



Circadian rhythms in *Neurospora crassa*: Dynamics of the clock component frequency visualized using a fluorescent reporter

Ernestina Castro-Longoria^{a,*}, Michael Ferry^{b,1}, Salomón Bartnicki-Garcia^a, Jeff Hasty^b, Stuart Brody^c

^a Department of Microbiology, CICESE, Ensenada, B.C., Mexico

^b Department of Bioengineering, University of California, La Jolla, CA 92093-0116, USA

^c Division of Biological Sciences, University of California, La Jolla, CA 92093-0116, USA

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ABSTRACT

The frequency (*frq*) gene of *Neurospora crassa* has long been considered essential to the function of this organism's circadian rhythm. Increasingly, deciphering the coupling of core oscillator genes such as *frq* to the output pathways of the circadian rhythm has become a major focus of circadian research. To address this coupling it is critical to have a reporter of circadian activity that can deliver high resolution spatial and temporal information about the dynamics of core oscillatory proteins such as FRQ. However, due to the difficulty of studying the expression of circadian rhythm genes in aerobic *N. crassa* cultures, little is known about the dynamics of this gene under physiologically realistic conditions. To address these issues we report a fluorescent fusion to the *frq* gene using a codon optimized version of the mCherry gene. To trace the expression and accumulation of FRQ–mCherryNC (FRQ–mCh) during the circadian rhythm, growing vegetative hyphae were scanned every hour under confocal microscopy (100×). Fluorescence of FRQ–mCh was detected only at the growing edge of the colony, and located in the cytoplasm and nuclei of vegetative hyphae for a distance of approximately 150–200 μm from the apices of leading hyphae. When driven by the *frq* promoter, apparently there was also a second FRQ entrance into the nucleus during the circadian cycle; however the second entrance had a lower accumulation level than the first entrance. Thus this fluorescent fusion protein has proven useful in tracking the spatial dynamics of the *frq* protein and has indicated that the dynamics of the FRQ protein's nuclear trafficking may be more complex than previously realized.

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1. Introduction

Circadian rhythms are a widely-occurring part of the biological landscape, particularly for eukaryotic organisms. The generally agreed upon features of a circadian rhythm are: (1) a period of ~24 h (circa-dian); (2) an endogenous or self-sustaining rhythm when all external stimuli, such as light/dark cycles, are removed; (3) entrainability by external signals such as a daylength of 24 h; and (4) a period that remains relatively constant with respect to ambient temperature, particularly in those organisms that cannot regulate their internal temperature. The occurrence of circadian rhythms has been documented from micro-organisms both prokaryotic (Dunlap et al., 2004) and eukaryotic all the way up the taxonomic scale to humans (Aschoff, 1965). The occurrence of

circadian rhythms in fungi has been known for some time (Sargent et al., 1966; Bell-Pedersen et al., 1996a; Loros and Dunlap, 2001; Lakin-Thomas and Brody, 2004; Dunlap and Loros, 2006).

For circadian rhythms, the most widely-studied fungus has been *Neurospora crassa*, primarily because of its well-known genetics and biochemistry. Significant advances in understanding the mechanism of a core oscillator as well as its input and output components have been extensively documented (Aronson et al., 1994a; Lee et al., 2000; Liu et al., 2000). The *Neurospora* clock is expressed on solid agar media as a series of conidiating rings (asexual spore formation) designated as “bands” alternating with areas of thin filamentous growth. The macroscopic effect of this binary developmental lifestyle is to produce on agar media, either in long linear glass tubes (race tubes) or in Petri plates, bands that develop ~22 h apart. Moreover, since periods can be measured for many days, a large number of cultures can be simultaneously monitored and manipulated. The *Neurospora* clock system has all of the properties of the circadian rhythm definition listed above and is one of the handful of organisms where a reasonable amount of molecular understanding is known (Bell-Pedersen et al., 2001a,b; Loros and

Abbreviations: FRQ, frequency; RFP, red fluorescent protein; FRQ–mCh, FRQ–mCherryNC; FI2, future interband 2; FB2, future band 2; CT, circadian time.

* Corresponding author. Fax: +52 646 175 05 95x27052.

E-mail address: ecastro@cicese.mx (E. Castro-Longoria).

¹ These authors contributed equally to this work.

Dunlap, 2001; Dunlap, 2006). All of the formal aspects of the *Neurospora* clock have been worked out from cultures growing on the surface of an agar medium, either in race tubes or plates, and most of the details of the molecular biology of the *Neurospora* clock system have been obtained from a mycelial disk system in a shaking liquid culture (Nakashima, 1981). Despite the differences in culture conditions in liquid and solid cultures, the clock keeps the same time in both instances (Perlman et al., 1981). However, since growth is often limited in liquid cultures, the traditional *Neurospora* clock output phenotype (conidiation) is suppressed by the submerged growth environment. While developmental pathways are suppressed intentionally to examine only the circadian cycling of clock components, the conditions of the disk are unlike any fungi normally encounter. Consequently this has led some to question whether the data gained through such experiments can be directly applied to those when *Neurospora* is cultured on agar media. Furthermore, although macroscopic development is suppressed, it is interesting that many clock-controlled genes are still expressed in the disk system, including those involved in the formation of conidia (*ccg-2*). Thus although the final morphological output of the clock, conidiating regions of a culture, is not seen in the disk culture system, many of the downstream processes still are.

Increasingly it will be important to address what differences, if any, occur in the *Neurospora* clock between the surface and submerged systems if the clock regulation of the output processes, such as conidiation, are to be understood. To gain insight into the dynamics of the *Neurospora* clock gene components, newer online reporter tools (Morgan et al., 2003; Gooch et al., 2008) have been developed allowing the assessment of some clock components while the culture is growing on the surface of an agar media. Other studies on the *Neurospora* clock (Brody and Harris, 1973; Ramsdale and Lakin-Thomas, 2000) have involved extracting and analyzing components directly from surface-grown cultures. However, although the output of the circadian rhythm can be followed at a macroscopic level using clock-controlled production of luciferase, for example, no previous tool has been able to follow the dynamics of a *N. crassa* clock gene at the cellular level. Consequently our fusion protein has allowed us to determine the cellular localization of the FRQ protein in aerobic conditions with higher temporal resolution than previous techniques could achieve.

The system we have developed is based on a fluorescent protein fused in frame to the core oscillatory protein Frequency (FRQ). FRQ was chosen due to its critical importance in generating the *Neurospora* clock's rhythms since deletion of the *frq* gene abolishes all circadian rhythms in *Neurospora* (Aronson et al., 1994a,b). Moreover certain mutations in the *frq* gene retain expression but can alter the period of the rhythm from as low as 16 h to as much as 29 h (Feldman and Hoyle, 1973). Due to its importance, the temporal profile of the expression of FRQ has been extensively studied in the liquid disk system. However the spatial and temporal distribution of FRQ in an actively-growing, aerobic *Neurospora* culture has been difficult to determine due to the previously mentioned problems. The fluorescent fusion system we describe here is an attempt to overcome these limitations and is useful for determining *frq* gene dynamics both spatially and temporally. Advantages of this system include reporter output for more than 2 days of an actively-growing culture, allowing correlations with the visible conidiation rhythm. The system is an aerobic one, has high sensitivity, and can be sampled with high temporal resolution. Furthermore the response of the clock to phase shifting stimuli or other environmental changes can be rapidly observed.

