WC-2 mediates WC-1–FRQ interaction within the PAS protein-linked circadian feedback loop of *Neurospora*

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Eukaryotic circadian clocks comprise feedback loops where PAS domain-containing transcriptional activators drive gene expression of negative elements. In *Neurospora*, clock models posit a White Collar complex (WCC) containing WC-1 and WC-2 that activates expression of the central clock gene *frequency* (*frq*); FRQ protein is hypothesized to feed back to block the activity of the WCC. We have characterized the WC-2 protein and its role in this complex: WC-2 is an abundant constitutive nuclear protein, in contrast to rhythmically expressed FRQ and WC-1. WC-2 interacts with WC-1 and FRQ but, significantly, WC-1 and FRQ do not interact in the absence of WC-2. By quantifying the relative numbers of WC-2, FRQ and WC-1 proteins and complexes in cell extracts, both the numbers and types of complexes at different circadian times were estimated, yielding results consistent with the model. Constitutive and abundant WC-2 appears to provide a scaffold allowing for the interaction of two limiting and rhythmically out-of-phase proteins, FRQ and WC-1, and this temporal and physical relationship may be responsible for rhythmic expression of *frq*.

Keywords: circadian/complexes/frequency/white collar

Introduction

Circadian rhythms are endogenous daily cycles that orchestrate temporal control of physiological events in a wide variety of organisms. Enormous progress has been made in the past few years in the identification of the molecular components that make up the circadian oscillator. In *Neurospora*, a circadian oscillator regulates, among other things, the timing of sexual and asexual spore development. The molecular organization of the *Neurospora* circadian clock, as seen with other organisms (Loros, 1998; Dunlap, 1999; Iwasaki and Dunlap, 2000), involves a transcription–translation based autoregulatory feedback loop comprised of positive elements (heterodimers of PAS domain-containing proteins that act as transcription factors) and negative elements that down-regulate the activity of the positive elements.

The positive elements in *Neurospora* are the White Collar proteins WC-1 and WC-2 (Crosthwaite *et al.*, 1997) as well as the Frequency (FRQ) proteins, recently shown

to play a role in promoting the synthesis of WC-1 (Lee *et al.*, 2000). FRQ also acts as the negative element in the loop (Aronson *et al.*, 1994b; Garceau *et al.*, 1997). Circadian oscillation is manifest as a rhythmic change in the amount of the *frq* transcript that is essential for the overt circadian rhythm. Proteins encoded by the *wc-1* and *wc-2* loci are required for the activation of *frq* and, at the other end, FRQ expression has been shown to depress steady-state levels of the *frq* transcript. The biochemical means by which FRQ executes this negative regulation is not known. Although successful circadian oscillation requires both negative and positive feedback, the molecular connection between these two forms of regulation has not been established and the complete biochemical function is not known for any elements in the loop.

Both *wc-1* and *wc-2* encode GATA-type DNA binding proteins (Orkin, 1992; Ballario *et al.*, 1996; Linden and Macino, 1997; Marzluf, 1997). In addition, both contain PAS domains, which are protein–protein interaction domains seen in proteins involved in a variety of biological pathways (Taylor and Zhulin, 1999). In this same vein, studies have shown that WC-1 and WC-2 heterodimerize and that this interaction is an important mechanism that mediates light responses in *Neurospora* (Ballario *et al.*, 1998; Talora *et al.*, 1999). However, to date, no other proteins interacting with the WC proteins have been described. A strong and central prediction of the present model for the transcriptional/translational feedback loop in the *Neurospora* clock is that FRQ, acting as the negative element, must feed back and physically interact with the complex of WC-1 and WC-2. In this study, we took a biochemical approach to test this prediction. We developed novel antisera and by quantifying the relative levels of FRQ, WC-1 and WC-2, determined that at all times of day WC-2 is in excess. Furthermore, WC-2 is capable of interacting independently with FRQ and WC-1, and appears to mediate the interaction of these different proteins in a multimeric complex. Thus, although not rhythmically regulated itself, WC-2 mediates the protein–protein interactions that lie at the core of this clock-associated feedback loop.

Results

WC-2 is a constitutively expressed nuclear protein

As a first step to probing the role of WC-2 in the circadian system, a specific antiserum was generated against the full-length WC-2 expressed from bacteria (see Materials and methods). The specificity of the antiserum was assayed on western blots using cellular extracts from several *wc-2* mutant alleles in addition to a *wc-2* knockout strain of *Neurospore crassa* (data not shown). Consistent with its role as a transcription factor, WC-2 appears to be found almost exclusively in the nucleus (Figure 1) in

Fig. 1. WC-2 is a nuclear protein that does not display robust rhythmic expression. Western blot analysis of subcellular localization of WC-2, FRQ, CYS-3 and CCG-1 proteins and throughout a circadian cycle. WC-2 is detected in the total lysate (left) and is enriched in the nuclear (right) fractions. Conversely, CYS-3, a known nuclear protein, is only detected in the nuclear fraction, whereas CCG-1, a cytoplasmic protein, is detected in the lysate only. In contrast to FRQ, which cycles robustly, WC-2 levels are relatively constant; these data are representative of two separate experiments. Two different exposure times are shown for WC-2.

agreement with a recent report that appeared while this work was in preparation (Schwerdtfeger and Linden, 2000).

