WHITE COLLAR-1, a Multifunctional Neurospora Protein Involved in the Circadian Feedback Loops, Light Sensing, and Transcription Repression of wc-2

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WHITE COLLAR-1 (WC-1) and WC-2, the two PAS domain-containing transcription factors, are the positive elements of the circadian feedback loops in Neurospora. In addition, both proteins are essential components for the light input of various blue light responses, including the light entrainment of the circadian clock. Recently, we identified WC-1 as the blue light photoreceptor responsible for these light responses. In this study, we show that the formation of the FRQ-WC complex in vivo, a step critical in closing the circadian negative feedback loop, requires WC-1. In addition, we show that WC-1 negatively regulates the expression of wc-2 at the level of the transcription, forming another interacting loop. In a wc-1 mutant, we demonstrate that there is alternative protein initiation of WC-1, and the requirements of WC-1 for the light induction of frq and other genes differ significantly, suggesting the existence of different WC complexes in the cell. Consistent with this interpretation, our results show that there are at least two different types of WC-1/WC-2 complexes in vivo, and that the larger WC-1/WC-2 complex contains more than one WC-1 molecule. Using a series of wc-1 mutants, we show that the WC-1 PASC domain and its C-terminal region are essential for the formation of the WC-1/WC-2 complex. Functional analyses reveal that the DNA-binding domain of WC-1 is required only for the activation of frq in the dark and not for the light function of the protein, confirming that WC-1 is a multifunctional protein with separable protein domains.

Endogenous circadian clocks control a wide variety of daily physiological, behavioral, cellular, and biochemical activities in most eukaryotic and certain prokaryotic organisms. The circadian oscillators are networks of positive and negative elements that form the core circadian feedback loops generating the basic circadian rhythmicity (1, 2). The positive elements of the loop activate the transcription of the negative elements, whereas the negative elements feedback to block their own activation. In Neurospora, Drosophila, and mammals, the positive elements of the loops are all heterodimeric protein complexes consisting of PER-ARNT-SIM (PAS) domain-containing transcription factors (1, 2). In each system, the heterodimeric complex activates the transcription of the negative elements, and the protein products of the negative elements close the feedback loop by inhibiting their own transcription through direct physical interaction with the positive elements (3–9). In these systems, the negative elements of the oscillators also activate the expression of one or two of the positive elements, forming positive feedback loops that interlock with the negative ones (10–13).

In the Neurospora frq-wc based circadian feedback loops, WHITE COLLAR-1 (WC-1) and WC-2, the two PAS domain-containing transcription factors (containing GATA type zinc-finger DNA binding domains) form heterodimeric complexes (14) and act as the positive components (15, 16). On the other hand, two alternatively translated FREQUENCY (FRQ) protein forms are the negative elements (7, 15, 17, 18). In constant darkness, the WC-1/WC-2 heterodimeric complex binds to two light-regulated elements (LREs) in the promoter of frq and activates the transcription of frq (13, 16, 19). In either wc-1 or wc-2 mutants, the levels of frq RNA and FRQ protein are very low in the dark, and the circadian clock is not functional under normal conditions (13, 16, 20, 21). In addition, WC-1 is the limiting factor for the formation of the WC-1/WC-2 complex (13, 18).

After the transcription of frq, two forms of FRQ protein (large and small FRQ forms) are made (17, 22), and they exist in homodimeric complexes (15). After their amounts reach a certain level, they feedback to repress the transcription of frq by interacting with the WC-1/WC-2 complex (7, 15, 18, 23, 24), thus closing the negative feedback loop. In addition to its role as a negative element of the loop, FRQ positively regulates the expression of both WC-1 and WC-2 through two different mechanisms, forming positive feedback loops interlocked with the negative feedback loop (12, 13, 20). Our previous data suggest that the positive feedback loops are important for the robustness and stability of the clock (13).