The approach employed for the fluorescent labeling of clock proteins was a multi-faceted one. The first step was to use a fluorescent protein, mCherry, that emits in the red wavelengths of the visible spectrum and whose excitation wavelength is far away from the blue light range, which would have reset the *Neurospora*

clock (Liu et al., 2003). Unfortunately, the more commonly used GFP (green fluorescent protein) variants have this as a built-in limitation. The second step was to optimize the codons of mCherry for expression in *Neurospora* since there have been numerous reports about poor translation efficiencies for foreign genes in this organism (Kinnaird et al., 1991; Gustafsson et al., 2004). The third step was to drive the mCherry protein with various promoters in order to assess the time course, strength, and localization of the signal. The fourth step was to localize the FRQ protein and observe its kinetics by fusing the mCherry reporter to the C-terminal end of the FRQ protein (we call this fusion FRQ-mCh). The fifth step was to put FRQ-mCh under the control of different promoters, i.e. *ccg-2* or *frq*. The *ccg-2* promoter was chosen since it is one of the strongest promoters of *N. crassa* and would serve as a useful control to ensure that FRQ-mCh was detectable. However an argument could be made that data obtained with the *ccg-2* promoter driving FRQ-mCh is complicated to decipher since there may be feedback from a clock protein being regulated by a clock promoter. Therefore our final step was to fuse the mCherry gene to the native *frq* gene using a novel technique based on the “tk-blaster” method (Pratt and Aramayo, 2002). This technique allowed us to study the dynamics of the *frq* gene in its native genomic context with no disruption to its promoter, 5' or 3' UTR. Consequently our results with this strain removed any doubt about the functionality of our fusion protein since the circadian rhythm had a period near that of the wild type.

At the cellular level, our objective was to analyze the dynamics and spatial localization of the FRQ-mCh fusion protein to determine possible novel organelle localization, localization in tips vs. older areas, and if these processes were under circadian control. We were particularly interested in this promoter's spatial specificity (i.e. aerial hyphae vs. mycelia) and when it was activated during the circadian cycle and the conidiation process. Furthermore we have described previously (Castro-Longoria et al., 2007) some of the intracellular details of the *Neurospora* conidiation rhythm and these fluorescent strains gave us a new dimension to employ for this type of study. Using these tools we have discovered that the FRQ protein makes a second, previously-unknown entry into the nucleus during the circadian cycle. This entry appears to be related to the conidiation process due to its timing and thus is evidence of the utility of our system for studying the *Neurospora* clock in an aerobic environment.

2. Materials and methods

2.1. Strain and culture conditions

Neurospora crassa strains used in this study are listed in Table 1. Unless otherwise stated, *N. crassa* strains were cultured and maintained in Vogel's minimal medium N (VMM) (Vogel, 1956) with 2% sucrose as a carbon source and 1.5% agar. Strains MFNC23 and MFNC30 were grown in Petri plates at 28 °C to obtain a higher expression of fluorescence. To determine the fluorescence signal from whole Petri plate cultures, the plates were illuminated using a halogen spot light bulb and a band pass excitation filter (580 nm ± 20 nm). The fluorescent emissions signal was captured using a standard digital camera (Canon A510) with an emission filter (610 nm ± 10 nm) placed in front of the lens. Hyphal extension rate and period of strains were calculated from cultures on race tubes. Cultures in special chambers were grown at 25 °C as previously described (Castro-Longoria et al., 2007). All cultures were incubated in a VWR® Diurnal Growth Incubator, Model 2015. Cloning steps necessary to produce the Pccg2-mCherry, Pccg2-FRQ-mCherry and Pfrq-FRQ-mCherry constructs are given in the supplementary methods. Moreover complete lists of the strains and

Table 1
Period length and hyphal extension rate of the *N. crassa* strains used in this study.

Strain	Promoter	Protein	Period (h) (n)	Growth rate (mm/h)
<i>bd</i> (control)	<i>frq</i>	FRQ	22.4 ± 3.2 (41)	1.2
<i>bd</i> (MFNC9)	<i>ccg-2</i>	mCherryNC	21.1 ± 2.0 (17)	1.0
<i>bd</i> (MFNC23)	<i>ccg-2</i>	FRQ–FLEX–mCherryNC	16.4 ± 1.8 (26)	0.9
<i>bd</i> (MFNC22)	<i>frq</i>	FRQ–FLEX–mCherryNC (ectopic integration)	19.6 ± 2.1 (21)	1.0
<i>bd</i> (MFNC30)	<i>frq</i>	FRQ–FLEX–mCherryNC	24.5 ± 2.0 (18)	1.1

plasmids produced in this study are given in [Supplementary tables S3 and S4](#) respectively.

2.2. Insertion of the mCherryNC gene at the native *frq* locus using a modified “tk-blaster” technique

Since the regulation of the FRQ gene is complex, containing regulatory elements both 5' and 3' of the actual FRQ coding sequence (Crosthwaite, 2004; Colot et al., 2005), we decided to fuse mCherryNC to the FRQ gene in its native genomic locus. We reasoned this fusion gene might capture the intricate regulation of FRQ more completely than an ectopic FRQ construct like that described in the supplementary methods section. Moreover our strategy allowed for the selectable marker gene's complete removal, resulting in a minimum of DNA being added to the genome (essentially being only a linker and mCherry sequences). Our strategy was similar to the “tk-blaster” strategy described in Pratt and Aramayo (2002), which itself is based on the URA-blaster strategy of *Saccharomyces* (Alani et al., 1987). The “tk-blaster” strategy is essentially a technique to knockout a gene without a permanent marker being left behind. This enables the recycling of the marker gene in subsequent knockout experiments and is therefore useful in building strains with multiple gene knockouts. We have extended this technique beyond knockouts by modifying it to fuse fluorescent protein genes to the genomic copy of a gene of interest. Unlike a knockout, this strategy retains the function of the native gene and allows the fusion gene's regulation to be studied in its native context with a minimal amount of inserted DNA. Importantly this removes any ambiguity about regulation being altered by an ectopic genome location.

To accomplish this fusion, our method, like the “tk-blaster” technique, makes use of the hygromycin–thymidine kinase fusion gene from the plasmid pHyTK (Lupton et al., 1991). This construct confers resistance to hygromycin B and sensitivity to 5-Fluoro-2'-deoxyuridine (FUDR) and thus enables dominant positive and negative selection of transformants (Pratt and Aramayo, 2002). In the “tk-blaster” technique a sequence is placed flanking the marker sequence on both the 5' and 3' ends, creating a direct repeat. This construct is then targeted to the genomic region of interest, selecting for hygromycin resistance. In the next step, marker removal is selected by plating cells on media containing FUDR. This removal process occurs due to the high frequency of mitotic recombination between direct repeats in the genome of *Neurospora* and other organisms (Pratt and Aramayo, 2002). In the “tk-blaster” strategy (Pratt and Aramayo, 2002), one of the repeat sequences remains as essentially unwanted DNA after the marker's removal (Pratt and Aramayo, 2002) this sequence was known as a “lambda scar”. In our strategy we have made the mCherry fusion sequence itself the repeat, hence when the marker gene is removed all that remains is the desired fusion sequence and no extraneous DNA is leftover. Importantly, the minimal disturbance to the native gene is made: the end result is an insertion of the fluorescent protein gene *only* between the second to last codon of the gene and the stop codon; no other part of the gene, including the promoter, 5' or 3' UTR, is altered. In our case with the *frq* gene, we ended up inserting only 711 base pairs into the *N. crassa* genome at the

end of *frq*. An overview of our strategy is shown in [Fig. 1](#) and a detailed overview of the cloning steps taken to produce the required construct is given in the supplementary methods.