To test whether the abundance of WC-2 in the nucleus changed over time, both total and nuclear WC-2 levels were followed in the dark from extracts harvested at different circadian times. WC-2 protein did not display significant cycling (Figure 1). This is in sharp contrast to the FRQ profile which, consistent with previous studies (Garceau *et al.*, 1997; Luo *et al.*, 1998), displays a robust rhythmic oscillatory pattern in both the total lysate as well as the nuclear fractions. Furthermore, the WC-2 electrophoretic mobility does not appear to change in a time of day-specific manner under these conditions.

WC-2 exists in a multimeric complex with WC-1 and FRQ

Although it is well established that FRQ negatively regulates its own expression and that the expression of *frq* gene requires *wc-2* (Crosthwaite *et al.*, 1997), the molecular mechanisms governing both the activation of *frq* and the feedback loop in general are unknown. A very strong prediction of the existing model for this FRQ/WC feedback loop in the *Neurospora* clock (Crosthwaite *et al.*, 1997; Dunlap, 1999; Collett *et al.*, 2000; Iwasaki and Dunlap, 2000) is that the regulation of *frq* transcription occurs via an association of WC-2 with other clock proteins, FRQ and WC-1. To address this question, extracts from subjective night (CT 15) were subjected to sucrose gradient analysis. As shown in Figure 2A, WC-2 was detected in two peaks, a low molecular weight peak (~60 kDa) that corresponds to its monomeric form, and a high molecular weight peak, migrating close to the β -amylase size marker (~200 kDa), indicating that WC-2 participates in a multimeric complex in the cell (Figure 2A). For comparison, the migration profile of two other clock proteins, WC-1 and FRQ was also assessed. WC-1 signal was detected in a single peak, greater than the predicted monomeric size of 120 kDa, and was coincident with the high molecular weight WC-2 peak (Figure 2A). FRQ protein migrated as a single broad band

Fig. 2. WC-2 enters complexes in the cell and sediments in two distinct peaks on a sucrose gradient. (A) Western blot analysis of WC-2, WC-1 and FRQ from non-denatured extracts size fractionated on a 10–30% sucrose gradient. The asterisks indicate the sedimentation peaks of four standard proteins and their native molecular weights. Fraction 2 corresponds to the top of the gradient and fraction 19 corresponds to the bottom of the gradient. (B) Representative densitometric analysis of WC-2, WC-1 and FRQ signals in each fraction obtained from the sucrose gradients of cultures grown for 4 h in the dark.

peaking at ~250–275 kDa (Figure 2A). This size is substantially greater than the expected molecular weight of 108 kDa predicted by the primary sequence (Aronson *et al.*, 1994a; Garceau *et al.*, 1997), or the 140 kDa observed in SDS gel electrophoresis (Garceau *et al.*, 1997). The FRQ peak shows substantial overlap with the WC-2 tail, also consistent with the FRQ proteins complexing with themselves or with another protein(s) including WC-2. Although the WC-2 signal was found in a broad range of fractions, the close correspondence between WC-2 and WC-1 is apparent, consistent with published data showing *in vivo* and *in vitro* that these two proteins interact to form WCC (Ballario *et al.*, 1998; Talora *et al.*, 1999). More importantly, the three protein profiles overlapped, consistent with WC-2, WC-1 and FRQ all existing together in a large protein complex (Figure 2B).

Two independent methods were used to confirm that WC-2 specifically and directly associates with FRQ. First, bacterially expressed and purified glutathione *S*-transferase (GST)–WC-2 fusion proteins or GST alone (see Materials and methods) were incubated with *in vitro* translated, 35 S-labeled FRQ or, as a negative control, the irrelevant protein luciferase (Figure 3A and B). SDS–PAGE analysis of the bound proteins indicates that FRQ binds to GST–WC-2 but not to GST alone. In contrast, no detectable binding of luciferase to either GST–WC-2 or GST was seen (Figure 3A).

