Transcriptional regulation and function of the *Neurospora* clock gene *white collar 2* and its isoforms

Andrea Neiss, Tobias Schafmeier & Michael Brunner⁺

University of Heidelberg Biochemistry Center, Heidelberg, Germany

FREQUENCY (FRQ) and the White Collar Complex (WCC), consisting of WC1 and WC2 subunits, are crucial components of positive and negative feedback loops of the circadian clock of *Neurospora*. In the positive limb, FRQ supports the accumulation of WC1 on a post-translational level and activates transcription of *wc2*. We analysed the transcriptional regulation of *wc2*. The WCC indirectly inhibits *wc2* by controlling expression of a putative repressor. FRQ activates *wc2* transcription by inhibiting WCC. A putative transcriptional activator binds to the *wc2* promoter and antagonizes the repressor function. Furthermore, an internal promoter in the *wc2* coding region drives expression of an amino-terminally shortened isoform, sWC2. Full-length WC2 and sWC2 are expressed in an antagonistic manner; thus, sWC2 expression seems to be a fail-safe mechanism that maintains total WC2 levels above a threshold.

Keywords: circadian clock gene transcription; positive feedback loop; *Neurospora*

EMBO reports (2008) 9, 788–794. doi:10.1038/embo.2008.113

INTRODUCTION

Circadian clocks are time-keeping devices that organize the rhythmic expression of a large number of genes. They allow organisms to adapt their physiology and behaviour in anticipation of daily changes associated with rotation of the earth. Circadian clocks generate an endogenous, cell-autonomous rhythm of about 24 h that is synchronized with the astronomical day by environmental cues such as light and temperature. Circadian oscillations in eukaryotes are based on interconnected feedback loops on a transcriptional, translational and post-translational level (Brunner & Schafmeier, 2006; Ko & Takahashi, 2006; Liu & Bell-Pedersen, 2006; Gallego & Virshup, 2007). The network of feedback loops is organized in a similar manner in fungi (*Neurospora*), insects (*Drosophila*) and vertebrates (mice). In the core of the clock is a heteromeric PAS domain-containing transcription factor that

drives clock output by directly and indirectly activating transcription of clock-controlled genes.

In mice, CLOCK/BMAL1 activates transcription of *Period 1/2* and *Cryptochrome 1/2* (*Per* and *Cry*, respectively), and of the nuclear receptor genes *Rev-erb α* and *Rora*. PERs and CRYs inhibit CLOCK/BMAL1 activity (Ko & Takahashi, 2006), whereas *Rev-erb α* and *Rora* encode a transcriptional repressor and activator of the *Bmal1* gene, respectively (Preitner *et al*, 2002; Sato *et al*, 2004). In *Drosophila*, CLOCK/CYCLE (dCLK/CYC) activates transcription of *per* and *timeless* (*tim*), and that of *vri* (*vri*) and *pdp1* (Bae & Edery, 2006). PER and TIM inhibit dCLK/CYC, whereas *vri* and *pdp1* encode a repressor and activator of *dClk* transcription, respectively (Cyran *et al*, 2003).

In *Neurospora*, WHITE COLLAR 1 (WC1) and WC2 form the WC complex (WCC), a circadian transcription factor and light receptor (Dunlap & Loros, 2004; Liu & Bell-Pedersen, 2006). The WCC activates transcription of *frequency* (*frq*), which encodes an inhibitor of WCC activity. The WCC supports transcription of *wc1* and thereby forms a positive loop stabilizing the clock (Káldi *et al*, 2006).

Less is known about the transcriptional organization of the *wc2* gene. Genetically, *wc1* is a repressor and *frq* an activator of *wc2* (Cheng *et al*, 2001, 2003); however, the molecular basis of this regulation is not known. Here, we have characterized the *wc2* promoter region and analysed the regulation of *wc2* transcription. Our data indicate that the WCC represses *wc2* transcription indirectly by activating expression of an unidentified repressor. FRQ inactivates the WCC and thereby activates *wc2* transcription by repressing expression of the repressor, similar to the regulation of *Rev-erb α* and *Vri* described above. In addition, a transcriptional activator supports transcription of *wc2* independent of WCC and FRQ. Furthermore, we identified a promoter in the coding region of *wc2*, which directs expression of an amino-terminally truncated, partly functional isoform of WC2.

