings and Sweeney, 1958; Pittendrigh, 1981). Studying PRCs obtained from various experimental systems shows that light or temperature pulses reset circadian clocks in a universally predictable manner (Johnson, 1999). For example, a light pulse given during the early (subjective) night delays the clock to the previous day whereas a similar pulse given during late (subjective) night will advance the clock into the next day. As expected, during the subjective day, there is a more or less extensive 'dead zone' during which light pulses have little effect on the state of the oscillator. It is useful to exemplify this by looking at experiments in which cycles of short light or temperature pulses (skeleton cycles) are used to entrain organisms.

Circadian clocks entrain well to these conditions and their entrainment behaviour can often be predicted simply by knowing their free-running period (FRP) and light or temperature PRC. In Neurospora the PRC can be easily obtained by monitoring a 'hand' of the circadian clock, e.g. the timing of asexual spore production. This is done by inoculating conidiospores on growth medium contained in so-called race tubes (Fig. 3A). Spores are allowed to germinate and grow in the light for 24 h before cultures are transferred to constant conditions of darkness and temperature. This light to dark switch sets the circadian clock to subjective dusk (circadian time 12, CT12) and the first band of spore production occurs about 10–11 h after the transfer (CT0). A PRC is then obtained by systematically perturbing replicate cultures with brief light or temperature pulses at different times of the day and measuring the effect (in hours) these perturbations have on the phase of the subsequent bands of conidiation compared to an unpulsed control (Fig. 3B). The response to perturbations (advance or delay) is then plotted against the time when the pulses were given to yield the PRC (Fig. 3C). Studying the PRC will reveal at what time of day light or temperature pulses must strike to achieve entrainment in skeleton cycles (Fig. 3C). For example, if Neurospora cultures (FRP ~22 h) are exposed to 20- (T20) and 24-h (T24) skeleton cycles the pulse must advance (T20) or delay (T24) the clock by 2 h every day. In the 20-h skeleton experiment described above, light must hit during the late subjective night/early subjective morning for entrainment to occur. Because the peak of clock-controlled conidiation in Neurospora occurs during this time it is clear that the pulse will coincide with the peak of conidiation. On the other hand, if the skeleton cycle is lengthened to 24 h, light must strike at a time where the PRC predicts 2-h delay, i.e. 10–12 h before the peak of conidiation is reached (Fig. 3D).

It can be easily understood that light must have these effects in order for entrainment to occur but it is less obvious how the opposing effects of light pulses on clock resetting (delay, advance) can be explained at the molecular level. A simple answer emerged whilst studying the dynamics of *frq* transcript accumulation following short light pulses given at different times during the circadian cycle (Crosthwaite *et al.*, 1995). When pulsed with light, *frq* mRNA levels were shown to rapidly rise independent of the time of day when the light pulse was given. Thus, superimposed on the circadian rhythm of *frq* transcript levels in the unpulsed dark controls, a light pulse given during the rising slope of the *frq* cycle (i.e. during subjective dawn) would lead to a premature peak in *frq* transcript levels, i.e. advance the clock cycle. In contrast, inducing *frq* transcript at a time when mRNA levels are normally declining (at subjective dusk) will delay the fall in *frq* levels and hence delay the cycle. As expected, a light