This *in vitro* WC-2–FRQ interaction was confirmed with proteins made *in vivo* (Figure 3B). Lysates from cultures grown 24 h in the dark were subjected to

Fig. 3. WC-2 interacts with WC-1 and FRQ and mediates the complex association of WC-1 and FRQ. (A) *In vitro* binding assay, showing co-sedimentation of radiolabeled FRQ with GST-WC-2 but not with GST alone. Bacterially expressed GST (~30 kDa) or GST-WC-2 (~80 kDa) protein (left, Coomassie-stained gel) were incubated with either ³⁵S-labeled FRQ or LUC (right, Autoradiography, Input lanes). GST-containing proteins were sedimented, resolved by SDS-PAGE and visualized by autoradiography as described in Materials and methods. FRQ signal was detected on the autoradiogram in the GST-WC-2 but not the GST lane. Luciferase was not detected in either GST-WC-2 or GST lanes. (B) FRQ and WC-1 co-immunoprecipitate with WC-2. Extracts of wild-type (WT) strain grown 15 h in the dark were subjected to immunoprecipitation using the WC-2 antibody. Western analysis of FRQ, WC-2 and WC-1 signals from extracts immediately before precipitation (left, Input) and the recovered immune complex following precipitation (right, Precipitate) with either WC-2 antiserum (I) or pre-immune serum (PI). (C) WC-2 is required to mediate the association of FRQ and WC-1 in a complex. The left panel shows a control immunoprecipitation from a WT strain in which WC-1 antiserum (I) was used to precipitate both WC-1 and FRQ from whole-cell extracts of dark grown *Neurospora*; no proteins were detected in the precipitates of reactions containing WC-1 preimmune serum (PI). The experimental panel is on the right. Because FRQ is poorly expressed from its own promoter in the absence of WC-2, the inducible *quinic acid-2* promoter was used to drive FRQ expression in the *wc-2* knockout strain (*wc-2^{KO}::pBA50*). When QA was added, FRQ expression was substantially increased (right, Input). However, when these extracts bearing substantial amounts of FRQ were treated with WC-1 antiserum, FRQ did not co-precipitate with WC-1 (right, Precipitate).

immunoprecipitation with either WC-2 antiserum or pre-immune serum (see Materials and methods). Both FRQ and WC-1 co-immunoprecipitated with WC-2. These proteins were not detected in precipitates of extracts incubated with pre-immune antisera. Similar results were obtained in reciprocal experiments using either the FRQ or WC-1 antisera and checking for WC-2 co-immunoprecipitation (Figure 3C; data not shown).

WC-2 is required for WC-1-FRQ interaction

To define the organization of these interactions, we sought to determine whether WC-1 and FRQ interaction could be detected in the absence of WC-2 (Figure 3C). To test this, we performed immunoprecipitation experiments in a WC-2 knockout (*wc-2^{KO}::pBA50*) strain generated in our laboratory (M.A.Collett, J.C.Dunlap and J.J.Loros, submitted). Since WC-2 is required for the expression of FRQ

at physiologically normal levels (Crosthwaite *et al.*, 1997), we were initially unable to detect FRQ in the *wc-2^{KO}::pBA50* strain. To circumvent this, we utilized a construct (pBA50) that carried the FRQ open reading frame under the control of the inducible quinic acid (QA) promoter (Giles *et al.*, 1985; Aronson *et al.*, 1994b). The construct was transformed into the WC-2 knockout (*wc-2^{KO}::pBA50*). As expected, QA treatment resulted in a significant increase in steady-state levels of FRQ in the *wc-2^{KO}::pBA50* strain (Figure 3C, right). Lysates from either wild-type (WT) or WC-2 knockout strains treated and untreated with QA were subjected to immunoprecipitation using WC-1 antiserum. In WT control reactions, WC-1 and FRQ co-immunoprecipitated in the reactions containing WC-1 antiserum (lanes I) but were not detected in the reactions containing pre-immune serum (lanes PI) (Figure 3C, left). On the other hand, in the *wc-2^{KO}::pBA50* strain, in the absence of WC-2 protein and regardless of the FRQ expression level, FRQ did not co-immunoprecipitate with WC-1 (Figure 3C, right). These data establish an essential role for WC-2 in promoting the association of FRQ with WC-1 and, although it is possible that this role is indirect, given the interaction between *in vitro* expressed GST-tagged WC-2 and FRQ, a direct role for WC-2 in mediating the interaction seems likely.

To begin to localize the site of the FRQ-WC-2 interaction, a series of nested FRQ proteins were synthesized *in vitro* (see Materials and methods) such that all contained the 200 amino acids at the N-terminus of FRQ and increasingly longer amounts of the rest of the protein up to full length. In GST pull-down assays, all of these substrates were retained by GST-WC-2 (data not shown), suggesting that the interaction site is within the first 200 amino acids of long FRQ and the first 100 amino acids of short FRQ (Garceau *et al.*, 1997). Consistent with these data, the site of interaction has recently been mapped to this region by immunoprecipitation studies (see Cheng *et al.*, 2000).

