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33. This situation is familiar in mean field models of glasses, where α_d corresponds to a density of dynamical arrest, and α_c is the true transition point. For a review, see (*37*).
34. The explicit form is $P_i(H) = \int \Pi_a [du_a Q_{a\to i}(u_a)] \delta(H - \sum_a u_a) \exp(y \sum_a u_a)$, where the index a spans all the function nodes connected to the variable i.
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White Collar–1, a Circadian Blue Light Photoreceptor, Binding to the *frequency* Promoter

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In the fungus *Neurospora crassa*, the blue light photoreceptor(s) and signaling pathway(s) have not been identified. We examined light signaling by exploiting the light sensitivity of the *Neurospora* biological clock, specifically the rapid induction by light of the clock component *frequency* (*frq*). Light induction of *frq* is transcriptionally controlled and requires two cis-acting elements (LREs) in the *frq* promoter. Both LREs are bound by a White Collar–1 (WC-1)/White Collar–2 (WC-2)–containing complex (WCC), and light causes decreased mobility of the WCC bound to the LREs. The use of in vitro–translated WC-1 and WC-2 confirmed that WC-1, with flavin adenine dinucleotide as a cofactor, is the blue light photoreceptor that mediates light input to the circadian system through direct binding (with WC-2) to the *frq* promoter.

In *Neurospora*, a wide range of processes is light sensitive, including suppression and phase shifting of circadian rhythms, phototropism of perithecial beaks (*1*), and carotenoid biosynthesis (initially described in the first published study of *Neurospora* in 1843) (*2*). The photoreceptor(s) involved in these blue light–influenced processes has not been identified, but screens in *Neurospora* for mutants involved in light perception and signaling have repeatedly turned up two indispensable loci, *wc-1* and *wc-2* (*3*). WC-1 and WC-2 are nuclear transcription factors containing trans-activation and zinc-finger (Zn-finger) DNA binding domains (*4*, *5*). They form a White Collar Complex (WCC) by heterodimerizing via PAS (PER ARNT SIM) domains (*6*, *7*) and act as positive elements in light signaling, most likely through direct binding of DNA (*4*, *5*); in true *wc-1*^{KO} and *wc-2*^{KO} strains, all examined light responses are lost (*8*, *9*). This requirement suggested to us and others that either WC-1 or WC-2 is the photoreceptor, or that they both are required to mediate the response of an unidentified, perhaps duplicated, receptor (*1*, *10*, *11*).

In *Neurospora*, generation of circadian rhythms is dependent on WCC-mediated rhythmic production of *frq* transcript and protein, both of which are central clock components (*12*, *13*). Light causes a rapid induction of *frq* message, the central means by which light influences the clock (*14*). In the absence of WC-1 or WC-2, light induction of *frq* is completely abolished, highlighting the WCs' central role in light input to the clock (*8*, *9*, *15*).

Effects of light in vivo. To examine the contribution of transcription to the light-induced accumulation of *frq* transcript, the *frq* promoter was fused to a reporter gene, *hph*, and the resulting construct, pYL40B, was transformed into a *frq*$^{+}$ strain. Light treatment of transformants resulted in a marked increase in *hph* transcript level, similar to that of *frq* (Fig. 1A). Because only *frq* promoter sequence was fused to *hph*, light induction of the *hph* transcript, and consequently of endogenous *frq* message, is controlled at the transcriptional level.

We identified cis-acting elements mediating light induction of *frq* by transforming *frq* promoter deletion constructs into a *frq*^{KO} strain and the testing for light induction of *frq* message (Fig. 1B). Deletion of two light response elements (LREs) in the *frq* promoter decreased light induction of *frq* message. We noted an ~50% reduction with the distal LRE deleted (AF26) and an ~70% reduction with the proximal LRE deleted (AF33) (Fig. 1C). Deletion of both LREs (AF36) abolished light induction of *frq* transcript (Fig. 1C), which suggests that light induction of *frq* is controlled entirely at the transcriptional level. Both LREs were also sufficient to confer light inducibility on an *hph* reporter construct (pAF35), both individually (pAF43 and pAF44) and together (pAF45) (Fig. 1D).