In addition to their essential role in the circadian feedback loop, WC-1 and WC-2 are also essential components in the light input of the clock and other light responses in Neurospora (16, 25–27). In true wc-1 or wc-2 null mutants, most if not all light responses are abolished, including the light induction of frq (21, 28). Recently, we (28) and Froehlich et al. (19) identified WC-1, a flavin dinucleotide (FAD)-containing protein, as the blue light photoreceptor mediating these light responses. The result

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1 The abbreviations used are: PAS, PER-ARNT-SIM; WC-1, WHITE COLLAR-1; FRQ, FREQUENCY; al-3, albino-3; vvd, vivid; aa, amino acid; LRE, light-regulated element; QA, quinic acid; NLS, nuclear localization signal; LL, constant light.
of the in vitro DNA binding assay suggests that a large WC complex (that is different from the dark complex) binds to the frq LREs in a light-dependent manner to mediate light input of the clock (19). Thus, light irradiation triggers rapid induction of frq transcription, a process that leads to the resetting of the clock (29). Although the level of WC-1 determines the concentrations of the WC complex formed in vivo (13), the WC-2 PAS domain-mediated WC-1/WC-2 complex formation is important for maintaining the steady state level of WC-1 and its functions in the circadian clock and light responses (20). Without WC-2 or the formation of a WC-1/WC-2 complex, the level of WC-1 in the cell is low (20).

These previous studies indicate that WC-1 is a protein with at least two roles: it is a basic positive element in the dark and a photosensing transcriptional activator mediating light responses. Previously, WC-2 was proposed to function as a scaffold protein that mediates the FRQ-WC interaction to close the negative feedback loop (18); however, the involvement of WC-1 in this interaction is unclear. Although it is clear that WC-2 positively regulates WC-1 by forming the WC complex, it is not known whether WC-1 regulates the expression of WC-2. In addition, based on the data described above, there should be different forms of the WC complexes with distinct functions in the cell, but no in vivo evidence is available about the nature of the different WC complexes. Furthermore, we do not know which domain of WC-1 mediates its interaction with WC-2 and whether different regions of WC-1 have distinct functional roles. In this study, experiments were carried out to address these questions. Together, our results demonstrate that WC-1 is a multifunctional protein with separable protein domains.

EXPERIMENTAL PROCEDURES

Strains and Culture Conditions—The bd, a strain was used as the wild-type strain in this study. The wc-1(ER53) strain was made previously (28), and sequencing of the wc-1 locus revealed numerous G/C → A/T point mutations introduced by repeat-induced point mutation (RIP), resulting in several premature stop codons in the WC-1 open reading frame. Western blot analysis showed that no WC-1 protein was expressed in this strain. This wc-1(ER53) strain was used as the host strain for various his-3 targeting wc-1 constructs.

The wc-1 mutant (FGSC number 4401, wc-1(4401)) was obtained from the Fungal Genetics Stock Center. The wc-1 mutant strain, wc-1RIP described previously (16), produces a truncated WC-1 protein. The wc-1(qa-1wc-1) and frq(qa-1frq) strains were described previously (13).

Liquid culture conditions were the same as those previously described (7, 15, 22), except a lower glucose concentration was used in the media for strains used for quinic acid (QA) induction (1 x Vogel's, 0.1% glucose, 0.17% arginine, 0.01 M QA).

Plasmids—The Myc-WC-1 and Myc-WC-2 constructs were made previously (20). The Myc-WC-1 construct can rescue the circadian and light phenotype of the wc-1(ER53) strain, as described previously (15, 22). Equal amounts of total RNA (20 µg) were loaded onto agarose gels for electrophoresis, and the gels were blotted and probed with an RNA probe specific for frq, albino-3 (al-3), vivid (vd), or wc-2 mRNA.

RESULTS

WC-1 Is Required for the FRQ-WCC Interaction in Vivo—In the Neurospora circadian negative feedback loop, the closing of the loop is achieved by the direct physical interaction between FRQ and the WC complex (15, 18, 23). To examine whether WC-1 is important for the formation of the FRQ-WC complex in vivo, two FRQ expressing wc-1 mutant strains were used: wc-1(4401) and wc-1RIP. In the wc-1(4401) strain, as described below, the expression of WC-1 is very low, but the expression of FRQ and WC-2 is near normal in LL (Fig. 1). In the wc-1RIP strain, the expression of frq is under the control of the quinic acid-inducible promoter and can be induced by the addition of QA (Fig. 1). When the cell lysates of these two strains and a wild-type strain were subjected to immunoprecipitation with our WC-2 antiserum, although both FRQ and WC-1 were co-immunoprecipitated with WC-2 in the wild-type sample, no interaction between FRQ and WC-2 was found in the two mutant samples. This result demonstrates that the presence of WC-1 is essential for the FRQ-WC-2 interaction.