WC-2 multimeric complex is present throughout the circadian cycle

To see whether the formation of the complex was static or dynamically changing in a time-of-day-specific manner, sucrose gradient analyses were performed on dark-grown extracts harvested at the indicated times (Figure 4A). Although the width of the protein profiles varied, the overall locations of the WC-2, WC-1 or FRQ peaks were relatively constant, suggesting that formation of the complex was not dynamically regulated throughout the course of the day. On the other hand, the limitations of this method are such that we cannot rule out the possibility that the association and disassociation of FRQ and the WCs is a qualitative and not a quantitative event. For example, since the complex sizes would be similar, we would be unable to distinguish WC-2 interacting with WC-1 dimers from that of WC-2 interacting with FRQ dimers. Since it is possible that the swapping of factors is regulated in a time-of-day-specific manner, we looked to see whether there was a gain or loss of either WC-1 or FRQ from a WC-2-containing complex. WC-2 protein was immunoprecipitated from extracts harvested from several time points and the recovered complex was probed for the presence of FRQ and WC-1 (Figure 4B). We found that FRQ and WC-1

associated with WC-2 throughout the day. In addition, the amount of FRQ immunoprecipitated in the complex simply mirrored the amount of FRQ available in the total extract (Figure 4C), suggesting that there was little regulation of the complex formation beyond regulation of

the amounts of the constituent proteins. Similar findings were obtained for WC-1 (Figure 4B and C). In these immunoprecipitations, WC-2 and WC-1 were quantitatively cleared from the extracts, verifying the strength of the association between WC-1 and WC-2. FRQ, however, was not cleared. Since FRQ is found both in the cytoplasm and in the nucleus (Figure 1; Luo *et al.*, 1998), it seems plausible that the non-immunoprecipitated FRQ could correspond to the non-nuclear fraction. In any case, these data suggest that the association of WC-2 with WC-1 and FRQ is not particularly regulated in a time-of-day-specific manner.

WC-2 is abundant and is in molar excess to WC-1 and FRQ proteins

To understand better the relationship between the WC proteins and FRQ and determine to what extent the abundance profiles were informative of clock functions, abundance profiles of the proteins were assessed from total extracts as a function of circadian time. We first developed the tools for individually counting the numbers of WC-1, WC-2 and FRQ proteins (see Materials and methods; Figure 5). All of these proteins were *in vitro* translated in the presence of [³⁵S]methionine. The specific activity of the radioisotopes was known and the manufacturer provided the amount of unlabeled methionine present in the *in vitro* translation mix; therefore, it was possible to calculate the specific activity of the methionine in each *in vitro* translation. The results of the translations were electrophoresed and the radioactivity in the band corresponding to the full-length proteins determined by scintillation counting. Since the number of methionines in each protein was known from the primary sequence, it was then possible to calculate the number of individual polypeptides present in each gel band. Specifically then, serial dilutions of *in vitro* transcribed and translated radiolabeled proteins were used to standardize the WC-2, WC-1 and FRQ antisera. Based on the amount of label incorporated, equimolar amounts of radiolabeled WC-2, WC-1 and FRQ (Figure 5A, left) were electrophoresed on SDS-PAGE and used for western blot analysis (right). Densitometric analysis of the western blots indicated that

Fig. 4. The WC-2, WC-1 and FRQ-containing complex is present at all times of day. (A) Densitometric analysis obtained from dark-grown (DD) extracts harvested at indicated times (between 4 and 24 h after transfer to darkness, DD4–DD24) and fractionated on sucrose gradients indicates that the sedimentation profiles of WC-2, WC-1 and FRQ do not change significantly over time. The maximum value for each time point was set to 1. (B) Immunoprecipitations with anti-WC-2 at six times across the circadian cycle show that WC-2, WC-1 and FRQ are always associated in complexes. Whole-cell protein extracts were made from cultures grown as described in (A) (left, Input), subjected to immunoprecipitation with anti-WC-2, and this immunoprecipitate probed with antisera to WC-1 and FRQ (right, Precipitate). Duplicate cultures are shown for all time points. (C) Although total levels of FRQ and WC-1 are circadianly regulated, the association of FRQ and WC-1 with WC-2 in complexes does not appear to be regulated independently. Data from (B) and additional experiments were quantified so that the amount of FRQ (triangle) or WC-1 (circles) present in one-twelfth of the immunoprecipitates in (B, Precipitate) could be divided by the amount present in one-hundredth of the whole-cell extracts (B, Input). This mean ratio of immunoprecipitable protein to total is plotted as a function of time $\pm$ SEM ($n = 5$ for FRQ; $n = 3$ for WC-1).