The effects of the LRE deletions on circadian clock function were examined using race tubes to monitor *Neurospora*'s rhythmic conidiation (*11*). In a wild-type strain, transferring race tubes from light to dark results in a decrease in *frq* transcript that sets the clock to subjective dusk, after which the clock continues to run (*11*, *14*). Control ABC1 transformants, containing the entire *frq* locus, displayed a period and phase similar to those of

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the wild type (Fig. 2A) (*16*). The proximal LRE deletion strain (AF33) displayed a wild-type period, but the phase was dramatically changed, with the first strong peak in conidiation ~24 hours after transfer to constant darkness (DD24), ~12 hours later than occurred in the wild type (Fig. 2A). At the molecular level, loss of the proximal LRE resulted in a stark reduction in *frq* mRNA and in the protein products of *frq* (FRQ) in continuous light (DD0) relative to wild type (Fig. 2, C and D) (*8*, *14*, *17*). Upon transfer to DD, the low levels of *frq* and FRQ were interpreted as subjective dawn instead of subjective dusk, thereby causing the ~12-hour phase difference. The proximal LRE is therefore necessary for maintaining elevated levels of *frq*/FRQ in prolonged light as well as for eliciting the initial rapid light-induced increase in *frq* transcript. It is interesting that such a dramatic change in phase can result simply from reducing the number of binding sites (from two LREs to one) in a promoter. Using temperature instead of light as the entraining signal (*18*, *19*), the first peak in conidiation of both the wild-type and proximal deletion strains occurred as expected around DD22 (Fig. 2B), indicating that deletion of the *frq* proximal LRE specifically affects light input to the *Neurospora* clock.

The distal LRE deletion strain, AF26, was recently shown to be arrhythmic on race tubes using either light or temperature as the entraining signal (*20*). The arrhythmicity occurs because the distal LRE plays a second role as the cis-acting element mediating the WCC-driven rhythmic *frq* expression that is essential for overt rhythmicity (*12*).

Factors bound to *frq* LRE. Electrophoretic mobility shift assays (EMSAs) were used to identify the trans-acting factors that specifically interact with the *frq* LREs. Tests for LRE binding using nuclear protein extracts and radiolabeled LRE oligo probes (Fig. 1B, bottom) revealed the formation of two distinct complexes for each LRE—a faster migrating complex seen using extracts from dark grown cultures (Fig. 3A, lanes 2 and 7) and a slower migrating complex seen using extracts from light-treated cultures (Fig. 3A, lanes 3 and 8). Specificity of the complexes was demonstrated by competition using unlabeled LRE DNA (Fig. 3B, lanes 3 to 5), and by noncompetition for binding by unrelated or mutated DNA sequences (Fig. 3 B, lanes 6 to 11).

The addition of either WC-1– or WC-2–specific antiserum to a binding reaction resulted in a supershift of the complex consistent with the presence of both WC-1 and WC-2 in the complex (Fig. 3B, lanes 12 and 13). This supershift was seen for both the dark- and light-induced complexes using either the distal or proximal LRE; preimmune sera had no effect. The presence of WC-1 and WC-2 in the LRE-bound complexes was confirmed by the absence of complexes in binding reactions performed using nuclear protein extracts from *wc-1*^{ER45} (loss-of-function) or *wc-2*^{KO} strains (*8*) (Fig. 3B, lanes 15 and 16). The presence of both WC-1 and WC-2 in the complexes correlates well with previous studies that revealed WC-1 and WC-2 heterodimeric complex in vitro (*6*) and in vivo (*7*). Because WC-1 and WC-2 are known to complex with FRQ (*7*, *21*, *22*), we also assayed for the presence of FRQ in the com-