Because of the low level of WC-1 in wc-1 null strains (20), we were not able to confirm whether WC-2 is required for the FRQ/WC-1 interaction. However, because of the important role of WC-2 in maintaining the level of WC-1, both proteins should be important for the FRQ-WCC interaction in vivo, and FRQ can bind only the WC-1/WC-2 heteromeric complex.

WC-1 Negatively Regulates the Transcription of wc-2—Previously, it was shown that the levels of both WC-1 and WC-2 proteins are positively regulated by FRQ (12, 13, 23). Because of the low levels of FRQ in wc-1 strains, we expected that the WC-2 protein level would be low as well. However, we found comparable amounts of WC-2 in the wc-1(ER53) strain and in the wild-type strain (Fig. 2A). There are two possible explanations for this result. First, the low level of FRQ in the wc-1(ER53) strain might be able to support normal expression of wc-2. Second, WC-1 may negatively regulate wc-2; therefore, in the wc-1(ER53), the negative effect of WC-1 and the positive effect of
FRQ on wc-2 counterbalance each other, resulting in a normal WC-2 level.

To test the first possibility, the levels of WC-1 and WC-2 were monitored in the frq -qa-FRQ strain in which the level of FRQ was induced to different levels in the presence of various concentrations of the QA inducer (Fig. 2B) (13). Therefore, the levels of WC-2 in this strain should inform us about the importance of the FRQ level on the expression of WC-2. When the QA concentration was less than $1 \times 10^{-3}$ M, FRQ level was low, and the levels of WC-1 and WC-2 were comparable with those without QA (Fig. 2B) and those in a frq null strain (data not shown). As the concentration of QA increased, leading to an increase in FRQ amount, the levels of both WC-1 and WC-2 also increased. At $10^{-3}$ M QA, the induction of FRQ appeared to reach the peak, as did the levels of the WC proteins. This result suggests that the amount of FRQ determines the level of WC-2. Thus, it is unlikely that the residual amount of FRQ in the wc-1RIP strain is able to fully support the expression ofwc-2.

To test the second possibility, we examined the effect of WC-1 on WC-2 in a frq or wc-1 strain in which the expression of WC-1 is under the control of the QA-inducible promoter (frq -qa-wc-1 or wc-1RIP qa-wc-1). In the wc-1RIP qa-wc-1 strain, the level of WC-2 did not respond to the change in WC-1 level (Fig. 2C), probably because the induction of WC-1 in this strain also resulted in an increase of FRQ, so that the positive effect of FRQ on wc-2 counterbalanced that of the WC-1. However, in the frq -qa-wc-1 strain, the WC-2 level decreased slightly after the induction of WC-1 (Fig. 2C). This small but reproducible decrease in the WC-2 level is likely due to the low level of WC-2 in the frq -background; thus, further reduction of WC-2 by the induction of WC-1 was limited. In addition, the induction of WC-1 was limited in this strain because of the absence of FRQ, as FRQ post-transcriptionally regulates WC-1 expression (20).

To further examine the frq-independent role that WC-1 has on the expression of wc-2, a frq -wc-1 double mutant was made by crossing the frq -strain with a wc-1 -strain. Compared with the low level of WC-2 in the frq -strain, WC-2 levels in the double mutants were significantly higher, and their levels were comparable to those in a wild-type strain (Fig. 3A). To confirm this result, the qa-wc1 construct was introduced into the double mutant strain. As expected, the level of WC-2 decreased significantly when WC-1 was induced in the presence of QA (Fig. 3B). Northern blot analysis performed using this strain further showed that WC-1 negatively regulated wc-2 at the transcriptional level in a light-independent manner (Fig. 3C). Together, these results demonstrate that WC-1 negatively regulates the expression ofwc-2 independent of frq. WC-1 was previously regarded as a transcriptional activator in the circadian clock and in light responses, our results here suggested a novel light-independent repressor function of WC-1.