the signal intensities of all three proteins were within the linear range (Figure 5B), although normalization of the western blot data revealed differences in the detection limits of the FRQ, WC-2 and WC-1 antisera (Figure 5C). Using these data as a normalization factor, the absolute cellular levels of the three proteins were obtained directly from densitometric analysis of western blots of *Neurospora* extracts collected at different times of day (Figure 5D). We found WC-2 to be between 5 and 30 times more abundant than either WC-1 or FRQ depending on the time of day. During a circadian cycle, when FRQ is at the lowest level, WC-2 is ~20- to 30-fold in excess (Figure 5D). At the peak level of expression, FRQ is

5-fold lower than that of WC-2. WC-2 is also in molar excess to WC-1 at every time point examined. On the other hand, WC-1 and FRQ levels appear to be nearly equimolar relative to each other, although their rhythmic relationship shows the 8 h phase difference in peak times between FRQ and WC-1 previously reported (Lee *et al.*, 2000). On the basis of the large molar excess and constitutive expression of WC-2, these data support the hypothesis that WC-2 acts in the nucleus as a scaffold, mediating the WC-1 and FRQ interaction. At the same time, the rhythmic relationship of FRQ and WC-1 suggest that it is these essential and limiting factors that directly affect the transcriptional activation activity of the WCC (Figure 6).

Discussion

The data presented here confirm central predictions based on the present model for the PAS-protein-mediated circadian clock-associated feedback loop of *Neurospora*. We have shown that WC-2 is always present in the nucleus of the cell at levels greatly in excess of either WC-1 or FRQ. WC-2 provides the means for the interaction between the clock-regulated elements of the loop—WC-1 and FRQ—and thereby plays a central role in this aspect of the circadian system. In addition to its central role in the clock described here and elsewhere (Crosthwaite *et al.*, 1997; Collett *et al.*, 2000), WC-2 is also required for general light signal transduction (Degli-Innocenti and Russo, 1984; Linden *et al.*, 1997b; M.A.Collett, J.C.Dunlap and J.J.Loros, submitted). In this study, we provide biochemical details of the mechanism of action of these critical clock components.

The finding that WC-2 and WC-1 are nuclear proteins is consistent with their role as transcription factors and is supported by another recent study (Schwerdtfeger and Linden, 2000). Both genes contain GATA-type zinc DNA binding domains, and bind to the promoter regions of

Fig. 5. WC-2 protein is present at >5-fold molar excess compared with its interaction partners WC-1 and FRQ at all times throughout the circadian cycle, whereas WC-1 and FRQ are approximately equimolar with respect to one another. (A) *In vitro* translation of FRQ, WC-1 and WC-2 with methionine of known specific activity allows absolute quantitations of FRQ, WC-1 and WC-2 protein levels (see Materials and methods). Serial dilutions of known amounts of ^{35}S -radiolabeled *in vitro* transcribed and translated (IVT) FRQ, WC-1 and WC-2 were separated by SDS-PAGE and transferred to a membrane for western analysis; complete transfer was confirmed. Proteins were visualized by both autoradiography (left) and western analysis (right). (B) Densitometric analysis of the data from (A) shows a linear relationship between the amount of FRQ (triangles), WC-1 (circles) and WC-2 (squares) detected by western analysis and known to be present based on radioisotope incorporation (see Materials and methods). (C) The data from (B) are shown as a ratio of western blot signal intensity per fmol of protein (mean $\pm$ SEM, $n = 6$). These factors, determined separately for FRQ, WC-1 and WC-2, allow the absolute quantitative comparison of FRQ, WC-1 and WC-2 levels in cell extracts. (D) WC-2 is between 5 and 30 times more abundant than FRQ or WC-1. Whole-cell protein extracts were prepared from cultures grown in constant light (LL) or darkness for the times indicated over one and a half circadian cycles. The extracts were electrophoresed and analyzed by western analysis. The relative abundance of WC-2 (circles), WC-1 (triangles) and FRQ (open squares) in the extracts was determined by applying the data from (B) and (C) to these western blots. Data are plotted as the mean $\pm$ SEM, $n = 2$ for WC-1 and FRQ; $n = 3$ for WC-2.

Fig. 6. Temporal changes in WC-1 and FRQ levels dictate changes in the WC-2–FRQ–WC-1 complexes that drive changes in *frq* expression: A schematic model indicating a basis for WC-2 mediated rhythmic transcription of *frq*. Top: in the early morning hours *frq* RNA and FRQ levels are low. Cellular levels of WC-1 exceed the levels of FRQ. An excess of the heterodimeric WC-1–WC-2 complex (WCC) exists and is active resulting in an increase *frq* transcription. Bottom: through the day, FRQ protein accumulates in the cell and binds to the WCC complex preventing it from activating *frq* transcription. At the same time, cellular levels of WC-1 are declining. The combination of these actions, an increase in FRQ protein and a decrease in WC-1, limits the number of transcriptionally active WCCs, resulting in a decrease in *frq* expression and in steady state levels of *frq* mRNA.

