Roles for WHITE COLLAR-1 in Circadian and General Photoperception in *Neurospora crassa*

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ABSTRACT

The transcription factors WHITE COLLAR-1 (WC-1) and WHITE COLLAR-2 (WC-2) interact to form a heterodimeric complex (WCC) that is essential for most of the light-mediated processes in *Neurospora crassa*. WCC also plays a distinct non-light-related role as the transcriptional activator in the FREQUENCY (FRQ)/WCC feedback loop that is central to the *N. crassa* circadian system. Although an activator role was expected for WC-1, unanticipated phenotypes resulting from some *wc-1* alleles prompted a closer examination of an allelic series for WC-1 that has uncovered roles for this central regulator in constant darkness and in response to light. We analyzed the phenotypes of five different *wc-1* mutants for expression of FRQ and WC-1 in constant darkness and following light induction. While confirming the absolute requirement of WC-1 for light responses, the data suggest multiple levels of control for light-regulated genes.

LIGHT is one of the most important environmental cues for most living creatures, including humans. Collected light information is transferred to many different biological effectors. The proper perception of ambient light is essential for an organism to adapt to its environment, and one important recipient of light information is the circadian system. The circadian oscillator is composed of self-sustaining cellular feedback loops that, through their interactions, generate a 24-hr cycle (Dunlap 1999; Young and Kay 2001). Time-of-day (phase) information from the circadian oscillator influences many different biological activities. Molecular components of circadian oscillators have been characterized in eukaryotic model organisms including Neurospora and Drosophila and recently in mammalian systems, all of which show transcription/translation-based feedback loops in which heterodimeric complexes of PAS proteins act as transcriptional activators (Dunlap 1999; Young 2000; Loros and Dunlap 2001; Reppert and Weaver 2001; Young and Kay 2001).

One of the characteristics of a circadian oscillator is that the rhythm can be entrained by environmental cues, *e.g.*, changes in ambient light, temperature, and nutrient conditions (Crosthwaite *et al.* 1995; Liu *et al.* 1998; Sarov-Blat *et al.* 2000; Devlin and Kay 2001; Stokkan *et al.* 2001). Of these cues, light entrainment has been studied more intensively than any other environmental cue, and the molecular mechanisms governing how light information is transferred and how the circadian clock is reset are beginning to be understood in microbial, plant, and animal systems (Johnson and Golden 1999; Johnson 2001; Loros and Dunlap 2001; Reppert and Weaver 2001; Williams and Sehgal 2001; Young and Kay 2001). Plants use a complex array of multiple phytochromes and cryptochromes to detect light (Quail *et al.* 1995; Cashmore *et al.* 1999). For circadian control in higher plants, phytochrome B plays a major role in response to high-intensity red light, whereas phytochrome A plays a role in response to low-intensity red light, and phytochrome A and cryptochrome 1 act together to transmit low-fluence blue light to the clock (Somers *et al.* 1998). Recently, the bacteriophytochrom CikA was cloned and characterized as an important component in the light-input pathway that resets the Synechocystis circadian clock (Schmitz *et al.* 2000). Previous work in Neurospora has shown that the light induction of *frq* transcript by WHITE COLLAR proteins is sufficient for resetting the clock (Crosthwaite *et al.* 1995, 1997; Froehlich *et al.* 2002). Similarly, in other systems, light acts to alter the level of a negative element within the oscillator. Regulatory relationships within the feedback loops responding to light result in changing the level of a negative element and thereby resetting the clock (Shigeyoshi *et al.* 1997; Suri 1998; Yang *et al.* 1998; Young and Kay 2001).

Despite advances in understanding how light input impinges on the circadian clock mechanism, the issue of the molecular nature of circadian photoreceptors and their roles in regulatory gene expression remains a work in progress. Great progress has been made through studies in Drosophila in which the blue-light-absorbing molecule cryptochrome (CRY) has been shown

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to influence many biological activities (Hall 2000). Although the single CRY in Drosophila was suggested to be the unique light receptor for the fruit fly circadian oscillator (Stanewsky *et al.* 1998; Emery *et al.* 2000), in a cry^b loss-of-function mutant background, both behavioral rhythms and rhythms of PER and TIM expression within lateral neuron cells were still entrained by light (Stanewsky *et al.* 1998). This finding suggested that there are additional ways the fruit fly clock can see light. Consistent with this hypothesis, genetic ablation studies have suggested that at least three different light transduction pathways for the circadian oscillator exist (Helfrich-Förster *et al.* 2001). In contrast to Drosophila, the two CRY genes in mammals are believed to encode essential components of the circadian oscillatory machinery (Okamura *et al.* 1999; van der Horst *et al.* 1999). Although the expression of the mammalian CRYs in a cell is neither necessary nor sufficient for light induction of clock components (Okamura *et al.* 1999; Reppert and Weaver 2001), they may play a role in circadian photoreception in the eye (Selby *et al.* 2000), where melanopsin-based photoreceptors in retinal ganglion cells (Berson *et al.* 2002; Hattar *et al.* 2002) appear to be responsible for photoentrainment.

In Neurospora, all the known light-induced phenotypes are regulated by blue light, and all blue-light-induced phenotypes can be blocked in mutants of either *white collar-1* (*wc-1*) or *white collar-2* (*wc-2*; Ballario and Macino 1997). Further attempts to find loci other than *wc-1* and *wc-2* have failed (Degli-Innocenti and Russo 1984; Linden *et al.* 1997), suggesting that WC-1 and WC-2 are the only nonredundant key components for blue-light transduction in Neurospora. The *wc-1* and *wc-2* genes have been cloned (Ballario *et al.* 1996; Linden and Macino 1997) and their products shown to interact (Ballario *et al.* 1998; Cheng *et al.* 2001a; Denault *et al.* 2001). On the basis of these findings and sequence information showing that WC-1 and WC-2 contain PAS domains and GATA DNA-binding domains, it has been proposed that *wc-1* and *wc-2* are transcription factors mediating light-induced gene expression (Ballario *et al.* 1996, 1998; Linden and Macino 1997; Talora *et al.* 1999; Lee *et al.* 2000). Both WC-1 and WC-2 are regulated post-translationally through phosphorylation (Talora *et al.* 1999; Lee *et al.* 2000; Schwerdtfeger and Linden 2000, 2001), while light-induced phosphorylation of WC-1 is transient and influenced by VIVID (VVD; Heintzen *et al.* 2001) and phosphorylation of WC-2 is stable under constant light (LL). Consistent with these genetic and molecular analyses, WC-1, working with WC-2 and the cofactor FAD, has recently been shown to be a circadian blue light photoreceptor in Neurospora (Froehlich *et al.* 2002; He *et al.* 2002).

Although WC-1 is thus essential for light responses, it has become clear that the signaling pathway mediated by WC-1 is not simple. We created a definitive *wc-1* null strain and have examined an allelic series of *wc-1* mutants to better understand the roles of WC-1 both in the circadian oscillator in darkness and in light signal transduction to light-regulated genes. Our analysis of this series of *wc-1* mutant alleles suggests that WC-1 plays multiple roles for the proper expression of FREQUENCY (FRQ) in constant darkness and for the light induction of the light-inducible genes.