**Alternative Protein Initiation of WC-1 and Differential Requirement of WC-1 for the Light Induction of frq and Other Light-inducible Genes**—Previously, it was shown that WC-1 is required for the light induction of frq and other light-inducible genes (16, 25). However, we found one wc-1 mutant strain (wc-1A681) in which no detectable amount of WC-1 was produced, but it has a normal amount of FRQ in constant light (Fig. 4A). In addition, its FRQ level in the dark was low, a level comparable with that in a wc-1 null strain (Fig. 4A and data not shown). Phenotypically, this strain resembles other wc-1 mutant strains with white mycelia and exhibits arrhythmicity in constant darkness (data not shown). Northern blot analysis confirmed that the al-3 gene (a gene required for carotenoid biosynthesis) is not light-induced in this strain, although frq RNA was light-induced to a level that was about half of the wild-type induction level (Fig. 4B).

Recently, we and others identified WC-1 as the photoreceptor for circadian clocks and other light responses (19, 28). In addition, our analyses of the wc-1RIP strain and other wc-1 mutant
strains have shown that WC-1 is essential for the light induction of frq (28). To understand the nature of the wc-1 mutation in this strain, its wc-1 gene was cloned and sequenced. Sequencing of the wc-1 gene of this strain revealed 1 base pair deletion at nucleotide 184 (C) of WC-1, causing frameshift and premature protein termination (Fig. 4C). Because this mutant should produce only a small truncated form of WC-1 (66 aa) and behaves like a wc-1 null strain, why is frq still light-induced in this strain?

Three possible explanations might explain this result. First, WC-1 may not be the photoreceptor responsible for the light-activated transcription of frq. However, this possibility cannot fully explain the existing molecular and biochemical data (19, 28), nor can it explain the complete abolishment of frq light induction in other wc-1 mutants (16). Second, the 61-aa WC-1 N-terminal region may be capable of sensing light, but there is no obvious protein motif in this region of WC-1 to suggest such a role. Third, an undetectable amount of WC-1 may be produced in this strain as a result of alternative protein initiation using a downstream AUG, and this truncated WC-1 may be responsible for the light induction of frq. If this latter possibility is true, it will indicate that various light-inducible genes may require different amounts of WC-1 protein. Indeed, a small but detectable amount of light induction of vvd RNA (31) (less than 1% of the wild-type induction) was observed in this strain (Fig. 4B), and its conidiation rhythm can still be weakly entrained by light/dark cycles (32), suggesting that there is some residual WC-1 activity in this strain.

Alternative protein initiation can be caused by a “leaky scanning mechanism” in which the 40S ribosomes fail to start translation from the first AUG and use a downstream AUG instead (33). Upon examining the wc-1 sequence, we found another AUG at aa 86 with a good Kozak consensus sequence. To test this hypothesis, the same mutation in the wc-1null strain was introduced into a Myc-WC-1 construct (Myc-WC-1.4401), and this construct was transformed into the wc-1null strain. In the Myc-WC-1 construct, WC-1 is tagged by a 5-Myc epitope tag, allowing the detection of the tagged protein using a monoclonal c-Myc antibody (15, 20). In addition, the wild-type construct can rescue the circadian and light phenotype of the wc-1 null strain (13). As expected, the transformants carrying the mutant construct showed a level of FRQ expression in LL comparable with that in the wild-type strain (data not shown), whereas no WC-1 protein in the lysate could be detected using the monoclonal c-Myc antibody (Fig. 4D, left). However, by performing an immunoprecipitation assay with WC-2 antisera using 5 mg of the lysate of the Myc-WC-1.4401 strain, a smaller than the wild-type Myc-WC-1 band was detected (Fig. 4D). The apparent molecular weight shift indicated that WC-1 translation was reinitiated from a downstream AUG in the mutant, probably through a leaky scanning mechanism. We estimated that the amount of Myc-WC-1 in the Myc-WC-1.4401 strain is less than 1% of the Myc-WC-1 strain. Together, these data demonstrate that there is alternative protein initiation in the wc-1null strain, and the light induction of frq requires only a very low level of the WC complex. Therefore, the light induction of frq and other light-inducible genes was differentially regulated by WC-1, suggesting that there are different WC-1/WC-2-containing complexes in the cell to mediate light induction of frq and other genes.