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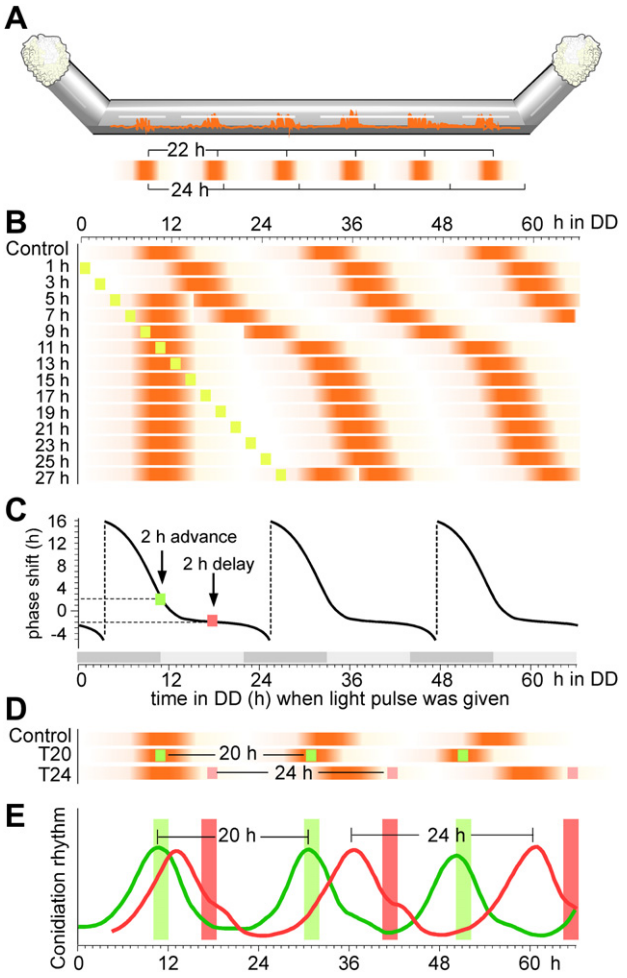

Fig. 3 – Circadian entrainment and the phase response curve (PRC). (A) The race tube assay. Schematic drawing of a race tube partly filled with solid growth medium. Neurospora is inoculated at one end and grows down the tube (left to right) at a fairly constant growth rate. In constant darkness (DD) the circadian clock throws the developmental switch that controls the formation of bands of aerial hyphae and asexual spores (dark orange bars) that repeat with characteristic 22-h periodicity. (B) Schematic representation of a perturbation experiment. Control cultures (top 'race tube') are kept in DD, whereas replicate cultures are exposed to brief light or high temperature pulses (yellow bar) at different times in DD as indicated. (C) Data from experiment in (A) plotted as a phase response curve (PRC). Phase advances or delays are plotted as a function of the time (in DD) when a pulse was given. Pulses given during the late subjective night/early subjective day (green box) or late subjective day (red box) lead to a 2-h phase advance or delay of the rhythm respectively. Grey bars, subjective night. (D) Predicted entrainment of cultures that are exposed to repeated and evenly spaced pulses that are either 20 (T20: green bars) or 24 h (T24: red bars) apart. A free-running control culture is shown (top). For entrainment to a T20 cycle to occur the clock cycle needs to be advanced/shortened by 2 h every day. This can be achieved when the pulse strikes repeatedly at times when such advances/delays are predicted from the PRC as shown in (C). In T20 cycles Neurospora should entrain with a phase of conidiation (onset, peak, and offset) that coincides with the time when the temperature pulse is given, whereas conidiation should precede the pulse in T24 cycles. (E) Densitometric traces of actual race tube cultures incubated at 25 °C in DD and exposed to temperature pulses (2 h, 30 °C) that were either 20 or 24 h apart. Colour coding as shown in (D). The observed behaviour closely follows the predictions depicted in (D).

pulse given during the subjective day, i.e. when *frq* levels are at their highest will not change the kinetics of the *frq* cycle and consequently has little or no effect on the clock cycle (Crosthwaite *et al.*, 1995).

The rapid induction or degradation of central clock components by brief light pulses has been used to explain clock resetting and entrainment to brief light pulses in other organisms (Shigeyoshi *et al.*, 1997; Young and Kay, 2001; Ashmore and Sehgal, 2003). However, several lines of evidence suggest that clock entrainment to continuous photoperiods, as encountered in the real world, is more complex (Johnson *et al.*, 2004; Tan *et al.*, 2004; Elvin *et al.*, 2005; Price-Lloyd *et al.*, 2005). In Neurospora the ablation of *qrf*, an antisense transcript arising from the *frq* locus, or *vivid* (*vvd*), a PAS/LOV-type photoreceptor, result in a much stronger resetting response to light pulses. Mutants in either component have a relatively normal circadian clock indicating that they influence the resetting response without affecting the central clockwork (Heintzen *et al.*, 2001; Kramer *et al.*, 2003; Crosthwaite, 2004). Experiments in which Neurospora wild-type and *vvd* mutants were either entrained in continuous or skeleton photoperiods (using light pulses) revealed that in the wild-type, the phase of clock-controlled conidiation in both entrainment protocols is different, i.e. skeleton photoperiods do not mimic entrainment to complete photoperiods. In a vvd^{KO} strain however, both protocols yield similar phases of entrainment, suggesting that VVD is involved in establishing clock phase in complete photoperiods (Elvin *et al.*, 2005).