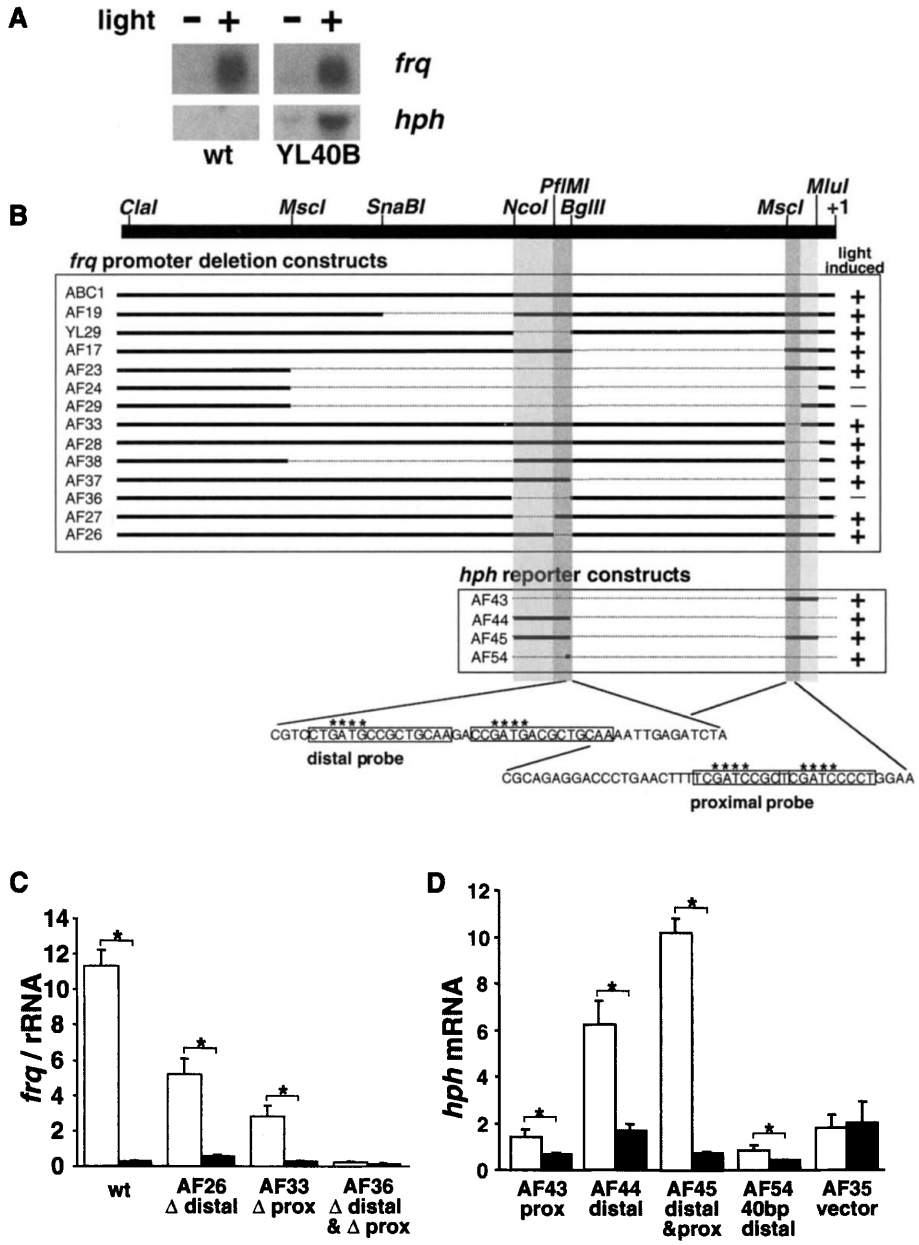

Fig. 1. Two cis-acting *frq* promoter elements are necessary and sufficient for light-induced transcription of *frq* transcript. (**A**) Northern analysis of *frq* and *hph* in control and light-pulsed tissue from wild type (wt) and strain YL40B. [(**B**), top] Schematic of *frq* promoter deletion constructs. The dashed lines indicate deleted regions. The ability to generate light-induced *frq* transcript is indicated by (+). The major start site of transcription is indicated by +1. [(B), middle] Schematic of *frq* promoter reporter constructs. The black lines indicate *frq* promoter regions in the *hph* reporter. The ability to generate light-induced *hph* transcript is indicated by (+). [(B), bottom] *frq* promoter sequence used as distal and proximal LRE probes in EMSA experiments. Imperfect repeats are indicated by boxes. The asterisks indicate bases changed in mutated LRE DNA. (**C**) Northern blot analysis of *frq* RNA in control (black) and light-pulsed (white) tissue from four strains: wt, AF26 (distal LRE deletion), AF33 (proximal LRE deletion), and AF36 (proximal and distal LREs deleted). (**D**) Northern blot analysis of *hph* RNA in control (black) and light-treated (white) tissue from five strains containing *frq* promoter/*hph* reporter constructs: AF43 (proximal LRE), AF44 (distal LRE), AF45 (distal and proximal LRE), AF54 (40-bp distal LRE), and AF35 (*hph* reporter construct alone). (C) and (D) The asterisk indicates $P < 0.05$, unpaired t test(1-tailed), $n = 3$ RNA samples ± SEM.