There Are Two Types of WC Complexes and a Portion of WC-1 Self-associates—Recently, the results of the in vitro DNA binding assay showed that there are two different WC-1-containing complexes that bind to the LRE elements of the frq promoter (28). Whereas the smaller complex that binds to the LRE in the dark is consistent with it being the WC-1/WC-2 heterodimer, the complex that binds to the LRE in a light-dependent manner is considerably larger. Because a similar larger complex was also found using in vitro translated WC-1 and WC-2 proteins, the WC proteins may form multimers. Previously, using the in vitro protein binding assay, it was shown that WC-1 or WC-2 mostly self-associates instead of forming a WC-1/WC-2 heterodimer (34). Although it is known that WC-1 and WC-2 form a tight complex in vivo (14, 28), there is no in vivo evidence to show the self-association of WC-1 or WC-2.

To examine this, the Myc-WC-1 and the Myc-WC-2 constructs were transformed into a wild-type strain. Either con-
struct has been shown to be able to rescue the clock and light phenotypes of either wc-1 or wc-2 mutants (20). As shown in Fig. 5A, Myc-WC-1 was expressed in the wt,Myc-WC-1 strain at a level that is about 50% of the endogenous WC-1. To examine the self-association of WC-1, the protein lysate of this strain was immunoprecipitated using the c-Myc antibody or our WC-1 antiserum. As shown in Fig. 5A, a small amount of WC-1 protein at the position of the endogenous WC-1 co-precipitated with the Myc-WC-1, and Western blot analysis using the c-Myc antibody showed that it was not the degradation product of the Myc-WC-1 protein. Similar results were obtained in four independent experiments. Comparison of the levels of two different WC-1 forms in the total extract and the immunoprecipitates showed that about 20–30% of WC-1 self-associated. As expected, both WC-2 and FRQ also co-immunoprecipitated together with Myc-WC-1. This result indicates that there are different forms of the WC-1-containing complexes in the cell. Whereas most of the WC-1 forms a heterodimer with WC-2, some WC-1 self-interacts to form larger complexes. This interpretation is consistent with the previous results of the sucrose gradient and in vitro DNA binding assay (18, 19).

In contrast to WC-1, WC-2 self-interaction was not detected in vivo. As shown in Fig. 5B, no endogenous WC-2 was found to co-precipitate with the Myc-WC-2 protein in the wt,Myc-WC-2 strain. This result suggests that the strong self-interaction observed in the in vitro protein binding assay is very likely an artifact. Because WC-2 is required for maintaining the steady state level of WC-1 through its PAS domain-mediated WC-1/WC-2 interaction (20), the existence of WC-1 self-association and the absence of WC-2 self-interaction suggest that one WC-2 molecule can form a complex with more than one WC-1 molecule in the cell.

The WC-1 PASC Domain and Its Immediate C-terminal Region Is Essential for the WC-1/WC-2 Interaction in Vivo—Previously, we showed that the PAS domain of WC-2 is essential for mediating the WC-1/WC-2 interaction in vivo (20). However, it is not known which region of WC-1 binds to the WC-2 PAS domain. Although the PAS domains of both proteins were proposed to interact with each other based on the in vitro protein result (34), as we have shown above, the protein-protein interactions observed in vitro do not necessarily exist in vivo. Therefore, a series of wc-1 deletion mutants were created and experiments were carried out to determine which region of WC-1 mediates the interaction with WC-2 in vivo.

Sequence analysis of WC-1 revealed that it contains two putative transcription activation domains, three PAS domains (including the LOV domain), one putative nuclear localization signal (NLS, aa 919 to 926), and a GATA-type zinc finger DNA-binding domain. A series of internal and C-terminal deletions of WC-1 were generated. The putative N-terminal transcription activation domain clearly is dispensable, because it is missing in the wc-1<sup>Δ507</sup> strain (Fig. 4). Fig. 6A is a schematic diagram of the WC-1 open reading frame and the deletion made in each mutant. Except for the three constructs at the bottom of the diagram that were made in the qa-wc-1 plasmid (allowing the expression of WC-1 in the presence of QA), the rest of the constructs were made in the Myc-WC-1 plasmid, so that the WC-1 protein can be monitored by the c-Myc monoclonal antibody. This is important when the level of WC-1 is low in the
mutants. All constructs were transformed into the \textit{wc-1}\textsuperscript{null} strain. Based on the structural predictions of the three PAS domains, small deletions predicted to abolish the formation of PAS structures were introduced into each domain.