RESULTS

The *wc2* gene contains several transcription start sites

The *wc2* gene is located on chromosome I, 2.6 kb downstream from the *uvs6* gene, which encodes a homologue of yeast *RAD50*. The genes are arranged in a head-to-head orientation (Fig 1A). To identify transcription start sites (TSSs), we performed 5' RNA ligase-mediated rapid amplification of complementary DNA ends (5' RLM-RACE; Table 1; Fig 1A). Seven TSSs for *wc2* were

University of Heidelberg Biochemistry Center, Im Neuenheimer Feld 328, 69120 Heidelberg, Germany

⁺Corresponding author. Tel: +49 6221 544207; Fax: +49 6221 544769;

E-mail: michael.brunner@bzh.uni-heidelberg.de

Received 23 January 2008; revised 14 May 2008; accepted 14 May 2008; published online 27 June 2008

Fig 1 | Characterization of the *white collar 2* promoter. (A) The *wc2* gene on chromosome I and *uvs6*, a homologue of *Saccharomyces cerevisiae* *RAD50*, are 2.6 kb apart and arranged in a head-to-head orientation. Boxes represent exons and flexed lines introns. Arrows indicate transcription start sites (TSSs) identified by 5' RNA ligase-mediated rapid amplification of complementary DNA ends at DD23 (constant darkness for 23 h). Distances of TSSs from ATG start codons of *wc2* (black) and *uvs6* (grey) are indicated. (B) *wc2* promoter constructs. Numbers refer to the length of the promoter fragment. The V-shaped dotted line indicates a deletion. (C) Western blot and real-time reverse transcription-PCR measurements showing expression of WC2 protein and RNA (data are means $\pm$ s.e.m. of 4–13 experiments) at DD24 in $\Delta wc2$ transformed with the constructs shown in (B). DD, constant darkness; WC, WHITE COLLAR; wt, wild type.

identified in the region between -370 and -1,080 bp and deviate only slightly from the consensus mammalian initiator sequence YYANT/AYY (Javahery et al, 1994). The broad region containing several TSSs might be due to the absence of a TATA-box (Carninci et al, 2006). The TSSs of *uvs6* were at -139 and -204. As the minimal distance between TSSs of *uvs6* and *wc2* is only 1,372 bp, expression of the two genes could be mutually affected by each other.

Table 1 | Sequences of *wc2* and *uvs6* transcription start sites

	Distance of TSS from ATG	Sequence
<i>wc2</i>	-1080	GCATGCG
	-797	CCACCAC
	-710	TCAAGAT
	-662	GCATTCC
	-654	CCAGGTC
	-483	GCATTTC
	-370	CCATCTT
<i>swc2</i>	+524	TTATTGC
	+533	CCAGCAT
	+536	GCATGTC
<i>uvs6</i>	-204	CCATGCT
	-139	TCATTCC
Consensus mammalian initiator sequence		YYAN ^T AYY

s, short isoform; WC, WHITE COLLAR; TSS, transcription start site.

We performed 5'-deletion analysis of the *wc2* promoter to identify *cis*-regulatory elements. *wc2* genes containing 2.2 kb (P 2.2), 1.2 kb (P 1.2) and 0.6 kb (P 0.6) upstream from the open reading frame (ORF) were integrated in a site-specific manner at the chromosomal *his3* locus of a $\Delta wc2$ strain (Fig 1B). Quantitative RNA and Western blot analysis showed no differences in the expression of *wc2* and WC2 between P 2.2, P 1.2 and P 0.6, suggesting that the main promoter activity is located within 0.6 kb upstream from the ORF. In accordance, a construct lacking this region (P 1.2-0.6) supported expression of only low levels of WC2 (Fig 1C). Furthermore, only residual amounts of WC2 were expressed under the control of a fragment corresponding to -3,200 to -1,300 (data not shown). In conclusion, positions -483 and/or -370 seem to be the main TSSs of the *wc2* gene.