plexes bound to the LREs using FRQ antiserum and extracts from a *frq*^{KO} strain. FRQ was not in the dark- or light-induced complexes bound to the LREs, nor was FRQ necessary for the formation of the complexes (Fig. 3B, lanes 14 and 17).

Alignment of each half of the imperfect repeats found in the distal and proximal LREs with the LRE from another *Neurospora* light-induced gene, *al-3*, (*23*), highlights the consensus sequence CGATN-CCGCT (Fig. 3C), with the GATN sequence having been shown for all three LREs to be necessary for binding of WC-1/WC-2 (Fig. 3B, lanes 9 to 11) (*4*, *5*). The presence of the two GATN sequence repeats in the LREs is consistent with the WCs binding as a heterodimer with the Zn finger of each WC interacting with one GATN. Although these Zn-finger DNA binding proteins are often referred to as GATA factors, the GATA sequence is altered within this consensus.

Effects of light in vitro. Extracts from dark grown cultures retained light sensitivity in vitro, forming the slower migrating "light" complex even when exposed to light hours after extraction in the dark (Fig. 3A, lanes 4 and 9). This experiment was possible because the extraction process, binding reactions, and gel running were performed under red lights, whose wavelengths (>550 nm) are not detectable by *Neurospora* and by extension did not affect extracts in vitro. In vitro light sensitivity of the extracts suggested that all factors required for light perception and signal transduction to the DNA-bound WCC were soluble nuclear factors. Establishing the in vitro light effect as biologically relevant would support using this assay to identify and study components involved in light perception and signaling in *Neurospora*. To this end, we determined in vitro the dose of light and effective wavelengths needed to obtain a response in extracts.

A dose-response curve was generated by exposing aliquots of identical dark-grown extracts to white light ranging from 0 to 18,000 μmol photons/m^2 before executing the binding reactions (Fig. 4A). As the amount of light increased, a gradual shift was seen from the faster migrating complex initially present in dark extracts to the slower migrating light-induced complex. A significant change ($P < 0.05$, unpaired t test) in the dark and light complexes occurs at 20 and 60 μmol photons/m^2, respectively, in close agreement with previously published in vivo data (reflecting activity of both LREs) showing a threshhold for circadian clock responses at ~8 to 24 μmol photons/m^2 (*14*, *24*). Extracts given identical light treatments more than 30 min apart generated the same amount of light-shifted complex (*17*), suggesting that the light complex, once formed in vitro, is stable.

An equal-intensity action spectrum was generated by exposing aliquots of identical dark-grown extracts to the same fluence of light at wavelengths varying from 410 to 540 nm before executing a series of binding reactions (Fig. 4B). The in vitro action spectrum revealed a peak in sensitivity around 455 to 470 nm and no response to wavelengths above 520 nm (Fig. 4B). This in vitro action spectrum is in close agreement with previously published in vivo data for circadian clock responses that identified a photoreceptor with a peak at ~465 nm, no response to wavelengths above 520 nm, and sensitivity extending into the ultraviolet range (Fig. 4B) (*24*, *25*). The close agreement among in vivo light studies, the in vitro action spectrum, and the in vivo and in vitro dose-response curves suggests that the in vitro light shift is a true reflection of the in vivo light responsiveness of *Neurospora*, that of a blue-light photoreceptor, potentially flavin-based, with peak activity at ~465 nm and no response above 520 nm.