These cloning steps eventually yielded plasmid pMFP18 containing appropriate targeting vector with repeating copies of mCherryNC flanking the HyTK marker. To transform *Neurospora* the pMFP18 vector was linearized with the XmnI restriction enzyme and used to transform *Neurospora* strain FGSC #9718 (Δ mus-51 bar a+) by electroporation. The resulting *Neurospora* colonies were transferred to 10 × 75 mm test tubes and screened for the presence of the pMFP18 derived construct by PCR. Conidia from positive isolates were streaked onto plates containing FUDR to select those that had undergone recombination between the mCherry repeats. Colonies were transferred to 10 × 75 mm test tubes and screened for the recombination event by PCR. The PCR products from isolates positive for the desired recombination product were then cloned into the pCR-Blunt vector (Invitrogen) and sequenced. The sequencing reaction indicated the desired recombination had occurred perfectly in at least some of the isolates. We named one of these isolates MFNC4 (FRQ–FLEX–mCherry, Δ mus-51 bar a+) and crossed it to the *bd* strain (*bd* A SG#2105) to remove the Δ mus-51 bar marker, add the *bd* allele, and generate a homokaryon. Please note that FLEX refers to the linker used to fuse FRQ to mCherry and is described in the supplementary methods. The cross yielded 72 isolates, which were screened for basta resistance (conferred by the Δ mus-51 bar marker), the *bd* allele and the presence of the FRQ–mCh fusion gene. One isolate was chosen from this cross and named MFNC29 (*bd*, FRQ–mCh, Δ mus-51 bar). This strain was then back crossed to *bd* A SG#2105 to generate MFNC30 (*bd* FRQ–FLEX–mCherryNC).

2.3. Fluorescence microscopy

Expression of fluorescence (strain MFNC9) was monitored from live cultures grown in special chambers (Castro-Longoria et al., 2007) under fluorescence microscopy (Carl Zeiss Axiovert 200 M inverted microscope) equipped with a Texas Red filter and in complete darkness. Observations of vegetative hyphae were done each hour at low magnification (using a 10× objective) once the colony had grown and formed the first conidiation band. Developing colonies were scanned and photographed from the growing edge inwards. Micrographs from cultures were assembled to obtain the whole region at which the mCherryNC fluorescence was expressed during the circadian cycle.

2.4. Confocal live-cell imaging

Vegetative hyphae at the edge of growing colonies of strains MFNC9, MFNC23 and MFNC30 were scanned every hour under confocal microscopy. Living hyphae were imaged at 25 °C (strain MFNC9) and 28 °C (strains MFNC23 and MFNC30) on VMM using an inverted Zeiss Laser Scanning Confocal Microscope LSM-510 META (Carl Zeiss, Göttingen, Germany). Cultures were scanned during subjective day and subjective night using the inverted agar block method (Hickey et al., 2005). Every hour an independent culture was scanned and at least three complete sets of observations were done

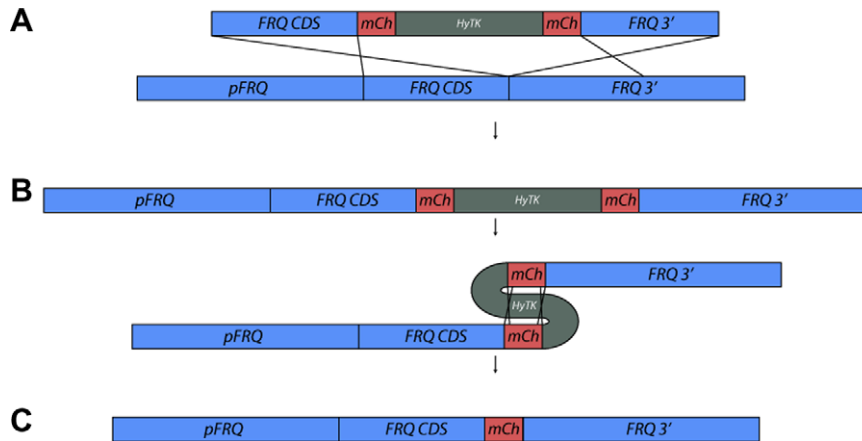


Fig. 1. Diagram depicting the modified “tk-blaster” technique. (A) The construct containing the HyTK marker gene flanked by two repeating sequences (in this case the mCh gene) is targeted to the genomic FRQ locus of *Neurospora*. Integration of this construct creates the FRQ–mCh fusion gene followed by the HyTK resistance gene and another copy of the mCh gene. (B) Mitotic recombination between the two mCh repeats is selected for by plating conidia on FUDR containing media. Only those cells which have removed the HyTK gene from their genome will be able to survive in this media. (C) The final result is an insertion of only the mCh gene following the FRQ gene. No other DNA, such as a marker is inserted. Moreover the promoter, 5' UTR and 3' UTR's remain intact.

for each strain. Also, additional observations were done at times at which the peak of fluorescence was detected in order to corroborate results. An oil immersion objective 100 \times (PH3) 1.3 N.A. plan neofluar was used. While scanning vegetative hyphae, the pinhole was set to 2.3 airy units. A photomultiplier module allowed us to combine fluorescence with phase-contrast channels to provide a simultaneous view of the fluorescently-labeled organelles and the entire cell. Confocal images were captured using LSM-510 software (version 3.2; Carl Zeiss) and evaluated with an LSM-510 Image Examiner (version 3.2). Some of the image series were converted into animation movies using the same software. Fluorescence intensity was measured with Image Pro Plus[®] and the obtained measurements were exported into Microsoft Excel spreadsheets and analyzed.

3. Results

3.1. Expression of the RFP mCherryNC under the control of the *cgg-2* promoter in *N. crassa*

All transformed *cgg-2*-mCherry strains (MFNC5 to MFNC9) were scanned under fluorescence microscopy and strain MFNC9 (*bd*, His3::Pccg2-mCherryNC-tTrpC) was chosen to continue further observations. The strain was first grown in standard Petri plates to monitor the circadian banding pattern (Fig. 2A) and then examined using a fluorescence lamp optimized for viewing the fluorescence signal from a Petri plate (see Section 2.1). We observed strong production of mCherry in bands of conidiation (Fig. 2B), while any accumulation of mCherry in the vegetative hyphae (inter-band region) was too low to detect using this setup. To further examine fluorescence at the microscopic level, fully-grown cultures in special chambers (Castro-Longoria et al., 2007) were scanned using confocal microscopy. Using this technique we observed a strong accumulation of mCherry in the conidiation bands as had been previously seen in culture plates (Fig. 2B). For example in Fig. 2C–E a conidiation band section examined using epifluorescence microscopy illustrates the strong fluorescence found in conidia.