WC1 and FRQ regulate *wc2* transcription indirectly

Genetic analysis has indicated that *wc1* acts as a transcriptional repressor and *frq* as an activator of *wc2* (Cheng et al, 2001, 2003). As the underlying mechanisms are not known, we analysed the levels of clock proteins and *wc2* RNA in wild-type (wt), Δfrq and $\Delta wc1$ strains (Fig 2A,B). As expression levels of FRQ and WC1 are mutually dependent on each other, we also generated and analysed Δfrq and $\Delta wc1$ strains that express FRQ constitutively under the control of the β -tubulin promoter. The levels of WC1 were reduced in Δfrq and elevated in the Δfrq ;tub-*frq* strain (Fig 2A), confirming that FRQ supports WC1 expression in a positive feedback loop (Lee et al, 2000; Káldi et al, 2006). WC2 levels were low in Δfrq and high in Δfrq ;tub-*frq*, $\Delta wc1$ and $\Delta wc1$;tub-*frq* (Fig 2A). Compared with wt, *wc2* RNA levels were reduced twofold in Δfrq , normal in Δfrq ;tub-*frq* and elevated 2.7-fold in $\Delta wc1$ and in $\Delta wc1$;tub-*frq* (Fig 2B). These data confirm that *wc1* genetically acts as a repressor and *frq* as an activator of *wc2*. In addition, the data show that *frq* affects *wc2* transcription only in the presence of WC1. As WC1 accumulates only in complex with WC2 (Cheng et al, 2002) and as FRQ is a negative regulator of the WCC, our data suggest that FRQ activates *wc2* transcription through inactivation of the WCC.

Fig 2 | Transcriptional regulation of *white collar 2*. (A) Western blot analysis of WC1, FRQ and WC2 in wild type (wt), Δfrq , $\Delta wc1$ and in Δfrq and $\Delta wc1$ constitutively expressing FRQ under the control of the β -tubulin promoter. (B) *wc2* RNA levels in the indicated strains. Quantitative real-time reverse transcription-PCR data are means $\pm$ s.e.m. of six experiments. (C) *wc2* RNA in $\Delta wc2$, $\Delta wc2$ Δfrq and $\Delta wc1$ transformed with *P* 3.2, *P* 2.2, *P* 1.9, *P* 1.5 and *P* 1.2 constructs (means $\pm$ s.e.m. of 4–13 experiments). All strains were harvested at DD24 (constant darkness for 24 h). (D) Site-directed mutagenesis of distal (dist) and proximal (prox) GATG repeats in the WCC-responsive region of the *wc2* promoter. (E) *wc2* RNA levels at DD24 in $\Delta wc2$, $\Delta wc2$ Δfrq and $\Delta wc1$ transformed with *P* 2.2 (*P* wt) or *P* 2.2 *mut dist* + *prox* (means $\pm$ s.e.m. of four experiments). DD, constant darkness; FRQ, FREQUENCY; WC, WHITE COLLAR; WCC, White Collar Complex.

Although the basic promoter activity is located within 0.6 kb upstream from the *wc2* ORF, regulatory elements could be located further upstream. As repression of *wc2* is maximal in the absence of FRQ, we transformed a $\Delta wc2$ Δfrq strain with *wc2* promoter constructs of different length (*P* 3.2, *P* 2.2, *P* 1.9, *P* 1.5 and *P* 1.2) to identify the location of repressing elements (Fig 2C, black columns). To identify the location of activating elements, the *wc2* constructs were expressed in a $\Delta wc1$ strain (Fig 2C, grey columns), as activation of *wc2* transcription is maximal in the absence of WC1. For control, a $\Delta wc2$ strain was transformed with the *wc2* constructs to mimic the wt situation (Fig 2C, white columns), as all

three clock proteins (FRQ, WC1 and WC2) are expressed in this strain. When these strains were transformed with *P* 3.2 and *P* 2.2, *wc2* was regulated in the same manner as endogenous *wc2* RNA (Fig 2C). With the shorter constructs *P* 1.9 and *P* 1.5, *wc2* RNA levels were still reduced in FRQ-deficient strains but no longer elevated in WC1-deficient strains, indicating that the truncated promoter retained the repressing element but was lacking an activating element (Fig 2C). This suggests that a transcriptional activator might bind between −1.9 and −2.2 kb. Despite the lack of the activating element, *wc2* transcription under the control of *P* 1.9 and *P* 1.5 was not significantly reduced in the transformed