To map the WC-1 domain that is essential for the interaction with WC-2, the lysates of the deletion mutants were subjected to immunoprecipitation using our WC-2 antiserum. As shown in Fig. 6B, in mutant that contains the deletion of the WC-1 N-terminal region (\textit{qa-wc-1.NH}) or the disruption of the first two PAS domains, the formation of the WC-1/WC-2 complex was maintained, indicating that the entire N-terminal half of the WC-1 protein is not required for the complex formation. However, the disruption of the PASC domain (Myc-WC1.PASC) or the removal of the C-terminal region downstream of aa 764 (Myc-WC1.NruI) completely abolished WC complex formation. Thus, the PASC domain and its C-terminal region are essential for the formation of the WC complex.

The low level of WC-1 expression in the PASC mutant is consistent with our previous conclusion that the formation of the WC complex is important for maintaining the steady state level of WC-1. Interestingly, although the deletion of the C-terminal of WC-1 in the Myc-WC1.NruI strain eliminated the WC-1/WC-2 interaction, its level of WC-1 was significantly higher than that of the PASC mutant. This could be due to the fact that the removal of the WC-1 C-terminal region stabilized the protein. Although the LOV and PASB domains are not required for the formation of the WC complex, their disruption, especially the mutation of PASB, did reduce the expression level of WC-1 significantly. Therefore, it is possible that they also influence the WC-1/WC-2 interaction.

To further map the region essential for the WC interaction, deletion mutants downstream of aa 764 were generated. Our results showed that the region upstream of the putative NLS is required for the interaction, but the NLS and its C-terminal region are dispensable. As shown in Fig. 7A, the removal of the C-terminal region downstream of aa 887 (WC-1.BamHI and WC-1.MluI) eliminated the WC-1/WC-2 protein interaction; however, in all mutants that contain the region upstream of the putative NLS (aa 918), the WC-1/WC-2 interaction could still be observed (Fig. 7B). In addition, the deletion of the NLS or its C-terminal region did not abolish the FRQ-WC interaction. Therefore, the minimal region that is required for the WC-1/WC-2 interaction appears to be from the PASC domain to aa 918.

Because WC-2 is a nuclear protein and its nuclear localization is independent of WC-1 (14, 20, 35), one possibility for the failure of the WC-1/WC-2 interactions in some of the mutants is that the mutant WC-1 proteins failed to enter the nucleus. To exclude this possibility and to examine the role of the putative NLS signal in the nuclear localization of WC-1, the localization of WC-1 in the NLS mutants and other mutants was examined. In these strains, WC-1 proteins were still found in the nucleus (data not shown). Therefore, the putative NLS signal and the regions required for the WC-1/WC-2 interaction are not required for the nuclear localization of WC-1. A putative NLS signal, located immediately upstream of the zinc finger domain, is also found in WC-2. It was previously shown that this signal was not required for the nuclear localization of WC-2 (35). The similarities of these two putative NLS signals in sequence and location suggest that they may be part of the domain involved in DNA binding rather than mediating nuclear import of the proteins, a notion that is supported by the functional study described below.

\begin{figure}[h]
  \centering
  \includegraphics[width=\textwidth]{fig7.png}
  \caption{The PASC domain and its C-terminal region of WC-1 are required for the WC-1/WC-2 interaction. (A) Immunoprecipitation assays using either WC-1 or WC-2 antisera showing that the C-terminal region of the PASC domain is required for the WC-1/WC-2 interaction. In the \textit{wc-1}\textsuperscript{null} \textit{qc-wc-1.BamHI} and \textit{wc-1}\textsuperscript{null} \textit{qc-wc-1.MluI} strains, \textit{10}^{-2} \textit{x QA was added into the liquid medium to induce WC-1 expression. PI, the wild type extract was immunoprecipitated with the preimmune antiserum. B, immunoprecipitation assays using WC-2 antisera showing that the putative NLS and the C-terminal part of the protein are not required for the WC-1/WC-2 and FRQ-WC interactions. The constructs indicated above are derived from the Myc-WC1 construct, and they were transformed into the \textit{his-3} locus of the \textit{wc-1}\textsuperscript{null} strain. PI, the \textit{wc-1}\textsuperscript{null} Myc-WC1.NLS protein extracts were immunoprecipitated with the preimmune antisera.}
  \end{figure}
There are required for the dark activation of FRQ but not for the light induction of frq and other genes. Therefore, it is likely that the putative NLS and the zinc finger domain act as one functional motif to activate gene expression in the dark.