light-induced genes (Ballario *et al.*, 1996; Linden *et al.*, 1997a). The unexpected finding was that WC-2 is present in the nucleus all the time, and that a robust rhythmic expression profile was not observed. It was possible that since WC-2 is required for rhythmic expression of *frq* transcript, WC-2 expression could be expected to be rhythmic. However, WC-2's constitutive expression suggests that the abundance level of WC-2 is not the primary factor responsible for clock regulation of *frq*, and in fact, since WC-2 is much more abundant than FRQ, it seems likely that some WC-2 in the cell is not involved in rhythm generation. The role of WC-2 as one of the PAS protein partners in the clock-associated feedback loop in *Neurospora* is similar to that of CLOCK in rhythmic

cells of the mammalian suprachiasmatic nucleus (SCN) and CYC in rhythm generating cells of *Drosophila*; interestingly, none of these proteins is rhythmically expressed, whereas expression of their PAS partners—WC-1, BMAL1 and CLK—is circadianly regulated (Darlington *et al.*, 1998; Hogenesch *et al.*, 1998; Honma *et al.*, 1998; Lee *et al.*, 1998, 2000; Shearman *et al.*, 2000). Based on the molecular, regulatory and sequence parallels among these the components of loops, it seems possible that CLOCK expression may be found to be elevated with respect to BMAL in the clock relevant cells just as WC-2 and CYC are elevated with respect to their partners.

Transcription factor activity may be regulated directly through protein–protein interactions. WC proteins both contain PAS domains that mediate heterodimerization (Ballario *et al.*, 1998; Talora *et al.*, 1999) as also demonstrated in PAS proteins of other systems (Huang *et al.*, 1993, 1995; Gekakis *et al.*, 1998; Honma *et al.*, 1998; Rutila *et al.*, 1998). Both WC-1 and WC-2 are required for transcriptional activation of *frq*, and their interaction via PAS domains is likely to be essential for their activation functions. Since it has been established that FRQ is the negative element in the feedback loop (Aronson *et al.*, 1994b), it seemed plausible that FRQ would directly interact with the WC complex as a method of repressing the WC activity (e.g. Crosthwaite *et al.*, 1997; Loros, 1998; Dunlap, 1999). Previous data on the association of FRQ with itself or other proteins, however, was ambiguous, with sucrose gradients consistent with little complexing and gel filtration data suggesting a complex, prompting the tentative (and incorrect) conclusion that FRQ was ‘probably not typically associated with a large protein complex’ (Garceau *et al.*, 1997). In this work, we have provided several lines of evidence showing that WC-2 is in a stable complex with WC-1 and FRQ. First, WC-2 directly interacted with FRQ *in vitro*. Secondly, better designed sucrose gradient experiments showed that all three proteins sediment at larger than expected molecular weight, and the sedimentation profiles overlapped one another. Thirdly, WC-2 antibodies co-immunoprecipitated WC-1 and FRQ. Taken together, we conclude that a multimeric complex containing interacting positive (WC-1 and WC-2) and negative components (FRQ) exists in the cell.

What is the nature of the multimeric complex? It is possible that there are several co-existing populations of multimeric complexes. By using the predicted size of each native protein and measuring the migration of the protein markers on the gradient, we can extrapolate the approximate size of the complex and predict the number of potential populations. For example, we calculated that the native form of FRQ exists in the cell in the form of a high molecular weight protein, ~250–275 kDa. This size is consistent with a trimeric complex containing WC-1 (~120 kDa), WC-2 (~60 kDa) and FRQ (~100 kDa), but is also consistent with a FRQ–FRQ–WC-2 complex. Thus, the exact arrangement and or stoichiometry of the complex cannot be determined from these experiments. For these reasons we utilized additional strategies to investigate the nature of the complex. Our *in vitro* binding data establish a direct WC-2–FRQ association. *In vivo* co-precipitation studies also substantiate WC-2 interacting with FRQ as

well as WC-1. The significant findings that WC-2 is necessary for the WC-1–FRQ interactions and, from reciprocal immunoprecipitation studies using WC-1 and FRQ antisera, that both proteins interact with WC-2 together, provide compelling evidence for the existence of a heterotrimeric complex containing WC-2, WC-1 and FRQ, and places WC-2 in the center of the action.