Fig 3 | Analysis of WHITE COLLAR 2 isoforms. (A) Expression of full-length, medium (asterisk) and short (s) WC2 isoforms in wild type (wt), Δfrq and $\Delta wc1$ at DD24 (constant darkness for 24 h). (B) WC2 isoforms in LL in $\Delta wc2$ expressing WC2 with a carboxy-terminal DHFR tag under the control of the *wc2* promoter *P 2.2*. Tagged and non-tagged WC2 isoforms are schematically outlined. Grey box: DHFR (D). (C) WC2 isoforms in $\Delta wc2$ expressing WC2 with an amino-terminal TAP tag under the control of the inducible *qa2* promoter in the presence (+) or absence (–) of quinic acid (QA). Cultures were grown in LL. Tagged and non-tagged WC2 isoforms are schematically outlined. Black box: TAP tag (T). (D) Transcription start sites of *P_{int-wc2}* identified by 5' RNA ligase-mediated rapid amplification of complementary DNA ends at DD16. Distances from +1 are indicated. Grey bars: putative ATG start codons for sWC2. (E) Expression of WC2 isoforms in LL in $\Delta wc2$ transformed with *P 0.6* and *P 1.2–0.6*. (F) Ratio of *wc2* RNA in $\Delta wc1$ versus Δfrq measured by quantitative real-time reverse transcription–PCR at DD24 using probes specific to exon 1 (E1) and exon 2 (E2). DD, constant darkness; FRQ, FREQUENCY; LL, constant light; WC, WHITE COLLAR.

control strain (Fig 2C, white columns), suggesting that the repressor, which is controlled by the WCC, is not abundant in strains that also express FRQ, which inhibits the WCC. When the promoter was further truncated (*P 1.2*), *wc2* RNA levels in FRQ-deficient and WC1-deficient strains were similar to those in wt control. Accordingly, the target for the repressor of *wc2* is located between –1.5 and –1.2 kb. Thus, *wc2* transcription is regulated by an activator and a repressor that bind to distinct regions. In a wt situation, the repressor and activator are obviously balanced such that they functionally neutralize each other.

As the repressor and activator are not known, we investigated whether the WCC could directly regulate *wc2* transcription. So far, WCC has been described as a transcriptional activator; however, it is possible that WCC directly acts as a transcriptional repressor of *wc2*. Alternatively, WCC could act indirectly by activating expression of a putative repressor of *wc2*.

The WC proteins are GATA-type zinc-finger proteins, which bind to tandem GATN repeats spaced by 5–17 bp (He & Liu, 2005). We identified putative WCC binding sites about 2 and 1.26 kb upstream from the *wc2* coding region (Fig 2D). Assuming that WCC could use similar sites for transcriptional activation and repression, we destroyed these GATN repeats by site-directed mutagenesis. $\Delta wc2$, $\Delta wc2 \Delta frq$ and $\Delta wc2 \Delta wc1$ strains were transformed with either *P 2.2* or *P 2.2mut* and *wc2* RNA was measured (Fig 2E). No obvious difference was observed between the strains harbouring and those lacking the distal and proximal GATN repeats, indicating that neither activation nor repression of *wc2* was dependent on the GATN repeats. The same results were obtained when the sites were mutated individually (data not

shown). Thus, WCC does not bind to these sites to directly regulate *wc2* transcription. These data suggest that WCC supports expression of a repressor of *wc2*, whereas expression of the activator is independent of WCC. However, the formal possibility that WCC binds to totally unrelated sequences cannot be excluded.

WC2 isoforms

Analysis of wt, Δfrq and $\Delta wc1$ showed, in addition to full-size WC2, shorter WC2 reactive bands of about 52 kDa (indicated by an asterisk) and 44 kDa (sWC2) that were absent in a $\Delta wc2$ strain (Fig 3A). The 52 kDa band corresponds to a carboxy-terminally truncated proteolytic cleavage product of WC2 (supplementary Fig 1 online). This cleavage product is, in particular, prominent in $\Delta wc1$ —that is, when WC2 is highly expressed and its assembly partner is absent (Fig 3A).