In organisms whose entrainment to full photoperiods can be faithfully replicated by skeleton photoperiods, light used for entrainment is apparently only required at the dawn and dusk transitions (Johnson *et al.*, 2004). When studying the PRC to brief light pulses it is clear that during the subjective day some but not all parts of the clock cycle are insensitive to light. This is in part due to a phenomenon known as circadian gating in which the circadian clock influences its own responsiveness to light to certain times of day (Fleissner and Fleissner, 1992; Millar and Kay, 1996; McWatters *et al.*, 2000; Heintzen *et al.*, 2001; Dragovic *et al.*, 2002; Gillette and Mitchell, 2002). One would expect that the shape of the PRC and the extent of its dead zone would influence the degree to which a complete photocycle can determine the phase of entrainment. Indeed, VVD is known to influence the shape of the PRC largely by influencing the dead zone of the light PRC and has profound effects on the phase of clock-controlled processes in photo entrainment (Heintzen *et al.*, 2001; Elvin *et al.*, 2005).

4. Photoreceptors and light responses in Neurospora

In Neurospora two blue-light photoreceptors, WC-1 and VVD, are important for circadian entrainment. Both of these

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photoreceptors have PAS/LOV domains that bind flavin adenine dinucleotide (FAD) as chromophore. WC-1 interacts with WC-2 to form a transcriptionally and photoactive White Collar Complex (WCC). As we have seen earlier the WCC is not only required for photosignalling but is also an essential component of the circadian clock (Ballario *et al.*, 1996; Crosthwaite *et al.*, 1997; Froehlich *et al.*, 2002; He *et al.*, 2002). VVD, unlike WC-1, is not essential for circadian rhythmicity in constant darkness (Heintzen *et al.*, 2001; Shrode *et al.*, 2001; Cheng *et al.*, 2003; Schwerdtfeger and Linden, 2003). At least one of the functions of VVD is to repress the activity of the WCC, thus influencing light resetting and entrainment of the circadian clock (Heintzen *et al.*, 2001; Elvin *et al.*, 2005). In *vvd* mutants the resetting response to light pulses is much stronger and the phase of entrainment to complete or skeleton photoperiods is aberrant. By repressing WCC activity at dawn, VVD also effectively blocks clock resetting by light at this time and allows the clockwork to measure day length. However, VVD also promotes some aspects of light signalling, is necessary for sensing step changes in light intensity (Schwerdtfeger and Linden, 2003; Malzahn *et al.*, 2010) and probably modulates slow light responses in Neurospora (Chen *et al.*, 2009). Due to its general role in light sensing VVD is important for clock function in Neurospora. The structure of VVD was recently solved and VVD was shown to transiently dimerize in a light-dependent manner (Zoltowski *et al.*, 2007; Lamb *et al.*, 2008; Zoltowski and Crane, 2008). These studies provide a first understanding of the early events that trigger conformational changes in VVD that subsequently could relay light signals to downstream targets.

In addition to the White Collar proteins and VVD, a number of genes are present in the Neurospora genome that encode for proteins with similarities to light sensors, such as phytochromes (PHY-1, PHY-2), cryptochrome (CRY), and opsin (NOP-1) (Bieszke *et al.*, 1999a; Galagan *et al.*, 2003; Froehlich *et al.*, 2005, 2010). In addition to WC-1 and VVD, another PAS/LOV protein with similarities to plant phototropins (PHOT) has also been identified (Froehlich *et al.*, 2005). It seems possible then, that other light signalling pathways exist that enable Neurospora to sense a broader spectrum of light and that these pathways could affect its circadian system. However, deletion of these genes has only modest if any impact on the light and circadian phenotypes analyzed under standard conditions (Froehlich *et al.*, 2005, and Mark Elvin, Christian Heintzen, Ping Cheng, Yi Liu, unpublished results).