To trace the dynamics of Pccg2–mCherryNC in growing MFNC9 colonies and to assess circadian control of the construct, we scanned cultures every hour at low magnification using fluorescence microscopy after an initial 11-h period of continuous darkness. All observations were carried out in vegetative hyphae growing on an agar surface. Furthermore, to examine Pccg2–mCherryNC's circadian regulation (if any) we adjusted all time units to circadian time

(CT). Circadian time is a formalism used under constant conditions to normalize the rhythm lengths among organisms with different endogenous periods to 24 circadian hours per cycle. This adjustment allows for easier comparisons among different organisms clocks. By convention CT0 corresponds to subjective dawn and CT12 to subjective dusk.

Near subjective dawn (CT1–3) the hyphal fluorescence at the growing edge of the MFNC9 colony was low compared with fluorescence detected in conidia from the previous band (B1) (Fig. 3A). A rapid increase in fluorescence was detected from CT4 to CT7 (Fig. 3B and 3 lower panel) and after CT8 fluorescence gradually decreased. After CT11 fluorescence in vegetative hyphae remained at very low, but detectable levels for the following 11 h (Fig. 3C–D). During this time nascent aerial hyphae were monitored in the developing future band (FB2) and a very strong fluorescence signal was observed in aerial hyphae and conidia (Fig. 3E).

In addition to epifluorescence microscopy, we scanned growing MFNC9 colonies at higher magnification (100 \times) using confocal microscopy yielding similar results to those previously obtained. The mCherryNC protein was detected in vegetative hyphae at the growing edge of the colony and at CT1 fluorescence was low (Fig. 3, lower panel and Supplementary movie 1). The level of expression increased gradually and reached a maximum of intensity at CT4 (Fig. 3, lower panel, Fig. 4 and Supplementary movie 2). The intensity remained high for the following 5 h and then decreased rapidly during the next 3 h. During the following 11 h, the expression of mCherryNC in vegetative peripheral hyphae was very low. Furthermore the Pccg2 promoter displayed an impressively high dynamic range during the course of the circadian cycle, reaching a maximum expression level at CT3–6 of at least 240 times that during CT11–CT22 (Fig. 4). Moreover when the low state cultures (those between CT11 and CT22) were exposed to continuous light, we detected a very high production of mCherryNC after 1 h (data not shown).

3.2. Kinetics of the protein FRQ under the control of the *cgg-2* promoter in *N. crassa*

The use of the mCherryNC reporter proved to be a powerful tool for observing the Pccg2 promoter's dynamics at the cellular level. Moreover this strain served as a control, ensuring that the mCherryNC protein was detectable in *Neurospora*. Our next step was to expand upon this initial strain by having the Pccg2 promoter drive a FRQ–mCh fusion protein instead of the mCherryNC protein alone.

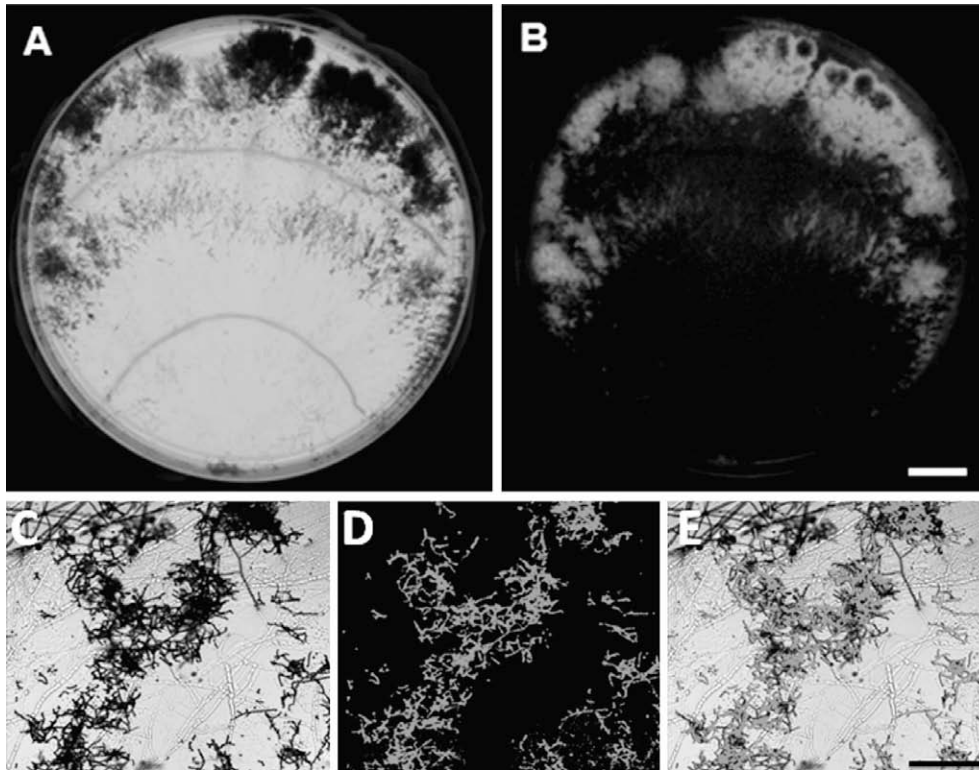


Fig. 2. Phenotype of strain MFNC9 (*Pccg2*-mCherryNC) of *N. crassa*. (A) Banding pattern on a culture plate viewed under transmitted light. (B) View of the same culture under a fluorescent lamp (see Section 2 for excitation and emission wavelengths); note the strong expression of mCherryNC on bands of conidiation. (C–E) Close up of a conidiation band from a fully-grown *Neurospora* strain MFNC9 colony grown in a special chamber and scanned under confocal microscopy. (C) Transmitted light channel. (D) mCherryNC fluorescence. (E) Merged images. (A–B) Scale bar = 2 cm. (C–E) Scale bar = 200 μ m.