By contrast, the levels of sWC2 were reduced when full-size WC2 was highly expressed ($\Delta wc1$) and elevated when full-size WC2 levels were low (Δfrq ; Fig 3A). To characterize the sWC2 isoform, we constructed $\Delta wc2$ strains that express N- and C-terminally tagged versions of WC2. One strain, *P 2.2 wc2-DHFR*, expressed a WC2 fusion protein with a C-terminal DHFR domain (Fig 3B); the strain did not express sWC2. Instead, a larger protein corresponding to sWC2-DHFR was detected. Both WC2-DHFR and sWC2-DHFR were phosphorylated. The second strain, *qa2-TAP-wc2*, expressed a WC2 fusion protein with an N-terminal TAP tag under the control of the inducible *qa2* promoter (Fig 3C); sWC2 was expressed in this strain. Together, the data show that sWC2 is an N-terminally truncated version of WC2. Expression of TAP-WC2 was dependent on quinic acid (QA), an inducer of the

Fig 4 | The short *WHITE COLLAR 2* isoform is partly functional. (A) Western blots showing the expression of WC2, sWC2 and WC1 in $\Delta wc2$ transformed with the indicated constructs. Cultures were grown in LL. M/L indicates the change of methionine residues 127 and 133 to leucine. (B) Race tube assay showing circadian banding, in constant darkness, of $\Delta wc2$ transformed with the indicated constructs. Two representative race tubes for each strain are shown. The $\Delta wc2$ *ORFi* strain shows a period lengthening of 3 h before the rhythm dampens. Period values (τ) are averages $\pm$ s.e.m. of 4–10 tubes. (C) Western blot showing overexpression of sWC2 from the constitutive *cpc1* promoter in a $\Delta wc2$ and wild-type (wt) background in LL. (D) Overexpression of sWC2 in wt causes the same period lengthening of circadian banding in DD as overexpression of sWC2 in $\Delta wc2$. Period values (τ) are averages $\pm$ s.e.m. of 9–14 race tubes. Two representative race tubes for each strain are shown. DD, constant darkness; LL, constant light; ORFi, open reading frame including introns; s, short isoform; WC, WHITE COLLAR.

qa2 promoter (Fig 3C, left lane). However, expression of sWC2 was independent of QA (Fig 3C, right lane), suggesting that a constitutive promoter is located in the 5'-region of the *wc2* ORF. To test this hypothesis, we analysed *wc2* RNA by 5' RLM-RACE. The analysis showed three closely spaced TSSs in the second exon of *wc2* (Table 1; Fig 3D), indicating the presence of an internal promoter (*P_{int-wc2}*). The TSSs were located upstream from two closely spaced in-frame ATG codons (codons 127 and 133 of WC2). The corresponding ORFs have the ability to encode shortened WC2 isoforms of about 44 kDa, which is in good agreement with the electrophoretic mobility of sWC2.

To characterize the regulation of WC2 versus sWC2 expression, we compared the levels and ratio of the WC2 isoforms expressed under the control of *P 0.6* and *P 1.2–0.6* (Fig 3E). Expression of WC2 was high in *P 0.6* and low in *P 1.2–0.6*, in which the main TSSs of the *wc2* promoter were present and absent, respectively. In contrast, expression of sWC2 was low in *P 0.6* and high in *P 1.2–0.6*, indicating that the isoforms were expressed in an antagonistic manner.

The same results were obtained when *wc2* RNA was measured. The probe used to quantify *wc2* RNA by real-time reverse

transcription-PCR (Figs 1,2) was specific to the first exon (E1)—that is, it detected specifically the long transcripts initiated at the promoters upstream from the *wc2* ORF (*P_{wc2}*). To characterize the transcriptional organization of *wc2* in more detail, we used a probe that was specific to the second exon (E2), which detects transcripts initiated at *P_{wc2}* as well as transcripts of *P_{int-wc2}*. The levels of E2-containing RNA (*P_{wc2}* + *P_{int-wc2}*) were less affected by FRQ and WC1 (supplementary Fig 2A online). Thus, the factor between maximal repression (Δfrq) and maximal induction ($\Delta wc1$) is 3.3 for *P_{wc2}* + *P_{int-wc2}* transcripts, as compared with 6.5 for *P_{wc2}* transcripts (Fig 3F). Together, the data suggest that *P_{wc2}* transcription interferes with transcription initiation at the downstream located *P_{int-wc2}*. In strains harbouring the promoterless *wc2* gene, *swc2* RNA levels were independent of FRQ or WCC (supplementary Fig 2B online).