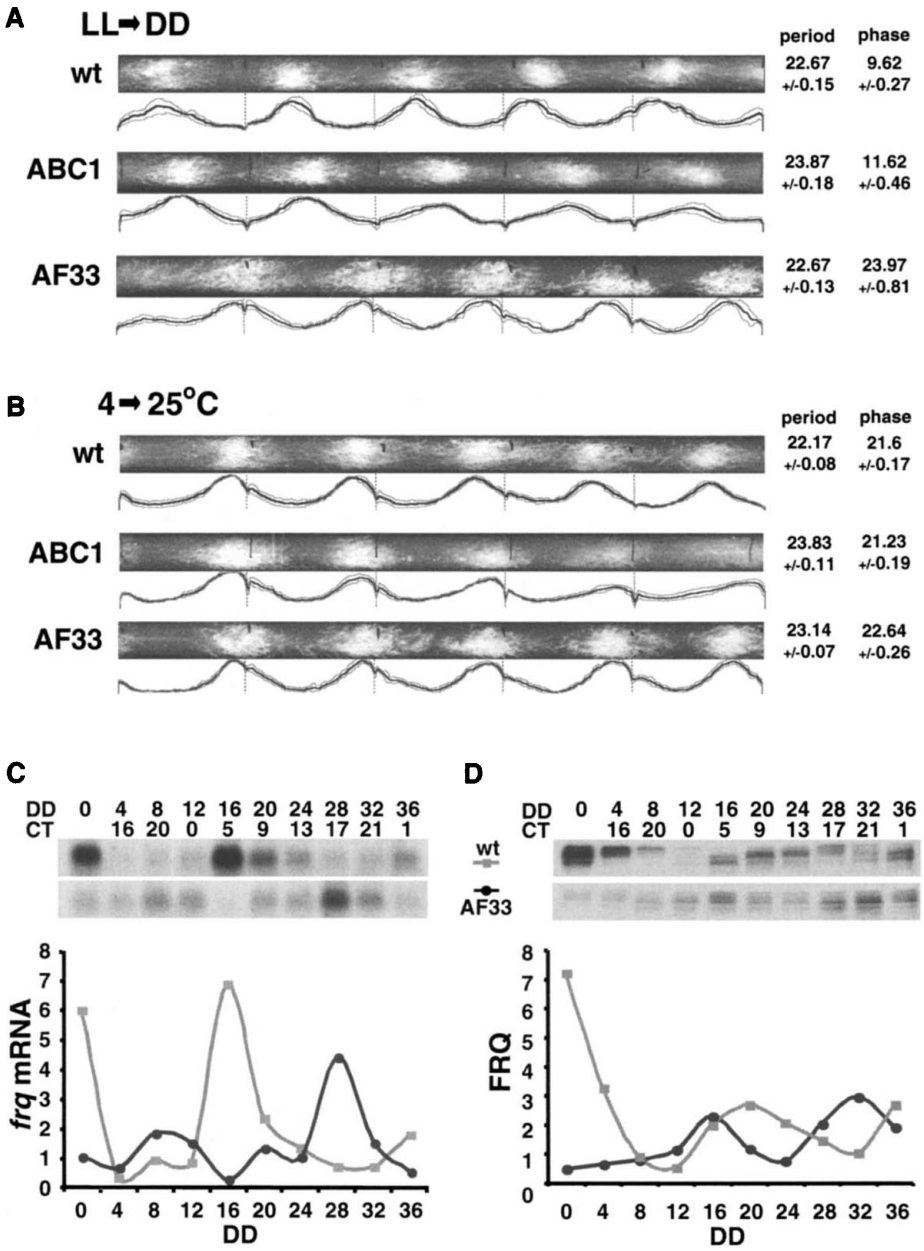

Fig. 2. Proximal LRE deletion results in a light-specific phase change of the clock. (**A** and **B**) Race tube analysis of wt (*bd*, *frq*$^+$), ABC1 [*bd*, *frq*^{KO} (pABC1)], and AF33 [*bd*, *frq*^{KO} (pAF33)]. Construct pABC1 contains the entire *frq* locus, pAF33, the *frq* locus minus the 45-bp proximal LRE (Fig. 1B). Single representative race tubes are shown with densitometric analysis of six plotted below; the bold line represents the average, and the thin lines indicate ± SD. The period and phase ± SEM are shown to the right of each race tube. (A) Race tubes entrained with light (LL) to dark (DD) transfer. (B) Race tubes entrained with temperature step. (**C** and **D**) Molecular rhythmicity in wt and AF33 strains. Liquid cultures were grown in DD for the number of hours indicated. Densitometry and Northern blot analysis of *frq* transcript (C). Densitometry and Western blot analysis of FRQ (D).