TABLE 1

N. crassa strains used in this study

Strain name	Genotypes	Source
87-3	*bd, wc-1*$^+$, *a*	Laboratory stock
87-74	*bd, wc-1*$^+$,*his-3, a*	Laboratory stock
FGSC#4395	*wc-1*^{ER45}, *A*	FGSC
FGSC#4397	*wc-1*^{ER53}, *A*	FGSC
FGSC#4401	*wc-1*^{MK1}, *A*	FGSC
FGSC#4399	*wc-1*^{ER57}, *A*	FGSC
308-1	*his-3, bd, wc-1*^{KO}, *a*	This study
308-2	*his-3, bd, wc-1*^{KO}, *a*	This study
308-3	*his-3, bd, wc-1*^{KO}	This study
230-7	*bd, wc-1*^{MK1}	This study
230-15	*bd, wc-1*^{MK1}, *A*	This study
230-17	*bd, wc-1*^{MK1}	This study
232-4	*bd, wc-1*^{ER45}, *a*	This study
233-2	*bd, wc-1*^{ER53}, *a*	This study
234-5	*bd, wc-1*^{ER57}, *A*	This study
KG5-7	(*his-3, bd, wc-1*$^+$ + *his-3, bd, wc-1::pKW103*)	This study
KG5-10	(*his-3, bd, wc-1*$^+$ + *his-3, bd, wc-1::pKW103*)	This study
KG5-14	(*his-3, bd,, wc-1*$^+$ + *his-3, bd, wc-1::pKW103*)	This study
241-6	*wc-2*$^{\Delta}$, *his-3, bd, a*	Laboratory stock
93-4	*his-3, bd, frq*^{10}, *a*	Laboratory stock

FGSC, Fungal Genetics Stock Center.

MATERIALS AND METHODS

Strains and growth conditions: General conditions for growth and manipulation of Neurospora are described elsewhere (Davis 2000). Culture conditions for rhythmic expression of FRQ and WC-1 were as described previously (Aronson *et al.* 1994b; Crosthwaite *et al.* 1995). Constant dark (DD) was 20 hr in the dark and LL was 20 hr in saturating light (40–50 μmol m^{-2} sec^{-1}). All the experiments were performed at 25°. Several *wc-1* alleles were obtained from the Fungal Genetics Stock Center (Kansas City). Strains used in this study are summarized in Table 1. The *frq* null strain, frq^{10}, was produced by gene replacement strategy as described earlier (Aronson *et al.* 1994a).

Sequencing *wc-1* mutant alleles: Neurospora genomic DNA was prepared from different *wc-1* mutant strains and the laboratory wild-type strain 87-3 (*bd*) by a standard phenol:chloroform extraction method with some modification. The mycelial samples were prepared by growing cells in 20 ml 1× Vogel/2% glucose liquid culture in 125-ml flask for 24 hr followed by vacuum filtration onto Whatman paper no. 1 (circles, 70 mm diameter). Mycelia were frozen by submersion into the

TABLE 2

Oligos used in this study

Oligo name	Sequence
w1-1	TCCCGTCTGCTTGAGTGACA
w1-5	CCATCGCGGAGGGCCAGGAG
W1-6	TCTCCGGCCTGTAAGAAAGT
w1-8	AGCTGTTGAAGGCGTCATCA
w1-18	AAACTCGGCTCTGGATTCCT
w1-19	CTTCGACATCCCGCCCATGA
w1-22	CTGACAGCGGGCCTTGATCT
w1-26	CCCTGTGATACCCGACCC
w1-27	CCAGTTGCCTAAATGAACCA
w1-33	ATGGGAAACACGGCTGGAC
w1-U1	CTGCGGTTCATTCTCATTC
al1-1	CGCGAGACCTTTGAAGATTT
al1-2	GCTTGCCGTCGGAGAACCAG
al2-1	CGACTCCGCATTGACCTGAT
al2-2	AGACCTCACGGCGAGATTTG
con10-1	CGAGAAACAGCGCGAAATTG
con10-2	CACCAGTGCCACCGGAAGCC
con6-1	TAATGTTTCCGAGGAAGCCA
con6-2	GGGTTCTTGTCGCCGTCGTC
vvd-1	GGATACAGCAATGCGGAGGT
vvd-2	CCGTCGGGTGACTGAAGAAA
L6-117F	CAGAAATGGTACCCTGCTGAGG
L6-176R	GCGGATGGTCTTGCGG
L6-140T	TGAGGCCCAGCCTCGCAAGG
wc1-879F	TGCACAAGGCATGGCTACA
wc1-940R	GGAAGGTTGTCGCGGGT
wc1-899T	CAGTCTCACAGGATGCGGCAAGCA
frq-352F	TCGACATCGCAGAGGAGAAA
frq-417R	CAACGAAACCCCAGACGAGT
frq-373T	ACTGGCAGACATGGAACGGCCG
c-ha-61	CAGCATCAGCAGCAACAAAAAACCAATCAG CGGATCCCCGGGTTAATTAA
c-ha(R1)	CGACGATGTAGTCAATGGGCCGAGTGGTT CGAATTCGAGCTCGTTTAAAC

liquid nitrogen and powdered using a mortar and pestle. The powder was resuspended in 2 ml of lysis buffer (50 mM Tris-HCl pH 8.0, 50 mM EDTA, 2% SDS, and 1% β-mercaptoethanol), and the mixture was incubated at 65° for 1 hr. The mixture was phenol extracted twice and chloroform extracted once. DNA was precipitated with 2 ml of isopropanol and 0.2 ml of sodium acetate and the pellet was collected by centrifugation (13000 × *g*) for 1 min. The DNA pellet was resuspended in 0.5 ml of TE buffer and 10 μg of RNAse A and incubated at 37° for 1 hr. The PCR products containing the *wc-1* open reading frame (ORF) were prepared from genomic DNA using two sets of oligos (w1-5 and w1-19 and w1-8 and w1-22; Table 2) with the following conditions: 94° for 2 min, 30 cycles of 94° for 30 sec, 68° for 3 min, and 68° for 7 min; the resulting products were used for subsequent sequencing reactions.

Plasmid constructs: To create a *wc-1* (MK1)-like strain from a wild-type strain, a "knock-in" construct was prepared. The plasmid pKW103 was designed to contain 2 kb of the 5′-untranslated region (5′-UTR) of *wc-1*, DNA encoding the first 58 amino acids of the amino-terminal portion of WC-1 followed by three repeats of a hemagglutinin (HA) epitope tag, the terminator sequence of the ADH1 gene of *Saccharomyces cerevisiae*, selection markers for kanamycin resistance and hygromycin resistance (*hph*, hygromycin B phosphotransferase), and 3.5 kb of the 3′-UTR region of *wc-1* (Figure 4B). The 2-kb fragment encompassing the 5′-UTR region of *wc-1* and the amino-terminal portion of the WC-1 ORF was created by PCR using two oligos, w1-u1 and w1-33 (Table 2), and genomic DNA as a template. The HA tag and the terminator sequences were produced by PCR using pFA6a-3HA-kanMX6 as a template and two oligos, c-ha-61 and c-ha-R1. The *Escherichia coli* strain CBB698, which harbors the plasmid pFA6a-3HA-kanMX6, was a gift from Dr. Stefan Otte (Dartmouth Medical School). The two PCR products described above were joined together by the PCR splicing by overlapping extension (SOE) technique (Horton *et al.* 1989). The SOE'd PCR product was ligated into pCR4-topo (Invitrogen, Carlsbad, CA) to make pKW65. The 4-kb *Eco*RI fragment of pKW65 that contains the engineered HA tag and kanamycin resistance gene was ligated into the *Eco*RI site of pKW4, which contains the 3′-UTR region of *wc-1*, to make pKW101. The 1.4-kb hygromycin resistance gene module (pCB1004/*Hpa*I; Carroll *et al.* 1994) was ligated into the *Pme*I site of pKW101 to make pKW103. The plasmid pKW103 was verified by sequencing, which revealed an unexpected repeat of DNA sequences at the WC-1 ORF portion of the knock-in construct. The duplication corresponding to the DNA encoding amino acids 43–58 in the endogenous WC-1 sequences probably arose from the long stretch of repetitive DNA sequence that encodes the amino-terminal portion of the *wc-1* ORF.