For further analysis, we generated strains that expressed full-length WC2, sWC2 or both proteins (Fig 4A). To generate the gene that encodes only full-length WC2, we changed the Met codons 127 and 133 to Leu codons. The corresponding strain (*P 2.2 M/L*) expressed only full-length WC2 and showed, similar to wt, a free-running period of 21.5 h (Fig 4A,B), suggesting that full-length

Fig 5 | Model for the regulation of *white collar 2* transcription. For details, see text. A, activator; CK1, casein kinase 1a; FRQ, FREQUENCY; P, phosphate; R, repressor; *wc2*, *white collar 2*; WCC, White Collar Complex.

WC2 is sufficient to support a circadian oscillation with a period similar to that of wt.

To generate a strain expressing sWC2, a $\Delta wc2$ strain was transformed with the promoter-less, intron-containing *wc2* ORF (*ORFi*). The strain expressed specifically sWC2 but not full-length WC2 (Fig 4A), indicating that sWC2 is expressed under the control of an internal promoter. When Met 127 and Met 133 in the *ORFi* construct were changed to Leu, sWC2 was not expressed (Fig 4A). The *ORFi* strain expressed almost normal levels of WC1, whereas WC1 did not accumulate in the *ORFi* M/L strain (Fig 4A, lower panel). This indicates that sWC2 assembles with WC1. *ORFi* showed a long-period (25 h) of rhythmic conidiation pattern on race tubes that dampened after several days in darkness (Fig 4B). Under entrained conditions (12h/12 h light–dark cycles), *ORFi* showed a robust rhythm (data not shown). The peak of conidiation (phase) of *ORFi* was delayed by 2.2 h compared with wt, which is also indicative of a long-period rhythm. Finally, we overexpressed sWC2 under the control of the *cpc1* promoter in $\Delta wc2$ or wt (Fig 4C). Both strains were rhythmic in constant darkness (DD) and showed a long-period (25 h) phenotype (Fig 4D). The data confirm that sWC2 is partly functional and competes with WC2.

DISCUSSION

WC1 and WC2 are crucial components of the circadian clock of *Neurospora*. Here we analysed the transcriptional organization of the *wc2* gene. Transcription of *wc2* is initiated at several sites in the region between –370 and –1,080 with respect to the *wc2* start codon. The main initiation sites are at –370 and/or –483. WC1, which activates its own expression (Káldi et al, 2006), represses transcription of *wc2* (Cheng et al, 2003; this work). As WC1 is degraded in the absence of WC2 (Cheng et al, 2002), both functions are exerted by the WCC rather than by WC1 alone. In *frq*-deficient strains, the levels of WCC are low but the transcription factor is highly active. The levels of *wc2* RNA were low in Δfrq , confirming that WC1 represses *wc2* transcription

independently of FRQ (Cheng et al, 2003) and indicating that *wc2* transcription is highly repressed when the WCC is active as a transcriptional activator. FRQ is genetically an activator of *wc2* and it has been suggested that FRQ might activate *wc2* transcription independent of WC1 (Cheng et al, 2003). However, our data show that FRQ activates *wc2* transcription only in the presence of the WCC, implying that FRQ exerts its activating function indirectly by inhibiting the activity of the WCC. We have shown that the *wc2* promoter region does not contain functional binding sites for WCC. Rather, the WCC might support expression of a transcriptional repressor of *wc2*. Our results can be summarized in the following model (Fig 5).

wc2 transcription is balanced by an activator and a repressor that bind to distinct sites. When FRQ is absent or low, active WCC supports expression of elevated levels of the repressor and thus *wc2* transcription is repressed. When FRQ is present (wt), it inactivates WCC by mediating its phosphorylation (Schafmeier et al, 2005, 2006) and hence expression of the repressor is attenuated, such that the repressor and activator functionally neutralize each other. In the absence of WCC ($\Delta wc1$), repressor levels are low and the *wc2* gene is transcribed under the control of the activator, which is expressed independently of WCC and *wc2* transcription is elevated. The transcriptional feedback might contribute to maintain stable expression levels and the ratio of WCC and WC2.