WC-1 contains a LOV domain, a subgroup of the PAS domain family associated with environmental sensing of cues that include light, oxygen, and voltage (*10*). Recently, the crystal structure of a light-sensing LOV domain from the chimeric fern photoreceptor PHY3 revealed 11 residues that interact with the chromophore, flavin mononucleotide (FMN) (*26*). These 11 residues are conserved in the WC-1 LOV domain, and the high degree of overall sequence conservation (*26*) suggests that the WC-1 LOV domain may exhibit the same overall secondary structure as the PHY3 LOV domain, ultimately sharing a common mechanism for flavin binding and possibly even light sensing. Supporting the light-sensing role of the WC-1 LOV domain are four "blind" alleles of WC-1, each with a single point mutation in one of the putative 11 FMN-binding residues (*6*, *9*), and the finding that bacterially expressed WC-1 sometimes copurifies with a yellow pigment suggestive of a flavin (*27*). Additionally, flavin-deficient mutants of *Neurospora*, *rib-1*, and *rib-2*, have greatly reduced photosensitivity for phase shifting and carotenogenesis (*28*, *29*).

To directly test the light sensitivity of WC-1 and any potential role for flavin cofactors, WC-1 and WC-2 proteins were produced in vitro using a coupled transcription/translation reticulocyte lysate system and then used in binding reactions. WC-1 and WC-2 together were able to bind to the LREs as not just one but two distinct complexes with mobilities similar to those seen using nuclear extracts (Fig. 5A, lane 13), suggesting that the light and dark complexes consist exclusively of WC-1 and WC-2. These complexes were not seen when either protein was used alone (Fig. 5A, lanes 3 to 10) or when unprogrammed lysate was used (Fig. 5A, lane 2). Sucrose gradient data further supported the notion that the dark complex contains only WC-1 and WC-2, because the WCs from dark nuclear extracts comigrated on gradients at the