Western and quantitative real-time PCR analyses: Protein analysis was performed as previously described (Lee *et al.* 2000). Immobilon-P (Millipore, Bedford, MA) was used for Western analysis. The relative intensities of bands on Western blots were analyzed using National Institutes of Health IMAGE 1.61. Total RNA was prepared by the following procedure. Frozen mycelial samples (50–100 mg) were powdered with a mortar and pestle, and the powdered samples were collected in 2-ml screw-cap tubes and stored in liquid nitrogen until all samples were processed. Then each tube was placed at room temperature and 1 ml Trizol (GIBCO BRL, Rockville, MD) was added and mixed well with a pipette tip. After an initial 10-min centrifugation to pellet cell debris, the samples were processed according to the manufacturer's recommendations. The RNA pellets were air dried and resuspended in water to a concentration of <1 μg/μl. One microliter of total RNA sample was treated with amplification grade DNase I (Invitrogen, Carlsbad, CA). The DNase I-treated RNA samples were stored at a final concentration of 50 ng/μl. To measure the amount of mRNA, the TaqMan Gold RT-PCR kit and SYBR Green PCR kit (PE Biosystems, Foster City, CA) were used. For the analyses of *frq* and *wc-1*, a TaqMan probe was used and, for the remaining genes, the SYBR Green method was used. A ribosomal protein gene in Neurospora, *L6*, was used for normalization in both TaqMan and SYBR Green RT-PCR analyses. Two gene-specific oligos per gene are used for these analyses: *frq*, frq-352F and frq-417R; *wc-1*, wc1-879F and wc1-940R; *L6*, L6-117F and L6-176R; *al-1*, al1-1 and al1-2; *al-2*, al2-1 and al2-2; *con-10*, con10-1 and con10-2; *con-6*, con6-1 and con6-2; and *vvd*, vvd-1 and vvd-2 (Table 2). The primers were used at 300 nM and the TaqMan probe at 100 nM. The reaction volume was 25 μl. All the TaqMan probes (frq-373T, wc1-899T, and L6-140T) were labeled with FAM (6-carboxyfluorescein) dye as a reporter and TAMRA (6-carboxy-*N,N,N′,N′*-tetramethylrhodamine) as a quencher. For the analysis of the remaining genes, a two-step SYBR Green approach was used. Random hexamers were used for the reverse transcription step. A total of 1 μl (50 ng) of the DNase I-treated RNA samples was used in a 50-μl reverse transcriptase reaction, and 1 μl of this was typically used in each 25-μl PCR reaction. The primers were used at 200 nM in the PCR step. The oligo pairs used for the SYBR Green approach were tested for any

nonspecific PCR products. All the quantitative real-time PCR was performed in an ABI Prism 7700 sequence detection system (PE Biosystems). We have found that the RT-PCR approach provides a more sensitive measurement of the level of transcript than does microarray or Northern analysis.

Polyclonal antibodies against WC-1: Two different antigens (WC1p2 and WC1p3) were used to raise the polyclonal antibodies for WC-1. WC1p2, corresponding to an internal region of WC-1 (amino acids 288–709), was expressed in bacteria as a glutathione S-transferase (GST) fusion protein and purified (Lee *et al.* 2000). During the purification, the GST portion of the fusion protein was removed by thrombin cleavage. To produce full-length WC-1 in bacteria, the intron in the WC-1 ORF region was eliminated by PCR. The intron-less WC-1 was ligated into pGEX-4T-1 (Amersham Biosciences, Piscataway, NJ) to make pKW31. The *E. coli* BL21 strain (Epicurian Coli BL21, Stratagene, La Jolla, CA) was used for transformation and expression of the full-length WC-1, WC1p3. WC1p3 was purified using an immobilized glutathione column (Pierce, Rockford, IL) and analyzed by SDS-PAGE and Coomassie Blue staining. This antigen was used to raise antibodies in rabbits (Pocono Rabbit Farm and Lab, Canadensis, PA). The antiserum raised against the bacterially expressed WC-1 protein was depleted against extracts from the bacterial strain that expresses GST (Harlow and Lane 1988), as well as against Neurospora strain 308-1 ($wc\text{-}1^{KO}$). All the Western analyses shown in this report used the antibody raised against full-length WC1p3 except that shown in Figure 1B, for which the antibody raised for WC1p2 was used. No qualitative difference in the WC-1 bands was seen with different antisera although some background bands were different. Both antisera detected a weak nonspecific band right above the WC-1 band, even in the MK1 strain. The intensity of the nonspecific band did not change after light pulse or in DD. Neither antiserum was able to detect the 60-kD *wc-1* fragment of MK1.

RESULTS

A series of *wc-1* mutant alleles: To better understand the nature of Neurospora photoreception and its relation to the clock, structure/function analysis of WC-1 was initiated through sequencing of genomic DNA from a series of *wc-1* mutant alleles (Figure 1A). Sequencing of $wc\text{-}1^{MK1}$ genomic DNA showed that $wc\text{-}1^{MK1}$ had a mutation at nucleotide +184 (from the translation start site), resulting in a putative polypeptide containing 61 of the 1167 amino acids of wild-type WC-1 and encoding only the N-terminal polyglutamine domain. In $wc\text{-}1^{ER53}$, a nonsense mutation at amino acid 1796 (T to G) yielded an early termination at amino acid 599 in the middle of the second PAS domain. In $wc\text{-}1^{ER45}$, a deletion of one nucleotide at +2048 (G) resulted in a frameshift at amino acid 684 preserving just the first and second PAS domains. Finally, in $wc\text{-}1^{ER57}$, a missense mutation at nucleotide +1499 (G to A) changed a glycine to glutamic acid at amino acid 500 within the first PAS (LOV) domain but leaves the remainder of the protein intact. Western analyses of the protein products arising from the *wc-1* mutant alleles using polyclonal antisera raised against bacterially expressed WC-1 protein (materials and methods) showed specific bands with the expected sizes (Figure 1B) with the exception of $WC\text{-}1^{MK1}$, which was either too small or too unstable, and $WC\text{-}1^{ER57}$, in which a single amino acid change at amino acid 500 apparently results in instability of the protein.

Because the relatively complex phenotypes of the mutants (see below) suggested the possibility of dominant-negative functions, we established a definitive null strain by replacing the endogenous WC-1 ORF, including adjacent 5′- and 3′-UTR regions, with the hygromycin phosphotransferase gene, *hph* (Figure 2A). Three primary transformants were characterized further to have the knockout construct in the right locus (data not shown). Homokaryotic knockout strains were obtained by backcrossing one of the characterized primary transformants to wild type, and the replacement of the endogenous *wc-1* with the hygromycin-resistance gene insert was confirmed by PCR (Figure 2B).