The proposed transcriptional organization of *wc2* is reminiscent of the regulation of *Bmal1* in mammals and *dClock* (*dClk*) in flies: in mice, CLOCK/BMAL1 (functionally equivalent to the WCC) activates transcription of *Rev-erba*, encoding a repressor of the *Bmal1* gene (Preitner et al, 2002). Similarly, the *Drosophila* transcription factor dCLK/CYC activates transcription of *vriille*, encoding a repressor of *dClk* (Cyran et al, 2003; Glossop et al, 2003). Regulation of the putative activator of *wc2* is, however, different from the regulation of *Rora* and *pdp1* in mice and flies, as its expression is independent of WCC. As FRQ is an inhibitor of

the WCC, it indirectly activates *wc2* transcription by repression of the putative repressor, similar to PERs and CRYs in mice, and PER in flies, which inhibit the activity of CLOCK/BMAL1 and dCLK/CYC, respectively (Preitner *et al*, 2002; Cyran *et al*, 2003; Bae & Edery, 2006). As the repressors REV-ERB α and VRILLE, and the activators RORA and PDP1 are not conserved between species, it will be difficult to identify their functional counterparts in *Neurospora*.

We have identified sWC2, an N-terminally truncated version of WC2 that is expressed under the control of the internal promoter $P_{int-wc2}$. Transcription by the upstream-located P_{wc2} interferes with $P_{int-wc2}$ transcription. Accordingly, expression of sWC2 is high when expression of full-length WC2 is low, and vice versa.

Interestingly, the *wc1* gene also contains an internal promoter that drives expression of sWC1, an N-terminally truncated, partly functional form of WC1 (Káldi *et al*, 2006). The N-terminal portion of *Neurospora* WC2, consisting of MGS/TG repeats and a proline-rich region, is not conserved in WC2 proteins from different fungi (supplementary Fig 3 online). It is therefore not obvious whether and how the N terminus contributes to WC2 function. The antagonistic regulation of P_{wc2} and $P_{int-wc2}$ might represent a fail-safe mechanism to ensure that total WC2 levels do not decrease below a critical threshold. It seems likely that expression of sWC1 has a corresponding function.

METHODS

Strains and growth conditions. All strains carried the *bd* mutation (Loros *et al*, 1986). Plasmid constructs were inserted into the *his3* locus and strains were grown as described in the supplementary information online.

Protein analysis. Protein was extracted from mycelia using previously described methods (Garceau *et al*, 1997). Total protein (200 μ g) per lane was subjected to SDS-polyacrylamide gel electrophoresis on 7.5% gels for FRQ and WC1, and 12% gels for WC2. Western blots were decorated with monoclonal antibodies against FRQ or affinity-purified rabbit antibodies against WC1 and WC2. Western blots were performed using enhanced chemiluminescence. To control uniform loading of the gels, nitrocellulose filters were stained with Ponceau S.

RNA analysis. RNA was prepared using peqGOLD TriFASTTM (peqLab, Erlangen, Germany), reverse transcribed using the QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany) and analysed by quantitative real-time PCR (Görl *et al*, 2001). 5' RLM-RACE was carried out according to the manufacturer's instructions (Ambion Inc., Austin, TX, USA; Invitrogen, Carlsbad, CA, USA).

Race tube analysis. Race tubes were inoculated, germinated in constant light (LL) for about 24 h and transferred to DD at 25 °C. Race tubes were scanned and the conidial densities were quantified using the Chrono program (Roenneberg & Taylor, 2000).

For further details, see the supplementary information online.

Supplementary information is available at *EMBO reports* online (<http://www.emboreports.org>).

ACKNOWLEDGEMENTS

This work was supported by the Deutsche Forschungsgemeinschaft grants BR 1375-1 and SFB 638 and by the Fonds der Chemischen Industrie.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