CRY, however, may have roles in blue-light signalling that were previously assigned to the WCC (Froehlich *et al.*, 2005, 2010; Olmedo *et al.*, 2010). Interestingly, the *cry* transcript and protein are both blue-light induced and the transcript shows circadian expression which is out of phase to that of *frq*. However, although these expression profiles suggest some involvement in blue-light and clock-controlled pathways, in strains where CRY is either absent or aberrantly expressed there are no apparent or only very subtle light signalling or clock defects (Froehlich *et al.*, 2010; Olmedo *et al.*, 2010). Furthermore, a complex stimulus–response relationship for *con-10*, a light- and clock-controlled gene that is up-regulated during asexual spore development in Neurospora, was suggested. The authors speculate that CRY-1, NOP-1, and PHY-2 may repress the activity of the WCC on the *con-10* promoter (Olmedo *et al.*, 2010). It is likely that the key roles of these putative photoreceptors will be revealed as the experimental protocols more closely mimic growth conditions encountered in the wild.

More recently, a detailed investigation into the expression of *nop-1* has shown that NOP-1 plays a role during late-stage conidiation (Bieszke *et al.*, 2007). Purified NOP-1 has previously been shown to bind retinal as a chromophore with maximum absorption in green light (Bieszke *et al.*, 1999b). It is therefore possible that NOP-1 acts as a green light photoreceptor that in cooperation with other sensors fine-tunes spore maturation in Neurospora (Bieszke *et al.*, 2007). Moreover, plant phytochromes have recently been implicated in the mediation of temperature responses (Heschel *et al.*, 2007) and work on the Neurospora photoreceptor VVD has revealed an unexpected function in temperature-regulated pathways that influence the phase of clock-controlled conidiation (Hunt *et al.*, 2007). Thus, it will be interesting to see if, in addition to VVD, some of the other Neurospora photoreceptors also play roles in temperature sensing.

5. Temperature responses

To date no receptors dedicated to the detection of temperature change and its transduction to Neurospora clock molecules have been identified. In poikilotherms (whose body temperatures vary with that of their surroundings) such as *Neurospora* the requirement for a temperature transduction pathway is not obvious. Temperature increases the rate of chemical reactions resulting in increased growth, thus the question is not whether the contents of the hyphae change in response to temperature but whether there is a need for a fast response system or a pathway that senses and responds to small changes in temperature. This is a pertinent point considering that the clock is known to entrain to cycles of high and low temperatures that differ by only 2 °C (Francis and Sargent, 1979). In addition to the photoreceptors mentioned above, the transient receptor protein family of ion channels that detect and respond to temperature change in homeotherms (organisms that are able to maintain a relatively constant body temperature independent of the surrounding environment) (Talavera *et al.*, 2008) are candidate thermoreceptors. Proteins with limited similarity are found in Neurospora but as yet their function is not known. Alternatively, direct effects of temperature on clock gene products or their synthesis, leading to alternative forms of RNA and protein and resulting in changes in protein abundance and activity might be all that is necessary.

While it is likely that temperature affects the levels and/or activity of most clock components, only its effects on *frq* and *vvd* gene products have so far been reported. In response to an increase in temperature there is a transient increase in *frq* RNA but, rather than altering transcription or RNA stability, temperature primarily acts post-transcriptionally to regulate levels and forms of FRQ protein. Two forms of FRQ protein, large FRQ (l-FRQ, 989 aa) and small FRQ (s-FRQ, 898 aa), have been identified and these arise from the use of two in-frame ATGs (Garceau *et al.*, 1997; Liu *et al.*, 1997). As the temperature increases the overall level of cycling FRQ protein is raised and the ratio of l-FRQ:s-FRQ increases (Fig. 4A–C). These changes are brought about due to temperature-regulated splicing of