WC-1 is necessary for the proper expression and modification of FRQ in DD: Reduced yet detectable levels of FRQ protein are present in all the strains harboring truncated *wc-1* alleles, and a reduced level of WC-1 expression was observed in the *wc-2* knockout strain, as shown previously (Collett *et al.* 2002; Figure 3A). No FRQ protein was detectable in the $wc\text{-}1^{KO}$ background. We then asked how different mutations in WC-1 influence the activator role of WC-1 on cyclic FRQ expression in DD. Two *wc-1* alleles were chosen for further analysis, $wc\text{-}1^{ER45}$, which produces a truncated WC-1 containing the LOV and PAS B domains, and $wc\text{-}1^{ER57}$, which produces a full-length protein with one amino acid change (Figure 1A). Compared to wild type, in which FRQ and WC-1 showed the expected rhythm in expression/modification (Garceau *et al.* 1997; Lee *et al.* 2000; Figure 3B), the levels of FRQ were much lower in $wc\text{-}1^{ER57}$ and $wc\text{-}1^{ER45}$ backgrounds. FRQ and WC-1 displayed no cycling in abundance and, moreover, were not progressively phosphorylated but remained in their highly phosphorylated forms. We analyzed the changes in the steady-state levels of *frq* and *wc-1* mRNA by quantitative real-time PCR (Figure 3C). The levels of *frq* mRNA were oscillating in the wild-type background as expected, whereas no change in *frq* levels in *wc-1* mutant alleles was observed (Figure 3C). The levels of *wc-1* mRNA were not oscillating as previously reported (Figure 3C; Lee *et al.* 2000).

MK1 knock-in strains have $wc\text{-}1^{KO}$ phenotypes: To look for evidence of additional possible photoreceptors as a measure of the residual activity in the *wc-1* alleles, we investigated the steady-state levels of FRQ in different *wc-1* mutant backgrounds in constant light. As expected, no FRQ protein was detectable in LL in $wc\text{-}1^{KO}$ (Figure 4A). Surprisingly, however, the steady-state level of FRQ in the $wc\text{-}1^{MK1}$ background was high in constant light (Figure 4A). This very unexpected observation suggested one of two possibilities:

1. The 60-amino-acid N-terminal fragment of MK1 is sufficient to interact with WC-2, perhaps eliciting an

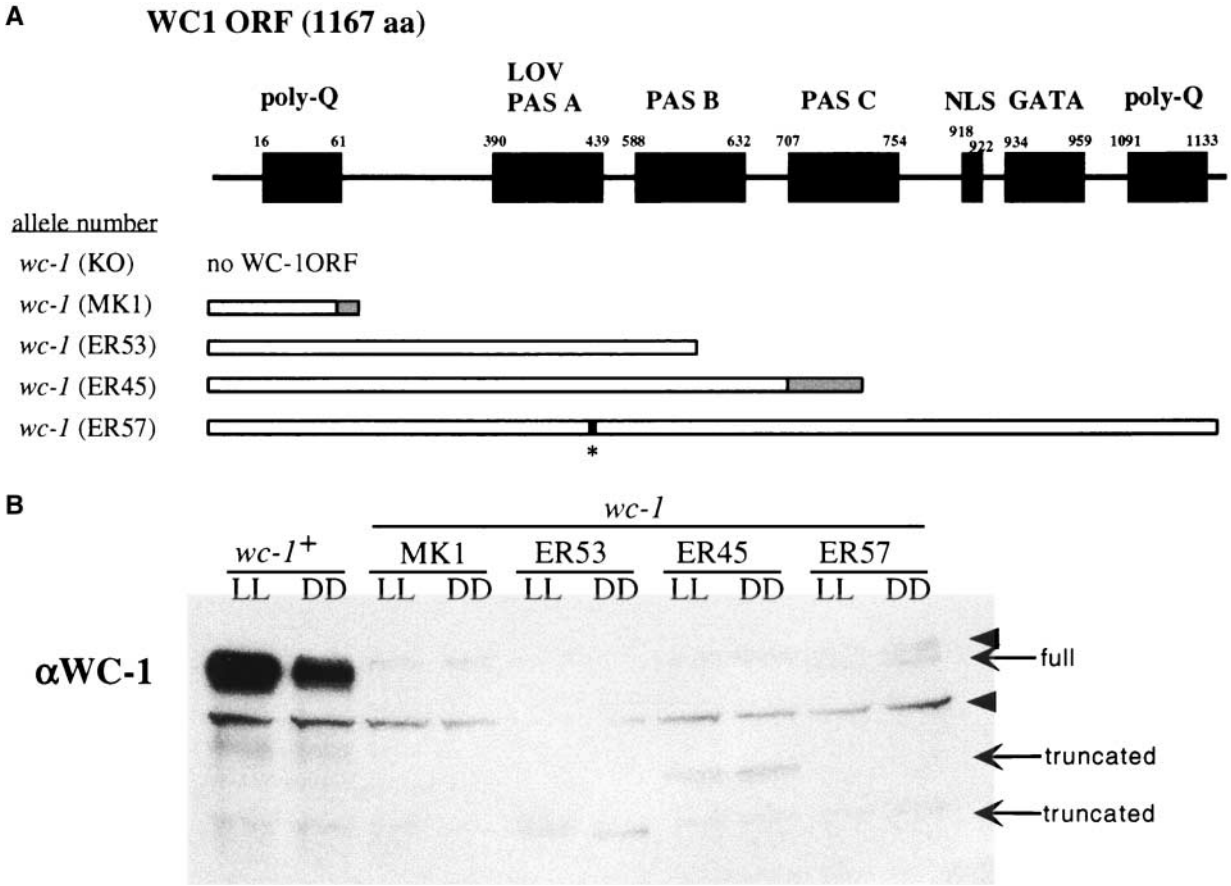

FIGURE 1.—Characterization of *wc-1* mutant alleles. (A) Protein domain structures of proteins encoded by *wc-1* mutant alleles. (Top) The WC-1 ORF with domains indicated: polyQ, glutamine-rich region; LOV, light-oxygen-voltage-sensing domain; PAS, protein-protein interaction domain; NLS, nuclear localization signal; GATA, GATA-DNA-binding domain. The numbers indicate the beginning and end of each domain. (Bottom) Open rectangles represent wild-type WC-1 ORF sequences, whereas solid rectangles represent amino acid sequences created by frameshifts. The asterisk indicates a glycine-to-glutamic-acid change at position 500 in ER57. (B) Western analysis of *wc-1* mutant alleles in DD confirms the sequencing data. WC-1 proteins (arrows) of the predicted sizes were detected in different *wc-1* mutant backgrounds. Arrowheads indicate background bands.

allosteric change or mediating interactions with a third factor and thus allowing light induction. In this case, WC-1 could not be the sole photoreceptor for *frq* light induction.

2. Translational reinitiation downstream of the MK1 stop codon results in a partially active protein lacking the N-terminal glutamine-rich region.

One obvious test of these alternatives is to use antisera to examine extracts of *wc-1*^{MK1} to see whether there is any evidence for translational readthrough or reinitiation, either of which should result in production of a protein that would contain several hundred amino acids and be visible on gels such as those in Figures 1 and 2. In repeated attempts using antisera directed against WC-1 (LEE *et al.* 2000), including a new antiserum directed against full-length WC-1 ORF including the N-terminal 60 amino acids of WC-1 (data not shown), we were unable to visualize any WC-1-specific proteins. However, since light induction in MK1 strains was both delayed and severely attenuated compared to that in wild-type strains, the possibility remains that a low level of WC-1 protein production can be sufficient to elicit light induction.