REFERENCES

- Bae K, Edery I (2006) Regulating a circadian clock's period, phase and amplitude by phosphorylation: insights from *Drosophila*. *J Biochem* **140**: 609–617
- Brunner M, Schafmeier T (2006) Transcriptional and post-transcriptional regulation of the circadian clock of cyanobacteria and *Neurospora*. *Genes Dev* **20**: 1061–1074
- Carninci P *et al* (2006) Genome-wide analysis of mammalian promoter architecture and evolution. *Nat Genet* **38**: 626–635
- Cheng P, Yang Y, Liu Y (2001) Interlocked feedback loops contribute to the robustness of the *Neurospora* circadian clock. *Proc Natl Acad Sci USA* **98**: 7408–7413
- Cheng P, Yang Y, Gardner KH, Liu Y (2002) PAS domain-mediated WC-1/WC-2 interaction is essential for maintaining the steady-state level of WC-1 and the function of both proteins in circadian clock and light responses of *Neurospora*. *Mol Cell Biol* **22**: 517–524
- Cheng P, Yuhong Y, Wang L, He Q, Liu Y (2003) WHITE COLLAR-1, a multifunctional *Neurospora* protein involved in the circadian feedback loops, light sensing, and transcriptional repression of *wc-2*. *J Biol Chem* **278**: 3801–3808
- Cyran SA, Buchsbaum AM, Reddy KL, Glossop NR, Hardin PE, Young MW, Storti RV, Blau J (2003) *Vrille*, *Pdp1* and *dClock* form a second loop in the *Drosophila* circadian clock. *Cell* **112**: 329–341
- Dunlap JC, Loros JJ (2004) The *Neurospora* circadian system. *J Biol Rhythms* **19**: 414–424
- Gallego M, Virshup DM (2007) Post-translational modifications regulate the ticking of the circadian clock. *Nat Rev Mol Cell Biol* **8**: 139–148
- Garceau NY, Liu Y, Loros JJ, Dunlap J (1997) Alternative initiation of translation and time specific phosphorylation yield multiple forms of essential clock protein FREQUENCY. *Cell* **89**: 469–476
- Glossop NR, Houl JH, Zheng H, Ng FS, Dudek SM, Hardin PE (2003) VRILLE feeds back to control circadian transcription of Clock in the *Drosophila* circadian oscillator. *Neuron* **37**: 249–261
- Görl M, Merrow M, Huttner B, Johnson J, Roenneberg T, Brunner M (2001) A PEST-like element in FREQUENCY determines the length of the circadian period in *Neurospora crassa*. *EMBO J* **20**: 7074–7084
- He Q, Liu Y (2005) Molecular mechanism of light responses in *Neurospora*: from light-induced transcription to photoadaptation. *Genes Dev* **19**: 2888–2899
- Javahery R, Khachi A, Lo K, Zenzie-Gregory B, Smale ST (1994) DNA sequence requirements for transcriptional initiator activity in mammalian cells. *Mol Cell Biol* **14**: 116–127
- Káldi K, Herreros González B, Brunner M (2006) Transcriptional regulation of the *Neurospora* circadian clock gene *wc-1* affects the phase of circadian output. *EMBO Rep* **7**: 199–204
- Ko CH, Takahashi JS (2006) Molecular components of the mammalian circadian clock. *Hum Mol Genet* **15**: R271–R277
- Lee K, Loros JJ, Dunlap JC (2000) Interconnected feedback loops in the *Neurospora* circadian system. *Science* **289**: 107–110
- Liu Y, Bell-Pedersen D (2006) Circadian rhythms in *Neurospora crassa* and other filamentous fungi. *Eucaryotic Cell* **5**: 1184–1193
- Loros JJ, Richman A, Feldman JF (1986) A recessive circadian mutation at the *frq* locus of *Neurospora crassa*. *Genetics* **114**: 1095–1110
- Preitner N, Damiola F, Lopez-Molina L, Zakany J, Duboule D, Albrecht U, Schibler U (2002) The orphan nuclear receptor REV-ERB α controls circadian transcription within the positive limb of the mammalian circadian oscillator. *Cell* **110**: 251–260
- Roenneberg T, Taylor W (2000) Automated recordings of bioluminescence with special reference to the analysis of the circadian rhythms. *Methods Enzymol* **305**: 104–119
- Sato TK, Panda S, Miraglia LJ, Reyes TM, Rudic RD, McNamara P, Naik KA, FitzGerald GA, Kay SA, Hogenesch JB (2004) A functional genomics strategy reveals Rora as a component of the mammalian circadian clock. *Neuron* **43**: 527–537
- Schafmeier T, Haase A, Káldi K, Scholz J, Fuchs M, Brunner M (2005) Transcriptional feedback of *Neurospora* circadian clock gene by phosphorylation-dependent inactivation of its transcription factor. *Cell* **122**: 235–246
- Schafmeier T, Káldi K, Diernfellner A, Mohr C, Brunner M (2006) Phosphorylation dependent maturation of *Neurospora* circadian clock protein from a nuclear repressor towards a cytoplasmic activator. *Genes Dev* **20**: 297–306