**DISCUSSION**

In constant darkness, WC-1 and WC-2, the two PAS-containing transcription factors, form a complex that binds the cis-acting elements of the frq promoter and activates the transcription of frq, leading to the cycling of frq and the overt rhythmicity (19, 36). Recently, WC-1 has also been identified as the first fungal blue light photoreceptor that binds FAD as a chromophore and (together with WC-2) binds to the LREs of the frq promoter in a light-dependent manner (19, 28). Thus, the rapid light-dependent induction of frq transcription mediated by the WC complex is the mechanism that allows the Neurospora clock to be entrained by light (16, 29). Therefore, both WC proteins are required for the clock function in the dark and for the light input of the clock. In addition, the WC complex is required for other light responses in Neurospora (27).

The physical interaction between the WC complex and FRQ is a crucial step that closes the Neurospora circadian negative feedback loop (15, 18). Although WC-2 was previously proposed as a scaffold protein that mediates such an interaction (18), here we show that the presence of WC-1 is essential for the formation of the FRQ-WC complex (Fig. 1). In mutants without WC-1, despite the normal expression of WC-2, no WC-2-FRQ interaction was detected in vivo. Because WC-1 needs WC-2 to form complexes to maintain its level and because WC-1 is the limiting factor in WC complexes (13, 18, 20), we think that WC proteins can interact only with FRQ in vivo as a WC-1/WC-2 complex. A similar situation may also exist in Drosophila, in which PER and TIM may only interact with the dCLK-CYC heterodimer and not with the free CYC protein (37).

Previously, we showed that FRQ positively regulates the expression of WC-2 and that the PAS domain of WC-2 is required for the formation of the WC complex and the maintenance of the steady state of WC-1 (13, 20). Here we show that WC-1 negatively regulates the expression of wc-2 at the level of transcription (Figs. 2 and 3), thus forming another interacting feedback loop. Interestingly, the repression of wc-2 by WC-1 is light independent, suggesting that this function of WC-1 does not require its role in light sensing (Fig. 3C). Such an interlocked nature of the expression of the two WC proteins may be important for maintaining an appropriate ratio of the two proteins in the cell, allowing them to function properly in the clock and in light responses. The opposite effects of FRQ and WC-1 on wc-2 may help explain the fact that WC-2 is not rhythmically expressed (13, 18). Although we could not exclude the possibility that WC-1 regulates wc-2 indirectly, these data suggest that in addition to being a transcription activator, WC-1 may function as a transcription repressor as well.

Consistent with WC-1 being a multifunctional protein, our data also demonstrate the existence of different forms of the WC complexes and the significantly different requirements of WC-1 for light induction of frq and other genes. The differential requirement of WC-1 for light-induced gene expression is highlighted by our result in the wc-1<sup>tap33</sup> mutant (Fig. 4). In this mutant, resulting from alternative protein initiation from a downstream AUG, less than 1% of the normal amount of WC-1 was expressed, and it was still associated with WC-2. However, its light induction of frq was near normal, whereas the light induction of al-3 and vvd was mostly abolished. A similar situation was also observed for a wc-2 mutant. In the wc-2<sup>de233</sup> mutant (a point mutation in the zinc finger DNA-binding domain), although its light induction of other genes are abolished, its light induction of frq was close to normal (16, 20, 21). Therefore, the light induction of frq, a step critical in the light resetting of the clock, does not require fully functional WC proteins, and needs only a very low amount of a WC complex.