The definitive test for activity residing solely in the short N-terminal fragment of WC-1 is to recapitulate this mutation through creation of a "knock-in" strain that would produce the N-terminal fragment under the control of the normal promoter and 5′-UTR but would lack sequences corresponding to the remainder of WC-1, thereby precluding any possibility of readthrough or reinitiation (MATERIALS AND METHODS; Figure 4B). The knock-in construct was inserted into the endogenous *wc-1* locus by homologous recombination and expresses only N-terminal sequences of the WC-1 ORF. Three independent transformants were confirmed by PCR as having the knock-in *wc-1*, *wc-1*$^{MK1\text{-}KI}$ construct at the endogenous *wc-1* locus (data not shown) and were chosen for further characterization. The *wc-1*$^{MK1\text{-}KI}$ strains behaved like *wc-1*^{KO}, not like *wc-1*^{MK1}, with regard to light-induced FRQ expression (Figure 4, C and D). The *wc-1*$^{MK1\text{-}KI}$-bearing mutants had faster growth rates and

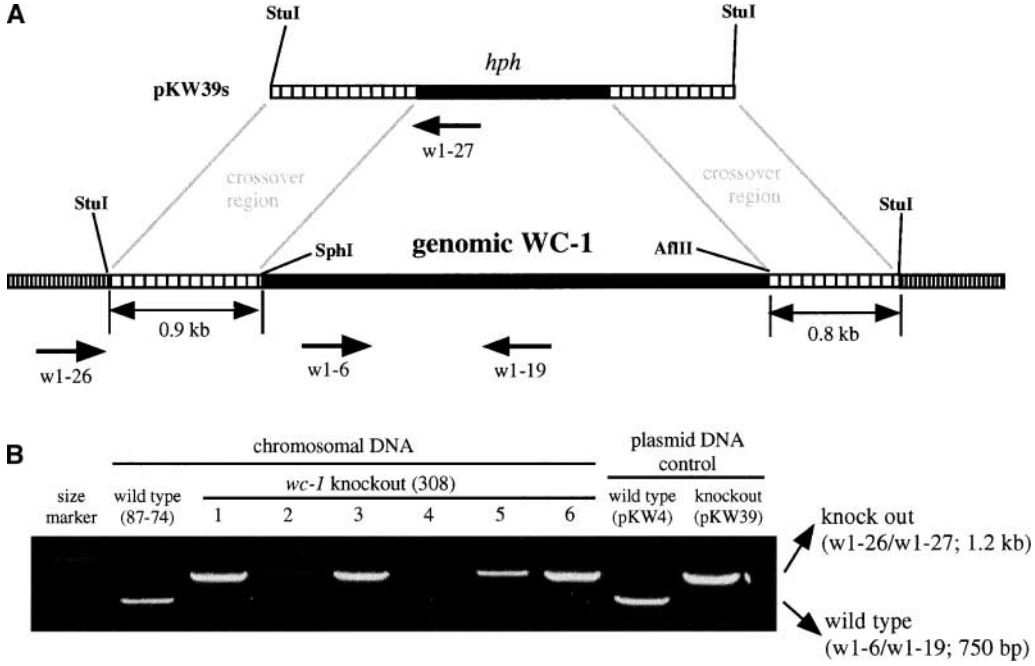

FIGURE 2.—Production of a definitive *wc-1* knockout strain. (A) Strategy used to create the *wc-1* gene replacement strain. Arrows represent oligos used to screen the transformant harboring a homologous integration at the endogenous *wc-1* locus. (B) Confirmation of the *wc-1* knockout by PCR. Chromosomal DNA from the *wc-1* knockout candidate progeny was prepared and analyzed by PCR using oligos w1-6, w1-19, w1-26, and w1-27 (Table 2). In the diagnostic PCR reaction, the oligo pair w1-6 and w1-19 produces a 750-bp product. The PCR product from wild-type chromosomal DNA and the oligo pair w1-26 and w1-27 produce a 1.2-kb PCR product from knockout chromosomal DNA. Chromosomal DNA from a wild-type strain (87-74), plasmid pKW4 containing wild-type *wc-1*, and the knockout construct plasmid pKW39 were used as controls.

an arrhythmic banding phenotype in race tubes resembling typical *wc-1* mutant phenotypes (Figure 4C). Western analysis was performed using the samples grown in constant light; surprisingly, no constant-light-induced FRQ was observed in the *wc-1*$^{MK1\text{-}KI}$ strains (Figure 4D). The MK1 knock-in experiment thus strongly suggested that the N-terminal amino acids cannot be responsible for the light-induction phenotype in *wc-1*^{MK1} and, by implication, that residual activity is due to readthrough or translational reinitiation. Coimmunoprecipitation experiments carried out elsewhere (P. CHENG and Y. LIU, personal communication) confirm that very low levels of WC-1 (<5%) were detected in *wc-1*^{MK1} strains, confirming our prediction.

The activator role of WC-1 in *frq* and *wc-1* light induction reveals complexity in the light-induction pathway: To characterize the ability of the *wc-1* strains to activate light-induced gene expression, we focused initially on the light induction of *frq* and *wc-1* mRNA using quantitative real-time PCR (MATERIALS AND METHODS; Figure 5A). The same data are presented in two different ways: as relative mRNA levels (Figure 5A, top) and as fold induction by light (Figure 5A, bottom). In a wild-type background, there was an ~12-fold light induction of *frq* and a 5-fold light induction of *wc-1*. Although ~3-fold more *frq* mRNA was present in wild type than in *wc-1*^{MK1} (Figure 5A, top), the fold increase of *frq* mRNA after light induction was comparable to that of wild type (Figure 5A, bottom). Following a dark-to-light transfer, the levels of *frq* transcript decayed much more slowly in MK1 than in wild type (Figure 5A, bottom). Qualitatively similar results were seen with *wc-1*, in that *wc-1* mRNA was still light induced in *wc-1*^{MK1} although only to ~40% of wild type (Figure 5A, bottom). The level of *frq* transcript was not light induced in the *wc-1*^{KO} background (Figure 5A, left), confirming previous data (CROSTHWAITE *et al.* 1995; FROEHLICH *et al.* 2002). Although the initial level of *wc-1* in the *frq* null background was low because FRQ is required post-translationally to promote WC-1 synthesis (LEE *et al.* 2000), the kinetics and fold induction of *wc-1* were relatively normal in a *frq* null background, thus confirming that *frq* does not have a significant role in light signaling (Figure 5A, right).

Analysis of the kinetics of FRQ light induction in the *wc-1* alleles yielded data largely consistent with the expectations from the RT-PCR analysis (Figure 5). First, in *wc-1*^{MK1} the FRQ protein synthesized *de novo* was detectable 30 min after the dark-to-light transfer; *i.e.*, it was significantly delayed compared to wild type. Second, although the full-length WC-1 protein in *wc-1*^{ER57} retained the ability to promote some synthesis of FRQ, neither the steady-state level of FRQ nor the FRQ phosphorylation profile changed greatly following the dark-to-light transfer (Figure 5B).