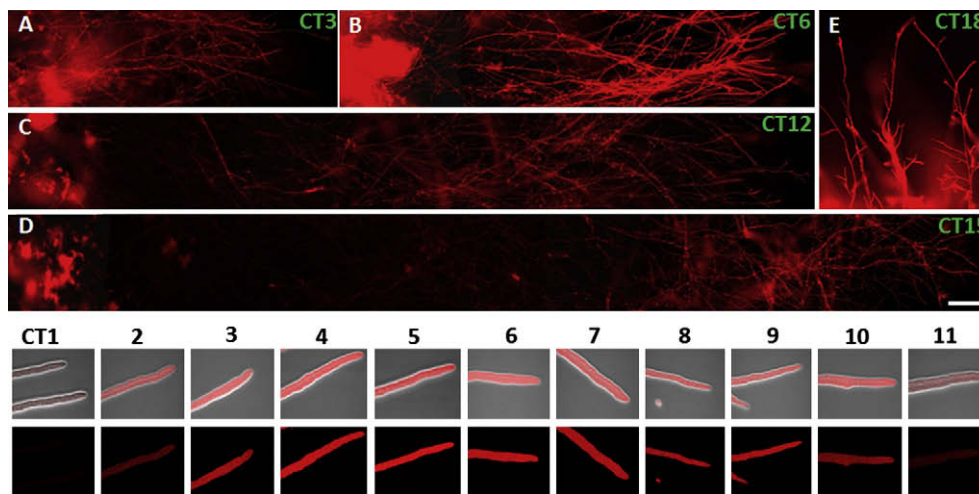


Fig. 3. Expression of mCherryNC driven by the *cgg-2* promoter during colony development of *N. crassa*, strain MFNC9. (A–E) Colony examined under fluorescence microscopy (10 \times) after the formation of the first band. (A–D) Vegetative hyphae. (E) Aerial hyphae. Lower panel: rhythmic expression of mCherryNC fluorescence in peripheral vegetative hyphae from CT1 to CT11 examined under confocal microscopy (100 \times). Upper row of images are the phase contrast and fluorescence channels merged; lower row, fluorescence channel. Scale bar upper panel 1 mm.

We then used this reporter to determine the spatial and temporal distribution of FRQ in living hyphae using the transformant strain MFNC23 (*bd*, *His3:Pccg2-FRQ-mCh*). This strain was monitored using confocal microscopy for an entire circadian cycle to follow the expression and intracellular localization of FRQ-mCh driven by the *cgg-2* promoter. The conidiation (banding) pattern of strain MFNC23 was clear with a period length of 16.4 h and hyphal extension rate of 1.0 mm/h.

Fluorescence was detected only in the colony's growth front, for a distance of approximately 150–300 μ m from the apices of leading

hyphae; beyond that distance only a diffuse signal was detected. Accordingly all observations were carried out in individual hyphae situated at the growth front's edge of the colony. Expression of FRQ-mCh was generally very low during most of the circadian cycle and some hyphae in the same culture did not demonstrate a detectable fluorescence signal. However, using only hyphae expressing detectable levels of fluorescence, a consistent pattern of FRQ-mCh localization was observed.

The nuclear entrance of FRQ-mCh was clear (Fig. 5) and during the first hours of subjective day (CT0–CT7) FRQ-mCh was localized

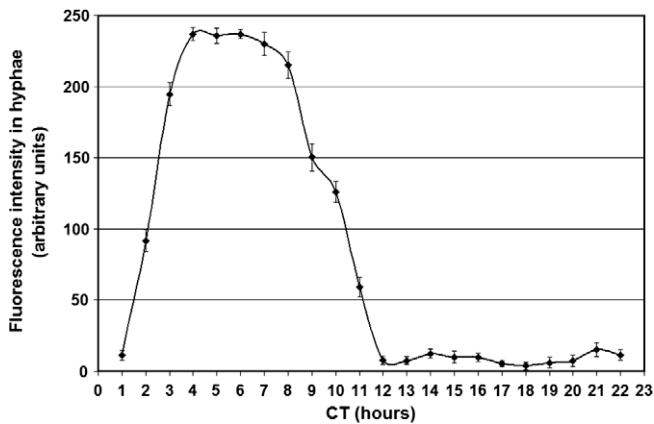


Fig. 4. Profile of fluorescence intensity of the protein mCherryNC driven by the *cgg-2* promoter during the circadian cycle of *N. crassa*, strain MFNC9. Only individual vegetative hyphae at the edge of the colony were considered. Mean and standard deviation were calculated from measurements on the subapical area of three different hyphae.

inside the nucleus at very low levels (Fig. 5A and Supplementary movie 3). A gradual increase of fluorescence inside the nucleus was detected from CT8 (Fig. 5B) until a strong accumulation was observed at CT11 (Fig. 5C and Supplementary movie 4). After the period of strong accumulation, levels of FRQ–mCh decreased and stayed at a low level until another nuclear entry cycle occurred, from CT18 to CT21, peaking at CT19. Both of these fluorescence peaks exhibited comparable signal levels. Moreover this nuclear entrance of FRQ–mCh was observed only in nuclei localized subapically; no entry was detected past 150–200 μm from the apices of leading hyphae.

3.3. Kinetics of the protein FRQ under the control of the *frq* promoter in *N. crassa*

Strain MFNC30 (*bd*, *bas*^S, FRQ–mCh) contains the mCherryNC gene inserted into the end of the native *frq* gene, enabling the

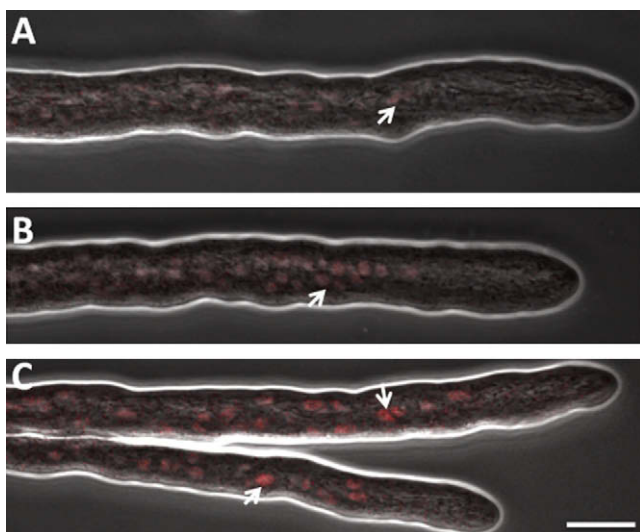


Fig. 5. Nuclear entrance of FRQ–mCh under the *cgg-2* promoter in vegetative hyphae of *N. crassa*, strain MFNC23. (A) A low accumulation of FRQ–mCh in nuclei was detected during early subjective day and night, image taken at CT2. (B) Gradual accumulation of FRQ–mCh in nuclei, image taken at CT9. (C) Maximum of fluorescence detected in nuclei at CT11 and CT19, image taken at CT11. White arrows point to selected nuclei. Images are the phase contrast and fluorescence channels merged. Scale bar = 10 μm .

production of the FRQ–mCh fusion by the native *frq* promoter, at the native *frq* locus. Confocal microscopy was again used to follow the production and localization of FRQ–mCh during the circadian cycle. The strain's growth was also monitored in race tubes and the circadian conidiation pattern was even clearer than that seen in MFNC23 with a period length of 24.5 h and hyphal extension rate of 1.06 mm/h.

Fluorescence in the colony was detected only in peripheral hyphae, as in strain MFNC23, therefore only individual leading hyphae in the growing region of the colony were examined. Very low accumulation of FRQ–mCh was detected inside the nucleus at CT1, followed by a period of rapid accumulation, which resulted in a high signal at CT4 (Fig. 6A and Supplementary movie 5). Following the strong accumulation of FRQ–mCh in nuclei, a gradual decrease in fluorescence intensity inside the nucleus was observed. At CT10 some fluorescence was also present outside the nucleus (Fig. 6B) in the subapical region. A strong fluorescence outside the nuclei was more evident after CT10 with almost undetectable levels inside the nucleus (Fig. 6C and Supplementary movie 6). Behind the subapical region (where FRQ–mCh was localized to the nucleus) an accumulation of fluorescence in the cytoplasm was present (as in Fig. 6C and Supplementary movie 7) and beyond this area the fluorescence signal was very low and diffuse (Supplementary movie 8). Prior to being released into D/D, the fluorescence signal in L/L was relatively strong but was localized completely to the cytoplasm.