The next pressing question revolves around how this trimeric complex functions in the cell. Specifically, how does FRQ-dependent inhibition of the WCC override *frq* gene activation? One possibility could be that FRQ directly binds to and physically disrupts an active WCC. Our data suggest that this is probably not the case. First, if the WCC dissociated in the presence of FRQ then the WC's sedimentation profile on a sucrose gradient would shift. We did not see a significant change in the peak profile of WC-2, WC-1 or FRQ at any of the time points studied. Similarly, our quantitative co-immunoprecipitation experiments suggest that the stoichiometry of the complex remains stable throughout the course of the day consistent with other findings that also describe a stable WCC (Talora *et al.*, 1999). In their work, the authors portray a stable WCC that does not dissociate in the dark or after exposure to light. Along these same lines, our studies show that even in the presence of a negative element, FRQ, the WCC is stable. Likewise, *in vitro* and *in vivo* studies in *Drosophila* have also shown non-disruptive interactions between positive elements (CLK–CYC) and negative elements (PER–TIM) forming a stable tetrameric complex (Lee *et al.*, 1999; Bae *et al.*, 2000).

An alternative hypothesis to the molecular mechanism of action of WC-2 in the cell, is that it is not the qualitative association of the complex that is important, but the quantitative relationship among its elements. Our data describe the abundance profiles of the three proteins and establish that WC-2 is always in molar excess to WC-1 and FRQ. From this we infer that WC-1 and FRQ, which are present at near equimolar levels relative to each other, may be the limiting factors regulating *frq* transcription. If so, the feedback model, illustrated in Figure 6, may be described as follows. At around midnight, *frq* RNA and FRQ levels are low. WC-1 levels exceed FRQ levels. WC-1 heterodimerizes with WC-2 through their PAS domains to form an active transcription complex resulting in an increase in the level of *frq* expression. Around midday, FRQ protein is at its peak, binds to and sequesters WC-2 and at the same time WC-1 levels decline. This combination causes a resultant decrease in the active transcription complex and *frq* expression begins to decline. Throughout the cycle, WC-2 acts as a nuclear organizing center bringing together both positive and negative acting elements.

In summary we now have strong biochemical evidence for the formation of a WC-2–WC-1–FRQ multiprotein complex in cells. All the components that form this complex are essential for maintaining circadian rhythmicity. The challenge that lies ahead is determining how this complex is regulated and how the complex mediates rhythmic transcription of genes. Answers to these questions will further our understanding of the molecular mechanism of action of these critical clock components,

and in our general understanding of the normal operation of this circadian clock.

Materials and methods

Strains and growth conditions

As a clock wild-type strain, we used *bd; frq⁺ A*, and general growth conditions that have been described previously (Davis and deSerres, 1970). Vogel's minimal medium supplemented with 2% glucose, and 50 ng/ml biotin and supplemented with 1.5% agar was used for the formation of solid media. For liquid culture experiments under circadian conditions, conidia from 7- to 10-day-old slants were suspended in 1 ml of sterile water and inoculated into plastic Petri dishes containing minimal media and incubated at 30°C in constant light for ~24–48 h until mycelial mats formed. Two to four millimeter disks of mycelia were cut from the mats and transferred to 50 ml of fresh media in 250 ml Erlenmeyer flasks. Flasks were shaken at 125 r.p.m. on rotary shakers in light at 24°C. For QA induction experiments, 100 mM QA (pH 5.2) was added directly to the liquid media and cultures were harvested 6–12 h later. Methods for growing rhythmic cultures have been published previously (Aronson *et al.*, 1992; Crosthwaite *et al.*, 1997). Briefly, liquid cultures were started as described above and shaken at 125 r.p.m. in the light at room temperature for 4–8 h. The cultures were then transferred into darkness and harvested at the indicated times.

Expression and purification of GST and GST–WC-2 proteins

Full-length *wc-2* cDNA (1–1590 bp) was amplified and directionally cloned in-frame into the pGEX-4-T (Pharmacia, Piscataway, NJ) vectors downstream from the GST gene. Correct pGEX-4-T–WC-2 construction was verified by sequence analysis. For the generation of bacterially expressed recombinant proteins, two plasmids, pGEX-4-T alone or pGEX-4-T–WC-2, were electroporated into BL21 cells. Briefly, 200 ml of transformed cells were grown to logarithmic phase while shaking at 37°C. Fusion proteins were chemically induced for 4 h with 1 mM IPTG. Next, cells were lysed by four cycles of freeze–thaw or sonication in phosphate-buffered saline (PBS) buffer (137 mM NaCl, 10 mM KH₂PO₄, 100 mM Na₂HPO₄, 27 mM KCl, pH 7.4) containing 1 mM EDTA, 1 µg/ml each pepstatin A and leupeptin, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1% Triton X-100, and 500 µM dithiothreitol (DTT). Cellular lysate was cleared by 20 min centrifugation (14 000 g) at 4°C. GST-containing proteins were batch or column purified by affinity chromatography using glutathione-coupled Sepharose. Proteins were either analyzed directly from the bacterial lysates by SDS–PAGE or purified and eluted in 1 ml fractions in the presence of 10 mM glutathione then analyzed by SDS–PAGE.