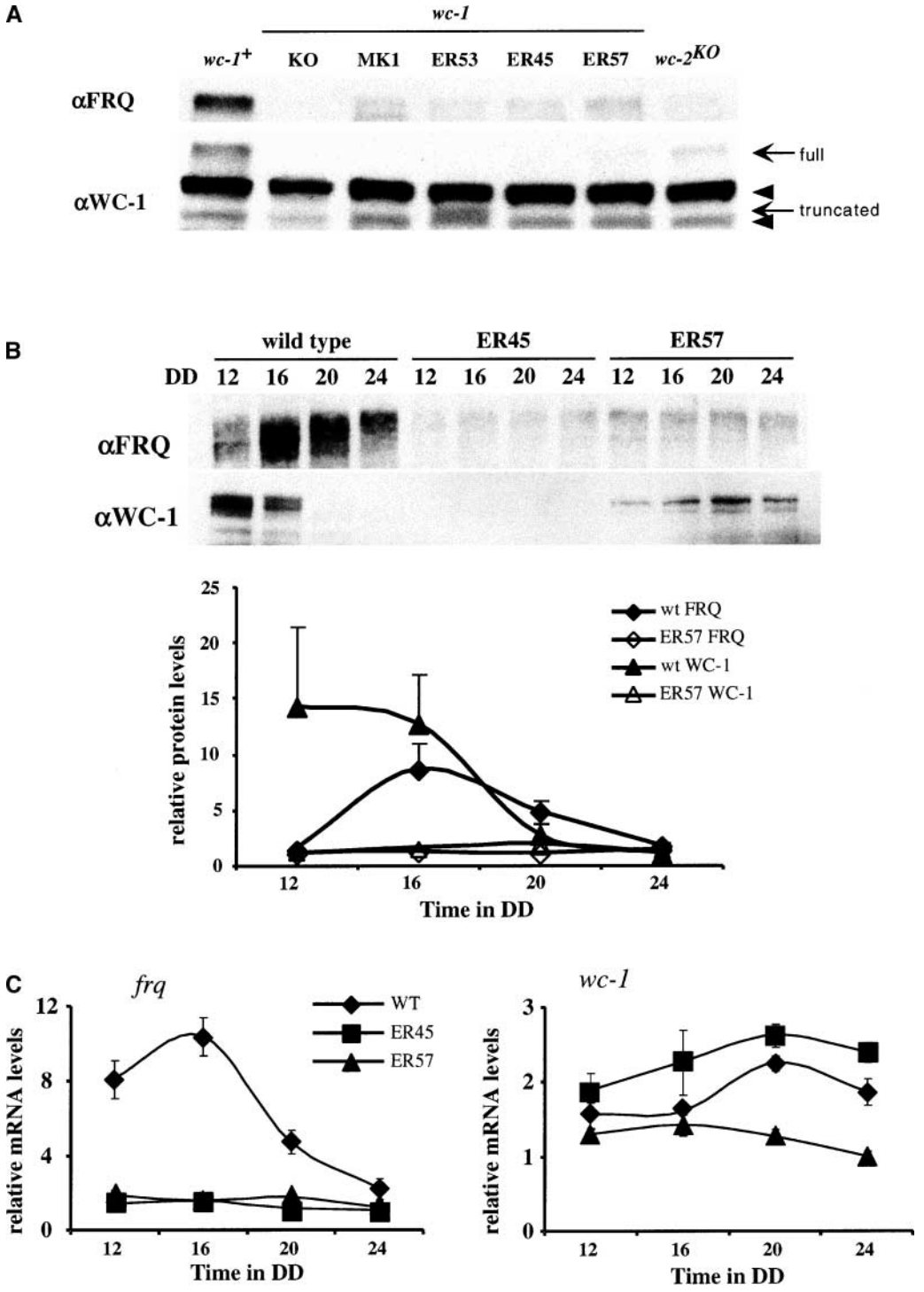

FIGURE 3.—WC-1 is necessary for proper expression and modification of FRQ in constant darkness. (A) Western analyses of WC-1 and FRQ in different *wc-1* mutant backgrounds. Arrows indicate the full-length and truncated WC-1 proteins. Arrowheads indicate background bands. (B) Western analyses of WC-1 and FRQ at different DD time points in different genetic backgrounds, *wc-1*$^{+}$, *wc-1*^{ER45}, and *wc-1*^{ER57}. The graph below is a densitometry reading of the Western image above. The error bar denotes SEM. Solid diamond, FRQ in wild type; open diamond, FRQ in ER57; solid triangle, WC-1 in wild type; open triangle, WC-1 in ER57. (C) Quantitative RT-PCR for (left) *frq* and (right) *wc-1* mRNA levels at four DD time points. Error bars denote SEM. Diamond, level in wild type; rectangle, level in ER45; and triangle, level in ER57.

To further characterize the MK1 strain as an example of a very low dosage of WC-1, we used real-time PCR analysis to examine light responses of several known light-inducible genes, *al-1*, *al-2*, *vvd*, *con-10*, and *con-6*, in three different genetic backgrounds, *wc-1*$^{+}$, *wc-1*^{MK1}, and *wc-1*^{KO}. In wild type, expression of all these genes peaked following 15–30 min of light, with induction ranging from 25-fold (*al-2*) to 1600-fold (*al-1*; Figure 6). In the *wc-1*^{MK1} background, results fell into two distinct groups: one in which there was a 10- to 100-fold reduced but still significant response (*al-1*, *vvd*, and *con-10*) and another in which the light response was completely abrogated (*al-2* and *con-6*; Figure 6). The reduced yet significant light induction of some genes could reflect a dosage effect of the low levels of truncated WC-1 produced in the MK1 allele background, or it could point to the importance of the amino-terminal activation domain for full activation function. Importantly, however, in all cases no light induction was observed in the absence of WC-1. Furthermore, even a 100-fold loss of