After the first nuclear entry, apparently a second entrance of FRQ into the nucleus was detected at CT19 as a low accumulation of nuclear fluorescence, compared with the levels found at CT4–5. This second entrance of FRQ to the nucleus coincided with the higher expression of cytoplasmic FRQ localized near the subapical region of the cell. Also some hyphae did not have much cytoplasmic FRQ in the subapical area and the fluorescence inside the nucleus was clearly observed (Fig. 7A–C). Although the fluorescence of FRQ–mCh detected inside the nucleus at CT19 was low, this entrance to the nucleus was also detected by measuring the

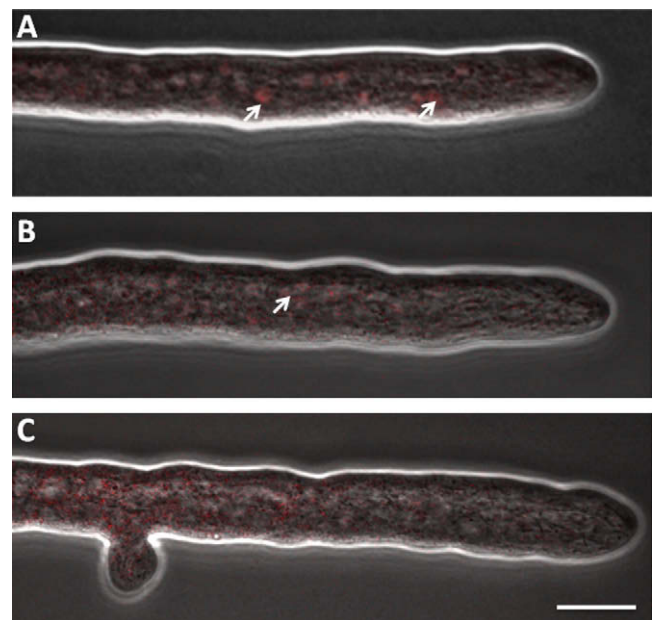


Fig. 6. Nuclear entry of FRQ–mCh expressed under the *frq* promoter in vegetative hyphae of *N. crassa*, strain MFNC30. (A) Peak of fluorescence detected in nuclei at CT4. (B) FRQ–mCh detected inside and outside the nucleus, image taken at CT10. (C) Accumulation of FRQ–mCh in cytoplasm at the subapical region of the cell, image taken at CT18. White arrows point to selected nuclei. Images are the phase contrast and fluorescence channels merged. Scale bar = 10 μm .

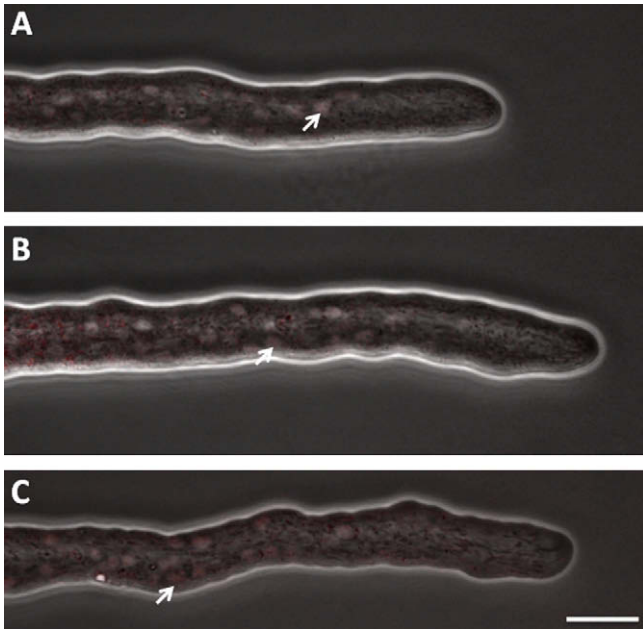


Fig. 7. Accumulation of FRQ-mCh in cytoplasm and nuclei of *N. crassa*, strain MFNC30. (A–C) Second entrance of FRQ-mCh in the nucleus, images taken at CT19. White arrows point to selected nuclei. Images are the phase contrast and fluorescence channels merged. Scale bar = 10 μm .

fluorescence intensity in individual nuclei (Fig. 8). The second accumulation of FRQ-mCh can be seen as a smaller peak of fluorescence intensity at CT19 (Fig. 8).

To ensure that FRQ-mCh localization was nuclear, we were able to obtain an image of two growing hyphae in which one of them had vacuoles showing no fluorescence. Furthermore both hyphae possess several nuclei with a strong fluorescence signal and in some the nucleolus can be clearly observed (Fig. 9A–C). Moreover as a control to ensure that mCherryNC alone does not enter the nucleus of *N. crassa* hyphae during the cycle, we used strain MFNC9 expressing mCherryNC alone driven by the *ccg-2* promoter. As demonstrated in Fig. 10, fluorescence seems to be dispersed in the cytoplasm without any visible nuclear accumulation. At CT4 fluorescence intensity is very high in strain MFNC9 (Fig. 4), and therefore we used a lower laser intensity to get a fluorescence

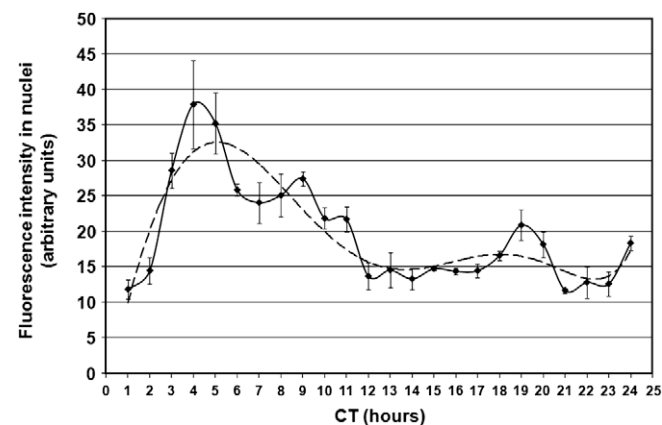


Fig. 8. Variation of fluorescence intensity in nuclei of the protein mCherryNC driven by the *frq* promoter during the circadian cycle of *N. crassa*, strain MFNC30. Note that only vegetative hyphae at the edge of the colony were considered. Mean and standard error were calculated from measurements on three different hyphae. Dotted line indicates the general trendline.