Recently, a study by Dragovic et al. (38) showed that in some wc mutants, conidiation rhythms could still be driven by light/dark cycles. Although this study suggested the existence of a wc-independent photoreceptor in Neurospora that regulates the conidiation process, the interpretation of the results is complicated by the fact that the wc-1<sup>tap33</sup> strain (generated by RIP) used in that study may not be a real wc-1 null strain. In that wc-1 mutant strain, although the light induction of other genes was eliminated, the light induction of frq could still be observed, a fact that is in conflict with the results obtained in true wc-1 null strains and other wc-1 mutants (16, 19, 28). Therefore, based on the results we presented here, we think that the wc-1 mutant used by Dragovic et al. (38) is most likely not a real null, and it could produce a low level of WC-1 (that could not be detected by regular Western blot analysis) because of alternative protein initiation from a downstream AUG.

The differential requirement of WC complex in the light induction of genes and the in vitro DNA binding data (19) suggest the existence of different WC complexes in the cell, a notion that was confirmed by the immunoprecipitation assay in a wt,Myc-WC-1 strain (Fig. 5). Using the extract of this strain, we found that there were at least two types of WC-1-containing protein complexes in the cell: ~20–30% of WC-1 was found to self-associate to form a large WC-1/WC-2 complex. The larger WC complex may be the large WC-containing complex identified by Froehlich et al. (19) using an in vitro DNA binding assay that binds to the LREs of the frq promoter in a light-dependent manner and regulates light responses. In contrast to WC-1, no WC-2 was found to self-associate in vitro, although strong self-association was previously observed in vitro (34). Because WC-1 needs WC-2 to form a complex to maintain its steady state level, one WC-2 molecule should form complexes with more than one WC-1 molecule in the cell. Our results also
confirm the previous results that the majority of WC-1 is in a WC-1/WC-2 heterodimeric complex (18, 19, 28). The function of the WC-1/WC-2 heterodimer may be important for the activation of *frq* and other genes in the dark (19, 39).

To map the WC-1 domain that mediates its interaction with WC-2 and to determine the function of various WC-1 domains, we made a series of *wc-1* mutants. Our results indicate that the PASC domain and its immediate C-terminal region (upstream of the putative NLS) are required for the formation of the WC-1/WC-2 complex, whereas the rest of WC-1 is not essential (Figs. 6 and 7). Although the requirement of the PASC domain for the interaction is consistent with the interaction mediated by the PAS-PAS interaction of the two WC proteins (20), the involvement of the PASC C-terminal region indicates that PASC alone is not sufficient for such an interaction. Consistent with our previous work of WC-2 (20), these results show that the formation of the WC complex is essential for their functions in the circadian clock and light responses and for maintaining the steady state level of WC-1. However, the existence of the WC-1/WC-2 complex is not sufficient to be a functional complex, because the PASB domain of WC-1 is required for both of its dark and light functions whereas it is not required for the complex formation (Fig. 8, A and B).

One surprising result from the deletion study is that the C-terminal part of the WC-1 protein (starting from the putative NLS and including the zinc finger) is not required for the light induction of *frq*, *al-3*, and *ucd* (Fig. 8). However, the NLS and DNA-binding domain are required for the expression of *FRQ* in the dark. Thus, by deleting this region of WC-1, we separated the light and dark functions of WC-1. This is in contrast with the mutant lacking the entire LOV domain, in which only the light function but not the dark function of WC-1 is defective (28). Without the DNA-binding domain of WC-1, how does WC-1 mediate the light-induced gene expression? Because WC-1 forms complexes with WC-2, the zinc finger DNA-binding domain of WC-2 must be responsible for the DNA binding in these mutants. Interestingly, as described above, in a *wc-2* mutant (ER33) that contains a point mutation in the DNA-binding domain, the light induction of *frq* was still observed, although the light induction of other genes was defective (16, 20, 21). Together, these data suggest that the light induction of *frq* only requires one DNA-binding domain from either of the WC proteins, but the DNA-binding domain of WC-2 may be essential for binding to the promoters of other light-inducible genes. On the other hand, the DNA-binding domains of both WC proteins may be required for DNA binding and transcription activation of *frq* in the dark.

In conclusion, our results show that WC-1 is required for the formation of the FRQ-WC complex and it negatively regulates the expression of *wc-2*. In addition, WC-1 differentially regulates the light induction of *frq* and other genes, and there are different forms of the WC complexes in the cell, possibly with distinct functions. Together, our data demonstrate that WC-1 is a multifunctional protein involved in the circadian clock, light sensing, and transcription repression in *Neurospora*.

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