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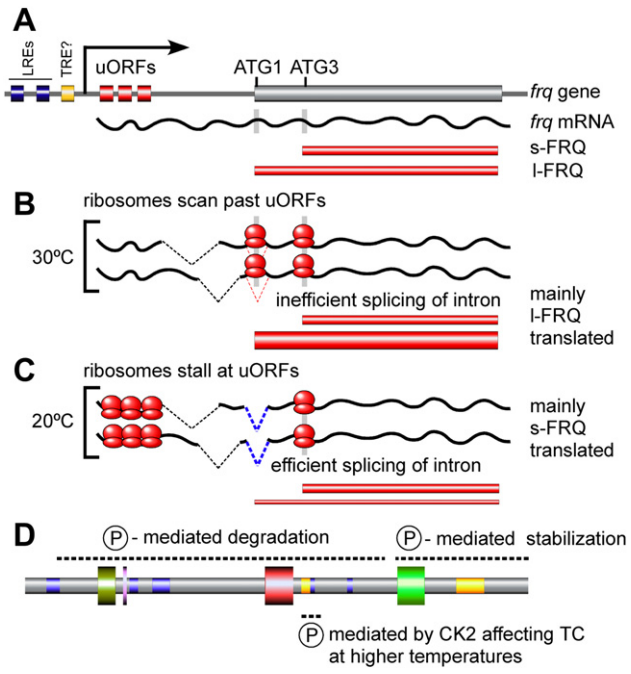

Fig. 4 – Temperature regulation of the *frq* locus. (A) Schematic of the *frq* locus and its products (not to scale). Key regulatory elements involved in *frq* transcription are shown. Two light-responsive elements (LREs) in the *frq* promoter are also shown (blue boxes). A putative temperature responsive element (TRE) is depicted in yellow, its location is speculative. uORF, short upstream open reading frames. ATG1 and ATG3 are used to produce a large (l-FRQ) and a small (s-FRQ) form of FRQ. (B) *frq* transcript is subject to multiple splicing events at the 5′ end. Only 3 splicing events are shown. Temperature-sensitive splicing surrounding ATG1 is depicted as a dotted red line. At 30 °C splicing of this intron is inefficient and ribosomes scan past the uORFs to initiate *frq* translation mainly from ATG1. Relatively more l-FRQ is produced. (C) At 20 °C the temperature-sensitive intron is efficiently spliced, removing the ATG1 from most *frq* transcripts. Ribosomes stall at the uORFs, leading to an overall reduction in *frq* translation that is now mainly initiated from the ATG3. Relatively more l-FRQ is produced. (D) Schematic drawing of the domain structure of FRQ. The dotted lines indicate regions within FRQ where phosphorylation either leads to FRQ degradation or stabilization. CK2-mediated phosphorylation events surrounding the N-terminal PEST sequence (yellow) are important for efficient degradation of FRQ at higher temperature and are implicated in temperature compensation. Blue bars, regions preferentially targeted for hyperphosphorylation. Dark green, coiled coil domain; lilac, nuclear localization signal; red, CKI-interaction domain; light green, FRQ–FRH interaction domain; yellow, PEST sequence.

frq transcript and the effects of upstream open reading frames (ORFs) on translation. At low temperatures non-consensus splice sites towards the 5′ end of the transcript are frequently used giving rise to *frq* mRNA containing only the ORF for s-FRQ. Moreover, at low temperatures, ribosomes scanning the RNA are more likely to initiate translation of the small upstream ORFs and less frequently reinitiate translation at s-FRQ or l-FRQ start sites (Colot *et al.*, 2005; Diernfellner *et al.*, 2005, 2007). As yet no dramatic difference in function of these two forms of FRQ has been shown. Both long and short forms of FRQ can support temperature compensated rhythms with indications that strains expressing only l-FRQ are slightly under compensated and those expressing only s-FRQ forms are slightly over compensated (Diernfellner *et al.*, 2007). Perhaps in natural conditions of light and temperature or when Neurospora is exposed to different carbon sources the relevance of the two forms of FRQ will become apparent.

As with light, temperature pulses and steps can reset the time on the clock, and the degree of resetting depends on the magnitude of the temperature change and the time at which the change takes place (Francis and Sargent, 1979). A 10 °C step up in temperature lasting 6 h has the same effect on the clock as a brief light pulse. Administered late in the day a phase delay is induced, administered late at night phase advances occur, and during the day there is little or no response (Francis and Sargent, 1979; Dharmananda, 1980; Hunt *et al.*, 2007). On transfer from low to high temperatures the clock is reset to CT0 while after a drop in temperature (40 °C to 30 °C) conidiation occurs 10 h later indicating that the change sets the clock to CT12 (Gooch *et al.*, 1994).