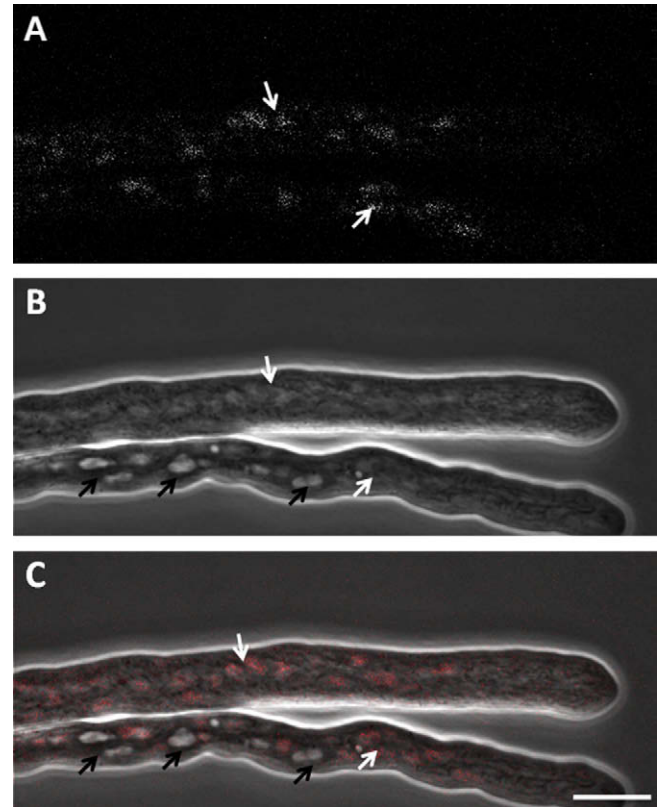


Fig. 9. Nuclear localization of FRQ-mCh in *N. crassa*, strain MFNC30. (A) Fluorescence channel, image taken at CT7. (B) Phase-contrast channel. (C) Merged images with fluorescence in red. White arrows indicate selected nuclei and black arrows point to selected vacuoles. Scale bar = 10 μm .

signal comparable to that seen in MFNC30. Nuclear size and distribution of strain MFNC30 (data not shown) were similar to those of a strain with nuclei labeled with GFP (Ramos-García et al., 2009).

While nuclear localization of the FRQ-mCh protein was clear in MFNC30, fluorescence intensity varied between the examined hyphae, possibly due to the disturbance created while being examined under the microscope. Despite such variability, a general trend in nuclear fluorescence was observed (Fig. 8). The first peak of fluorescence was the result of the strong accumulation of FRQ-mCh inside the nuclei and the second peak apparently was caused by a much lower accumulation. Cytoplasmic fluorescence accumulation

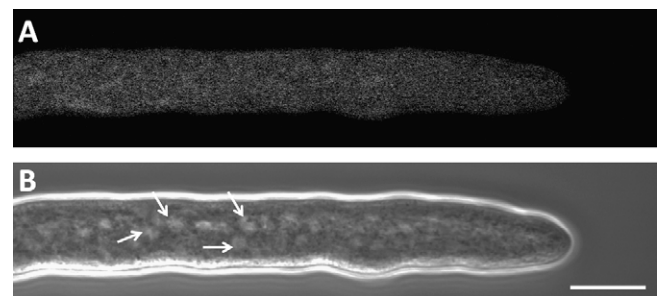


Fig. 10. Control showing the expression of mCherryNC alone driven by the *ccg-2* promoter in individual hyphae of *N. crassa*, strain MFNC9. (A) Fluorescence channel, image taken at CT4. (B) Phase contrast channel. Note that fluorescence is evenly distributed throughout the cytoplasm; there is no indication of accumulation in nuclei. Arrows indicate some nuclei. Scale bar = 10 μm .

also showed some variability; it was present during the complete circadian cycle, but followed a differential pattern of expression. At CT1–CT17 the accumulation of cytoplasmic FRQ–mCh was located further from the apices of leading hyphae (150–250 μm) and from CT17 to CT23 this accumulation was closest to the subapical region (Fig. 6C and Supplementary movie 6).

3.4. Clock effects in the *N. crassa* constructs

While the fluorescent reporters were useful for measuring the dynamics of the FRQ–mCh protein, we also wanted to ensure that the clock still functioned normally in these strains. Towards this end we measured the period and growth rate for each of the strains used in this study. We find that expressing mCherry under the control of the *cgg-2* promoter (strain MFNC9) had little effect on the free-running period of a *bd* culture when grown in constant darkness (Table 1). This result was expected since mutations in the *cgg-2* gene do not affect the period of the clock. Secondly, results with strain MFNC23 indicate that overexpressing FRQ–mCh using the *cgg-2* promoter, which is a strong promoter, reduces the *Neurospora* clock period by six hours.

Similar to the period effects observed driving FRQ–mCh with the *cgg-2* promoter, using the native *frq* promoter at an ectopic location to drive production also resulted in a shortening of the clock period. However, when mCherry was fused to the resident *frq* promoter in strain MFNC30, one could see a 2-h lengthening of the period. The period difference observed between strains MFNC22 and MFNC30 may be due to the two copies of the *frq* gene present in the former (native and FRQ–mCh integrated ectopically). The similarity in periods between MFNC30 and the *bd* control strain indicates that the FRQ–mCh fusion protein was generally capable of replacing the normal FRQ protein in terms of clock function under these conditions. This can be viewed as a validation of our approach of employing the mCherry protein as a useful reporter.

4. Discussion

One of the most ancient forms of biological regulation is the circadian rhythmicity found in almost all groups of organisms. The fungus *Neurospora crassa* has been used as a model organism to investigate the components of the circadian clock that regulate molecular, physiological and behavioral activities (Dunlap, 2006). Several properties of *Neurospora*, such as a straightforward handling in the laboratory, an easily-observable macroscopic rhythm (banding pattern) and the availability of the complete genome sequence, make this organism an ideal one for clock research (Bell-Pedersen et al., 2001a). To improve the understanding of how the clock operates in this organism we have created tools that have already proven useful for monitoring the dynamics of the *frq* gene under physiological conditions. The *frq* gene is one of the most important genes involved in the circadian rhythm of *N. crassa*, and consequently it has been intensely studied as one of the central components of the circadian negative feedback loop (Dunlap, 1999). The *frq* gene encodes central components of the circadian oscillator, the *frq* mRNA and two forms of the FRQ protein (Dunlap, 1993, 1996; Loros, 1995; Garceau et al., 1997; Liu et al., 1997). Each form of the FRQ protein enters the nucleus soon after its synthesis in the early subjective day and depresses the levels of its own transcript (Luo et al., 1998).

To elucidate the molecular details of the *frq* gene's dynamics in *Neurospora* a mycelial disk liquid culture has been extensively used (Nakashima, 1981). However some researchers have questioned the relevance this system has to understanding how the rhythm operates in an aerial environment, which is presumably the most similar to natural conditions. Now, by the use of a fluorescent fusion protein,

we were able to follow clock gene dynamics while the culture is growing on the surface of agar media. While others have used luminescent proteins to follow the circadian rhythmicity in *N. crassa*, these tools are limited to monitoring the cycle at a macroscopic level (Morgan et al., 2003; Gooch et al., 2008). Our tool, however, facilitates examination of rhythm components at the microscopic level, which allows us to gain insight into the intracellular transport dynamics of these proteins during the circadian cycle.