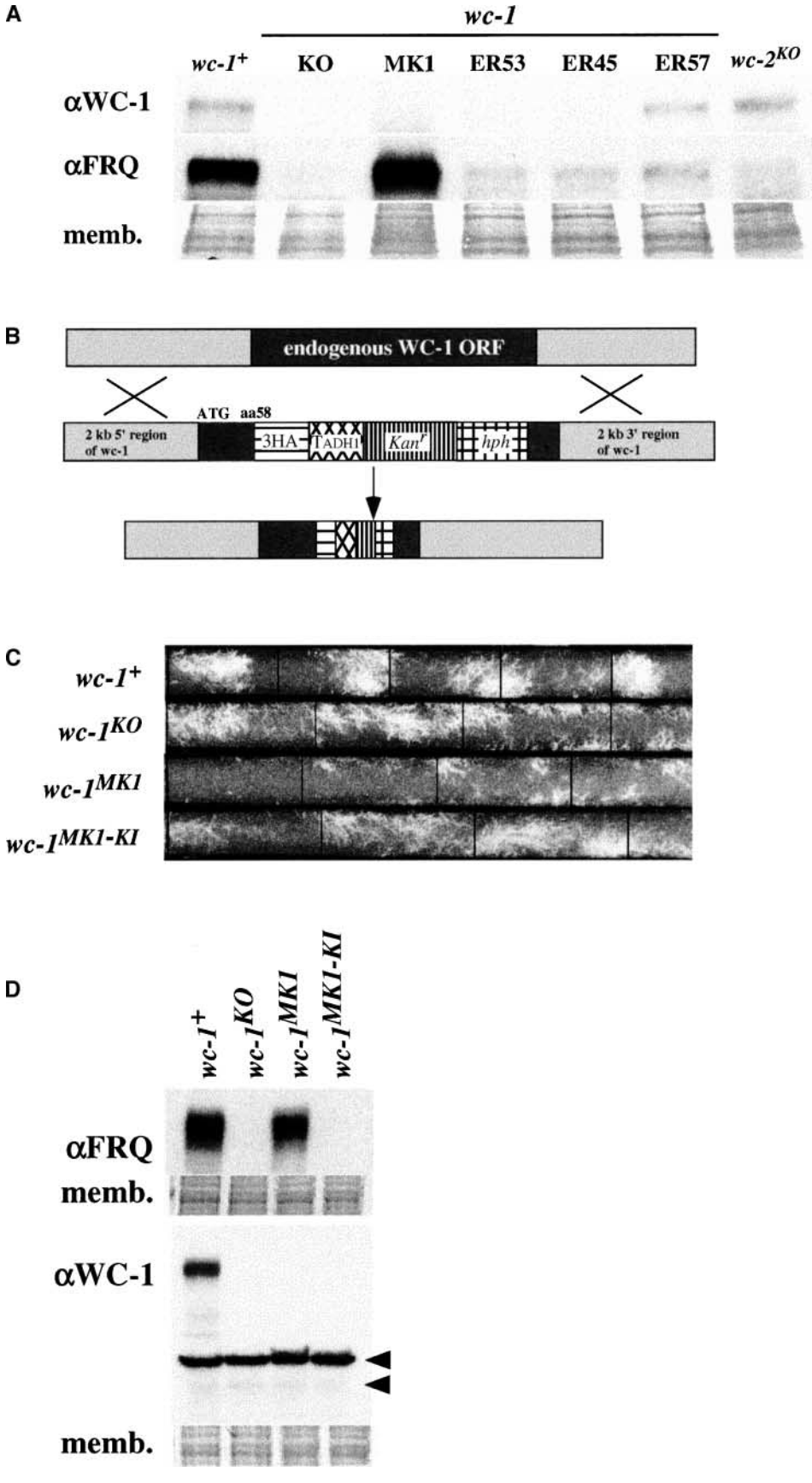

FIGURE 4.—*wc-1*$^{MK1\text{-}KI}$ mutants display *wc-1*^{KO} phenotypes. (A) Western analysis of WC-1 and FRQ in LL. (B) Strategy used to make the MK1-like knock-in mutant (*wc-1*^{KI}) in the wild-type chromosome. (C) Race tube analyses of *wc-1*^{KO}, *wc-1*^{MK1}, and *wc-1*$^{MK1\text{-}KI}$ show loss of rhythm in banding. (D) Western analysis of samples grown in constant light. Arrowheads indicate background bands.

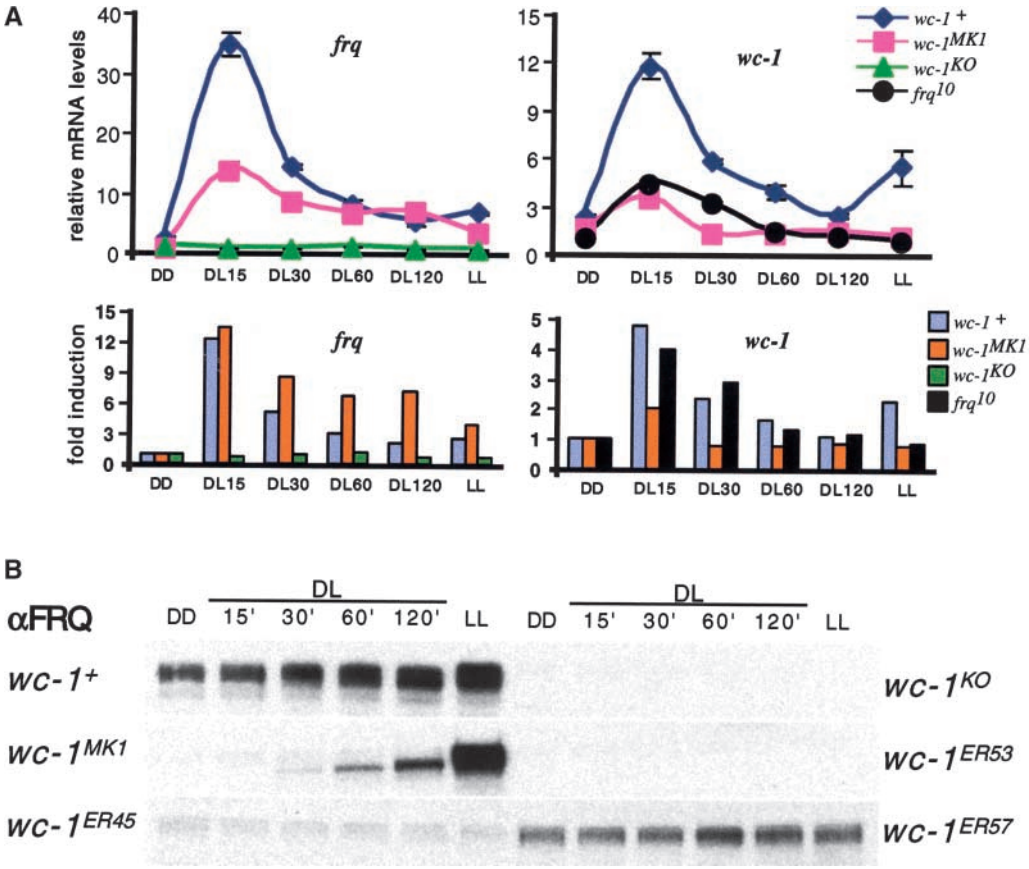

FIGURE 5.—The capacity for light induction of *frq* and *wc-1* is normal in the *wc-1*^{MK1} strain. (A) Analysis of light induction of *frq* and *wc-1* in different *wc-1* mutant backgrounds by real-time PCR. (Bottom) The fold induction after dark-to-light transfer, with the dark level normalized to 1. Standard error bars denote ±SEM. Each data point represents nine RT-PCR reactions from three different biological samples. (B) Western analysis of FRQ in different light conditions and in different *wc-1* mutant backgrounds. All lanes contain 100 μg protein and the exposures are equivalent.

signal in the *con-6* induction would have been detectable in the *wc-1*^{MK1} strain, and yet no signal was seen (partial loss *vs.* complete loss of light induction), suggesting the existence of additional factors affecting the light-induction pathway.

DISCUSSION

WC-1 was known to be important for light regulation in Neurospora, but unanticipated phenotypes among *wc-1* alleles have suggested unexpected roles for this transcription factor. These phenotypes were also reported in the strain that has been described as a *wc-1* null strain, Δ*wc-1* or *wc-1(Δ)*, which was not a definitive null but rather a mutant created by a unique genetic phenomenon in Neurospora, repeat-induced point mutation (RIP; TALORA *et al.* 1999; SCHWERDTFEGER and LINDEN 2000, 2001; MERROW *et al.* 2001). The totally blind phenotype of a definitive *wc-1* knockout strain (*wc-1*^{KO}) described here establishes the essential role of WC-1 in *frq* expression and in light regulation in general, demonstrating that FRQ has no direct role in the light input pathway (in contrast to MERROW *et al.* 2001), since the light induction of *wc-1* in a *frq* deletion strain, *frq*^{10}, was intact (Figure 5A).

There was no light induction in all the light-induced genes that we studied in the definitive *wc-1* null mutant background (Figure 6), suggesting that there are no additional functional photoreceptors for these genes. WC-1 not only is sufficient for light induction (FROEHLICH *et al.* 2002) but also is absolutely necessary for the circadian clock and other known light-induced genes in Neurospora. However, our analysis of the light-induced genes in the *wc-1*^{KO} background in this study was limited to a small number of known light-regulated genes. Genome-wide studies, *i.e.*, microarray analysis, will provide more informative data to determine whether WC-1 is the only light receptor in Neurospora. In plant and animal systems, the presence of multiple light receptors has been known for a long time, and recent studies have begun to uncover their redundant and/or distinctive roles (QUAIL *et al.* 1995; SOMERS *et al.* 1998; CASHMORE *et al.* 1999). In Neurospora, it was proposed that there is more than one light receptor (PAIETTA and SARGENT 1981, 1983). The Neurospora genome project (http://www-genome.wi.mit.edu/annotation/fungi/neurospora/) has revealed the presence of several orthologs of light receptors in Neurospora, orthologs of phytochromes (NCU04834.1 and NCU-05790.1) and cryptochromes (NCU00582.1). Whether they are biologically functional light receptors that have a physiological role in light signaling in Neurospora is yet to be determined.