Because FRQ oscillates at a higher level at high temperatures *versus* low temperatures, a step up in temperature results in FRQ levels that are indicative of subjective dawn at the high temperature, whilst a step down results in high FRQ levels similar to that present at subjective dusk at the low temperature (Liu *et al.*, 1997). However, though changing levels and activity of FRQ are clearly important, adjustments in the abundance and activity of other clock components and the phosphatases and kinases that regulate their phosphorylation state must act in concert to achieve the final change in clock time. Nevertheless, although adjustments in clock protein level and activity are achieved in different ways by temperature and light, the outcome of an increase in temperature and a light pulse is the same, and in a temperature cycle the high temperature is interpreted as day and the low temperature as night. Thus light and temperature cues reinforce each other (Dunlap and Loros, 2004).

6. Temperature compensation

Neurospora is able to survive −80 °C for prolonged periods, grows slowly at 4 °C, and on a good carbon source at 30 °C can achieve hyphal extension rates of 2 mm/h. As expected, with every 10 °C rise in temperature the rate of growth doubles (Sargent *et al.*, 1966) but importantly, over a range of physiological temperatures (18–32 °C) the period of conidiation is unchanged indicating that the circadian clock is temperature compensated. Above and below this range the rhythm in conidiation is lost but the state of the clock can be followed by assaying light emitted from a strain containing the firefly luciferase gene driven by the *frq* promoter (Gooch *et al.*, 2008). At 36 °C the rhythm has a 15-h period indicating loss of temperature compensation. Whether the clock functions below 18 °C is not reported.

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Since characterization of the first rhythm mutants it has been known that long period *frq* mutants have altered temperature compensation properties (Gardner and Feldman, 1980). Other period mutants with altered temperature compensation include: *chrono* (*chr*), *period-1* (*prd-1*), *prd-3*, *prd-4*, *prd-6* and *wc-2* (Gardner and Feldman, 1980; Collett *et al*., 2001). When the *chrono* and *prd-3* genes were cloned they were found to encode the βI and α subunits of CK2, a kinase that, depending on the temperature, phosphorylates different sites on FRQ. Importantly, when these sites are mutated temperature compensation is affected (Mehra *et al*., 2009). In a CK2 mutant FRQ is more stable than in the WT and its stability increases further as the temperature is raised, indicating that in the WT phosphorylation of FRQ by CK2 is required for its destabilisation (Mehra *et al*., 2009). Detailed examination of sites in FRQ phosphorylated over the course of a circadian day revealed that phosphorylation of one site promotes phosphorylation of neighbouring sites (Baker *et al*., 2009; Tang *et al*., 2009) (Fig. 4D). Mutation of some of these sites had no apparent effect on FRQ stability or period length, however phosphorylation of certain sites towards the C-terminus increased FRQ stability, whilst phosphorylation of a region towards the N-terminus had clear destabilizing effects (Tang *et al*., 2009). Whether these sites become accessible to the kinase *via* temperature-induced changes in the conformation of FRQ, or are phosphorylated owing to temperature-induced changes in the binding affinity of CK2 is not known (Mehra *et al*., 2009). Although studies to date concentrate on the effect of temperature on FRQ, whether FRQ is the primary target of temperature control or several clock components respond in parallel to adjust the clock to temperature change is an open question. The identity of *prd-1* and *prd-6* is unknown but their cloning will perhaps throw further light on this interesting property of the circadian clock.

7. Future perspective

In addition to the availability of the Neurospora genome sequence (Galagan *et al*., 2003) and its annotation, a number of tools are now available (Dunlap *et al*., 2007; Gooch *et al*., 2008; Honda and Selker, 2009) that will aid the identification of circadian gene expression networks in unprecedented detail. Eventually these data will further inform and refine computer models, allow us to evaluate the importance of each reaction within the circadian system, and help us to identify non-intuitive features of the network. Although many components of the Neurospora circadian system are already known and have been studied in some detail, the properties of these components that result in oscillations of hours rather than minutes and that are temperature compensated have not been fully resolved. It can be anticipated that cell biology and structural biology approaches will become increasingly important for a full understanding of circadian rhythm generation. Genetic approaches will continue to play a significant role in unravelling clock-controlled pathways and elucidating gene function including perhaps the identification of further non-protein coding RNAs that are required for a functional clock.

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