Specifically we used our first construct containing the *cgg-2* promoter driving the expression of the mCherryNC protein to gain insight into both the spatial and temporal dynamics of this promoter's activity during the circadian cycle. The *Neurospora cgg-2* (*eas*) gene encodes a fungal hydrophobin (Bell-Pedersen et al., 1992; Lauter et al., 1992) that is transcriptionally regulated by the circadian clock and other developmental pathways (Bell-Pedersen et al., 1996b,c, 2001b). Transcripts of the *cgg-2* gene accumulate during the late night to early morning in liquid cultures (Bell-Pedersen et al., 1996c).

At least two independent pathways are involved in the regulation of *cgg-2*, one from the endogenous clock to control rhythmic expression and another through an asexual development pathway (Bell-Pedersen et al., 1996c). The circadian rhythm and development are therefore major activators of the *cgg-2* promoter, however it is also induced by several additional factors, such as light and carbon source availability (Lauter et al., 1992; Arpaia et al., 1993; Bell-Pedersen et al., 1996b). Previous reports stated that endogenous *cgg-2* message levels increase and reach maximum accumulation after 60 min of continuous light (Bell-Pedersen et al., 1996b). We also detected a high production of mCherryNC from the *cgg-2* promoter after exposing cultures in the subjective night phase of the circadian rhythm (which normally has a low expression of *cgg-2* in vegetative hyphae) for one hour of continuous light (data not shown). Moreover the expression of the mCherryNC protein driven by the *cgg-2* promoter was rhythmic; leading us to conclude that the control of the *cgg-2* gene's expression could be observed using the mCherryNC fluorescence signal. In two developmental stages, aerial hyphae and conidia, there was a high expression of the mCherryNC protein. While we cannot rule out transport of mCherry from vegetative hyphae to the aerial hyphae and conidia using our microscopic results, previous reports on the *cgg-2* promoter make it highly likely that the *cgg-2* promoter is active in these regions (Lauter et al., 1992; Castro-Longoria et al., 2007). Since developmental regulation of the Pccg2–mCherryNC construct was so clear we expect this will become a powerful tool to study the dynamics of conidiation and other developmental processes. Once we determined that the mCherryNC protein reporter functioned well in *N. crassa*, we tagged the FRQ protein with mCherryNC to follow its intracellular localization during the circadian cycle. The expression of the FRQ–mCh fusion protein under the control of the *cgg-2* promoter was detected in nuclei of vegetative hyphae at very low levels during most of the circadian cycle. However two peaks of fluorescence in the nuclei were observed at CT11 and CT19 indicating that the fusion protein was being translocated into the nucleus. Although the same rhythmic pattern of fluorescence was generally observed, some of the examined hyphae did not express any fluorescence at all. This could have been due to the fact that this strain had two copies of the *frq* gene (the native copy and the ectopic fusion copy), which possibly affected its typical behavior. Moreover since the ectopic copy was driven by the *cgg-2* promoter, which is a much stronger promoter than the native *frq* promoter, we were surprised to see a macroscopic conidiation rhythm at all given previously-published reports on the over expression of *frq* abolishing the circadian rhythm (Aronson et al., 1994b). However this may be due to the fact that the *cgg-2* promoter is a clock-controlled promoter while the *qa-2* promoter used by Aronson et al. (1994b) was not.

When we compared the ectopic FRQ–mCh fusion (strain MFNC22) to our native FRQ–mCh fusion strain (MFNC30) we found that the macroscopic rhythm had a period of 24 h, closer to the typical 22 h of a wild-type strain, and that the banding pattern was very clear, at least for the first 4 days. While the signal was weaker than that observed using the *cgg-2* promoter driving the fusion protein (as expected due to the differences in promoter strengths) the nuclear localization of FRQ during the circadian cycle was similar to results previously obtained (Luo et al., 1998). The FRQ protein reached a maximum inside the nucleus at CT4 followed by a decrease through the rest of the cycle as previously reported (Luo et al., 1998). During the gradual decrease of nuclear FRQ that we observed, apparently another second smaller peak was detected at CT19. It has been suggested that the FRQ protein levels decrease after they are extensively phosphorylated, suggesting that phosphorylation of FRQ may lead to its degradation (He et al., 2003; He and Liu, 2005). Moreover recently the phosphorylation sites of FRQ have been shown influence its interactions with other components of the circadian oscillator in a phase dependent manner (Baker et al., 2009). Quantitative analysis of the FRQ protein by nuclear extractions during the circadian cycle revealed that the protein FRQ, together with FRH [FREQUENCY (FRQ)-interacting RNA helicase], enters the nucleus soon after its synthesis to repress its own transcript and that nuclear localization is required for its proper function (Aronson et al., 1994a; Garceau et al., 1997; Liu et al., 1997; Cheng et al., 2001, 2005; Denault et al., 2001; Froehlich et al., 2003). The protein FRQ enters the nucleus since a nuclear localization signal (NLS) exists for the small and large forms of FRQ and this signal is necessary and sufficient to direct the FRQ protein into the nucleus (Luo et al., 1998). The apparent second nuclear entrance of FRQ during the subjective night is a novel result and several possible explanations can be listed: (a) FRQ has a function outside the circadian feedback loops as previously suggested (Cheng et al., 2005); (b) FRQ has a role in the regulation of conidiation, independent of its role in the circadian rhythm; (c) FRQ enters in response to a signal from another oscillator, sort of a “cross-talk” with the FRQ-less oscillator; or (d) the second entrance of FRQ may be the long form of FRQ, since these experiments were done at 28 °C, where the long form of FRQ is at a higher level. Earlier studies also suggest that FRQ could be acting outside the feedback loop. For example, strain *frq*¹¹, which has only the small FRQ protein, exhibited different responses to light–dark cycles, suggesting that the two FRQ proteins have different functions in entrainment to light–dark cycles (Chang and Nakashima, 1997). It has been proposed that FRQ is part of a clock-regulated light input pathway to the temperature entrainable oscillator (Merrow et al., 1999). Other explanations are possible, and at this time, there are no convincing data to distinguish these alternatives.

In addition to nuclear entrance we observed differential accumulation of FRQ–mCh in the cytoplasm as well. Similar to recently published reports we detected that the subcellular distribution of FRQ shifted from mainly nuclear to mainly cytosolic as recently found (Diernfellner et al., 2009). In our results we generally, detected an accumulation of cytoplasmic FRQ–mCh 150 μm from the apices of leading hyphae. This accumulation of FRQ–mCh apparently was nearer the apices for several hours after CT17. By that time the FRQ–mCh expression levels in the subapical area of the cytoplasm were higher, compared with those observed during the first hours of the cycle. A possible explanation for this phenomenon could be that cytosolic FRQ behind the apices is entering the nucleus to inactivate the transcriptional activator WCC. On the other hand, an accumulation of hyperphosphorylated cytosolic FRQ is required to support the WCC accumulation (Schafmeier et al., 2006). It has been reported that while low levels of FRQ are required to drive efficient phosphorylation of WCC in the nucleus, a substantially higher concentration of FRQ is required to support accumulation and hyperphosphorylation of WCC in the cytosol (Schafmeier et al.,

2008); these findings are consistent with our observations. It has been shown that these conflicting functions are confined to distinct subcellular compartments and coordinated in temporal fashion (Schafmeier et al., 2006) as seen in our experiments.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.fgb.2009.12.013.

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