The MK1 allele revealed interesting regulatory details of WC-1 in FRQ expression; the roles of WC-1 in constant darkness and in response to light are genetically separable (compare Figure 3A, Figure 4A, and Figure

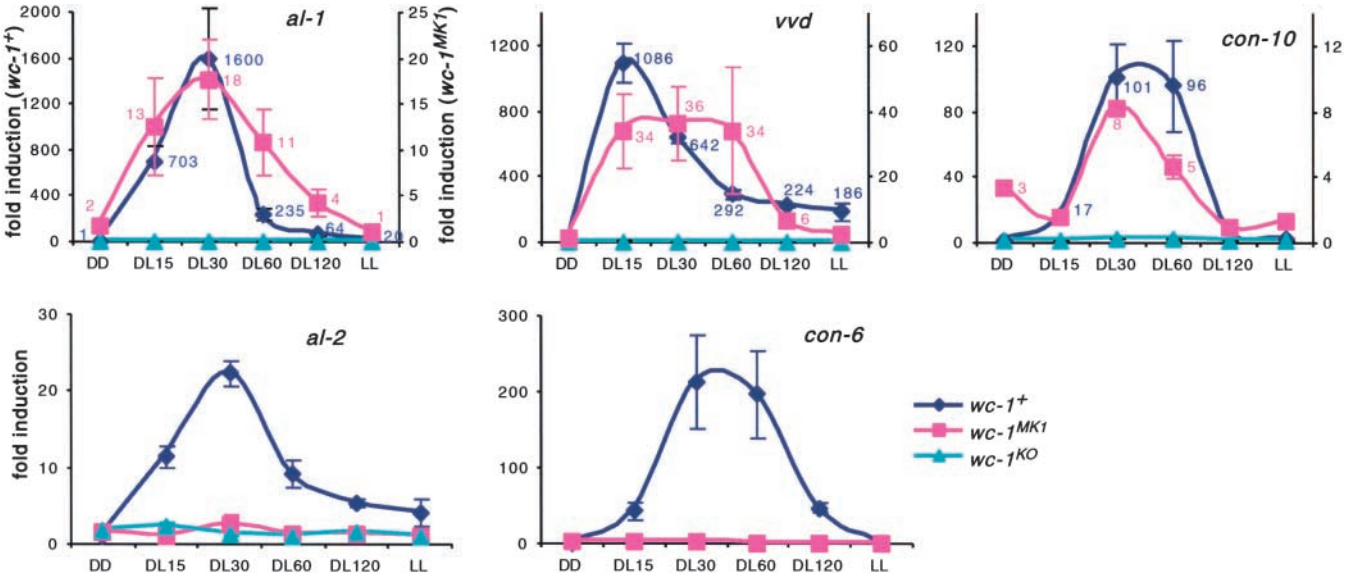

FIGURE 6.—Real-time PCR analysis of light-inducible genes in three different genetic backgrounds, *wc-1*$^{+}$, *wc-1*^{MK1}, and *wc-1*^{KO}, reveals diverse pathways for WC-1 regulation. When double *y*-axes are used (*al-1*, *vvd*, and *con-10*), the left-hand one is for wild-type and the right-hand one is for the MK1 strain.

5). FRQ induction in response to light and the steady-state level of FRQ in constant light were comparable in *wc-1*^{MK1} and wild type (Figure 4, A and D, Figure 5). One explanation is that the residual WC-1 activity present in the *wc-1*^{MK1} allele was sufficient to achieve the light-induction function but not sufficient to achieve the activation of FRQ in the dark. Alternatively, one can speculate that the activation domain present in the amino terminus is necessary for the dark function of WC-1 and the activation domain present in the carboxy terminus is sufficient for at least partial function in light induction. While we were preparing the revision of our manuscript, Nakashima's group reported a new *wc-1* allele, *rhy-2*, which can produce light-driven conidiation; however, the circadian rhythm of conidiation in continuous darkness is eliminated (TOYOTA *et al.* 2002). The *rhy-2* strain produces a truncated WC-1, which is very similar to the truncated WC-1 in the MK1 allele that we discuss in our article. The authors suggested that the amino-terminal polyglutamine domain in WC-1 plays an essential role for clock function and light-induced carotenogenesis (TOYOTA *et al.* 2002).

A systematic characterization of *wc-1* mutant alleles and the creation of a definitive knockout strain are important to fully appreciate the roles of WC-1. In the *wc-1*^{KO} background, no detectable FRQ protein was present by Western analysis, whereas a low yet detectable level of FRQ protein was present in other *wc-1* mutant alleles (Figures 3A, 4A, and 5B). This suggested that the truncated and unstable WC-1 protein was partially functional in FRQ expression, although we do not have sufficient data to explain how this was achieved. The low level of FRQ protein in a cell could have many significant physiological effects. The *frq*^{10} strain bearing a fusion construct with a FRQ ORF under the control of the inducible promoter, *qa-2*, produces a very small amount of FRQ protein in the absence of the inducer, quinic acid, due to the leakiness of the *qa* promoter (CHENG *et al.* 2001b). This trace amount of FRQ protein was sufficient to produce wild-type levels of conidia, whereas the *frq* null strain makes significantly lower levels of conidia (LOROS and FELDMAN 1986). FRQ can positively upregulate WC-1 levels by a post-transcriptional mechanism (LEE *et al.* 2000; CHENG *et al.* 2001b). WC-1 is the light receptor for the circadian clock (FROEHLICH *et al.* 2002; HE *et al.* 2002) and is also a limiting factor for the activation complex (white color complex, WCC) in constant darkness (CHENG *et al.* 2001b; DENAULT *et al.* 2001). FRQ is light induced in a RIP strain named Δ*wc-1* (DRAGOVIC *et al.* 2002), indicating that this strain, like several described here and in COLLETT *et al.* (2002), retains partial function and is not a complete null. The nature of the mutation and the FRQ light-induction profile in Δ*wc-1* (DRAGOVIC *et al.* 2002) is similar to *wc-1*^{MK1} (Figures 4 and 5). We propose that there is a residual WC-1 activity in the Δ*wc-1* strain, which is much like that in *wc-1*^{MK1}, as we have shown in this study.

The light-induction kinetics of *wc-1* was not changed in the absence of FRQ protein (Figure 5A, bottom; Figure 4A in MERROW *et al.* 2001). The difference observed in the relative levels of light-induced *wc-1* between the wild-type and *frq*^{10} backgrounds (Figure 5A, top) can be explained by the post-transcriptional regulation by FRQ; FRQ upregulates WC-1 protein levels (LEE *et al.* 2000) and WC-1 protein is necessary for the light induction of *wc-1* transcript (BALLARIO *et al.* 1996). Recently, it was shown that *frq* light signal transduction

differs from that of other light-regulated genes, *e.g.*, the alleles of *wc-2* that are photoinducible for *frq* are blind for another light-induced transcript, *al-3* (Collett *et al.* 2002). Interestingly, the kinetics of light induction of *frq* in the *wc-1^{MK1}* strain was similar to that of the wild type; however, the intensity of light induction of *wc-1* in *wc-1^{MK1}* was only 40% of wild type (Figure 5A). We wanted to investigate how other known light-induced genes respond to light in different *wc-1* allele backgrounds. The *wc-1^{MK1}* strain was used as an example of low residual WC-1 activity. We compared the light induction of known light-induced genes in the wild-type and MK1 backgrounds using quantitative RT-PCR (materials and methods). For *al-1*, *vvd*, and *con-10*, reduced yet significant levels of light induction were detected; however, in *al-2* and *con-6*, there was no detectable light induction in the *wc-1^{MK1}* background. These results suggest that the light-induction pathway is not as simple as light activating the WCC, which in turn activates all target genes. If this were so, *con-6* and *al-2* would respond to *wc-1^{MK1}* like other genes. This indicates that, while all light signaling begins with WC-1 in the WCC, the signal can be modified by additional factors for at least some target genes.

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