Antibody production and western analysis

The bacterially expressed and purified GST–WC-2 was used as an antigen for injection into rabbits following standard procedures (Pocono Rabbit Farm & Laboratory, Canadensis, PA). For western blot analysis, *Neurospora* proteins were extracted using essentially the same procedure as published previously (Garceau *et al.*, 1997), with minor modifications. Briefly, ground tissue was suspended in extraction buffer (137 mM NaCl, 50 mM HEPES pH 7.4, 10% glycerol, 1 mM EDTA, 1 µg/ml pepstatin A and leupeptin, and 1 mM PMSF). Fifty to one-hundred micrograms of clarified extracts were resolved on a 7.5% SDS–PAGE and transferred to PVDF membrane using semi-dry blotting apparatus (Bio-Rad, Hercules, CA). Immunoblots were incubated in the presence of anti-WC-2 (1:10 000) or with FRQ or WC-1 antibodies as described previously (Garceau *et al.*, 1997; Luo *et al.*, 1998; Lee *et al.*, 1999). Proteins were visualized by enhanced chemiluminescence (ECL; Amersham, Arlington Heights, IL). Signal intensity was determined by densitometric analysis using NIH image (v. 1.61). Membranes were stained with either Coomassie Blue or amido black to verify equal loading and transfer of the proteins.

In vitro transcription/translation and quantitation

Plasmids containing either WC-2, FRQ or truncated WC-1 placed under the control of either T7 or T3 promoter were used in combination with their respective polymerase and in the presence of [³⁵S]methionine in a coupled transcription/translation reaction of a rabbit reticulocyte system in accordance to the manufacturer's instructions (TNT, Promega, Madison, WI). The amount of protein synthesized in each reaction was determined by resolving an aliquot of the reaction on a 7.5% SDS–PAGE. After visualizing the proteins by autoradiography, the respective protein

products were excised and quantitated by liquid scintillation counting. The amount of protein synthesized was determined by measuring the incorporation of labeled methionine from the gel-purified proteins, considering the number of methionines contained in each protein and factoring in the molar ratio of unlabeled [endogenous methionine present in the lysates as assayed by the manufacture (Promega)] to labeled methionine present in each reaction. To assess the antisera sensitivities, a standard curve was generated by resolving serial dilutions of the calculated known molar amount of each synthesized proteins on a 7.5% SDS-PAGE and performing western blot analysis using ECL or ECL-plus (Amersham). Subsequent signal intensity/fmol of WC-2, WC-1 and FRQ protein was determined. For each protein, the exposure time was the same and each dilution series yielded a linear densitometric signal as more fully shown in Figure 5B. To ensure equal loading and transfer, the membrane was also subjected to autoradiographic analysis. For WC-2-FRQ mapping studies, a series of ³⁵S-labeled truncated FRQ protein was systematically synthesized in accordance to the manufacture's instructions (T7 & Quick, Promega). Briefly, PCR products were generated using a FRQ-containing plasmid as a template, in addition to T7-specific 5' primers in combination with a series of nested, *frq* sequence-specific 3' primers. Five microliters of the gel-purified PCR products were used directly as templates in a [³⁵S]methionine-containing reticulocyte reaction.

Immunoprecipitation

One to five milligrams of protein was extracted in immunoprecipitation buffer (PBS, 50 mM HEPES, 10% glycerol, 1 µg/ml pepstatin A, 1 µg/ml leupeptin, 1 mM PMSF) and incubated with anti-WC-2 antiserum (1:250) for 2 h at 4°C. Twenty microliters of washed protein A-Sepharose (Pharmacia) were added and extracts were incubated for an additional 60 min. Protein A-Sepharose was sedimented and washed twice with 1 ml immunoprecipitation buffer containing 1% Triton X-100 and twice with 1 ml immunoprecipitation buffer.

In vitro GST binding assay

In vitro radiolabeled proteins were synthesized using reticulocyte reaction (Promega). GST-containing proteins were batch purified using glutathione-coupled Sepharose. Labeled proteins were incubated 2 h with 10–20 µg of purified GST-WC-2 or GST proteins. Sepharose was sedimented by centrifugation, washed twice with PBS containing 1% Triton X-100 and twice with PBS. After the final wash, adherent proteins were electrophoresed on 7.5% SDS-PAGE and visualized by either Coomassie Blue staining of the gel or autoradiography.

Sucrose gradient centrifugation

Methods for sucrose gradient analysis have been published previously (Garceau *et al.*, 1997). Briefly, 200 µl (1–2 mg) of nondenatured cell extract was layered on a 12 ml 10–30% sucrose gradient made in extraction buffer lacking glycerol and fractionated (40 000 r.p.m.) for 16 h in SW-40 rotor. BSA, β-amylase and β-galactosidase were used as molecular weight standards in a parallel gradient. Four-hundred microliter fractions were collected from the top to the bottom and 90 µl of each fraction was used for western blot analysis.

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