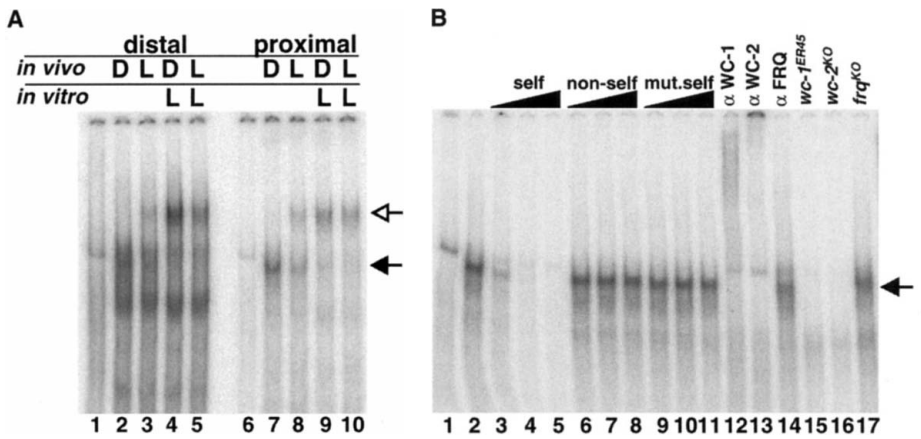

Fig. 3. Distal and proximal LREs are bound by distinct dark- and light-induced WC-1/WC-2 containing complexes. (**A**) EMSA of distal LRE probe or proximal LRE probe in binding reactions with *Neurospora* extracts from tissue grown with (in vivo L, lanes 3 and 8) or without (in vivo D, lanes 2 and 7) receiving light treatment. In vitro light treatment of the same extracts (in vitro L, lanes 4, 5, 9, and 10). The closed arrow highlights the complex predominant in the dark, and the open arrow highlights the complex induced by light, both in vivo and in vitro. Lanes 1 and 6 had no extract. The unbound probe has run off the bottom of the gel and wells are visible at the top. (**B**) EMSA of proximal LRE probe in binding reactions with dark grown *Neurospora* extracts and with antisera to identify proteins in complex. The arrow indicates the specific complex containing WC-1 and WC-2 bound to proximal LRE probe. Lane 1, no extract. Lanes 2 to 14, wt extract. Lane 15, *wc-1*^{ER45} extract. Lane 16, *wc-2*^{KO} extract. Lane 17, *frq*^{KO} extract. Lanes 3 to 5, unlabeled proximal LRE DNA as competitor (10×, 100×, 1000×). Lanes 6 to 8, unrelated, unlabeled DNA as competitor (10×, 100×, 1000×). Lanes 9 to 11, unlabeled mutated proximal LRE DNA (Fig. 1B) as competitor (10×, 100×, 1000×). Lanes 12, 13, and 14, antisera specific to WC-1 (*39*), WC-2 (*7*), and FRQ (*13*). Identical results seen using distal LRE probe with dark grown extracts and using either distal or proximal LRE probes with light-treated extracts (*17*, *18*). (**C**) The alignment of each half of the imperfect repeats found in the *frq* proximal and distal LREs and the *al-3* LRE (*23*).

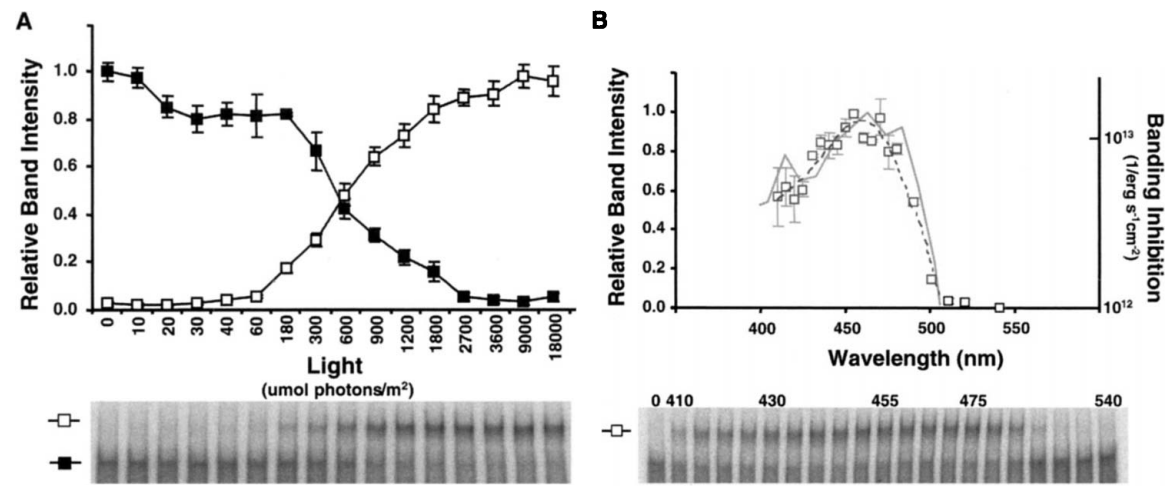

Fig. 4. The in vitro light-induced shift of the WCC occurs at biologically relevant fluence and wavelengths. (**A**) Dose-response curve generated for in vitro light shift. Aliquots of dark-grown *Neurospora* extracts were exposed to varying amounts of white light and then used in a series of binding reactions with distal LRE probe. Densitometric analysis of WCC/LRE complexes shows shift with increasing amounts of light from faster migrating (closed squares) to slower migrating complex (open squares). Bands corresponding to shifted complexes from three independent experiments were analyzed, and the average is reported ± SEM. A representative gel is shown. (**B**) Equal-intensity action spectrum generated for in vitro light shift. Aliquots of dark-grown extracts were exposed to the same fluence of light at wavelengths varying from 410 to 540 nm and then were used in a series of binding reactions with the distal LRE probe. Densitometric analysis of the slower migrating/light-induced complex (left axis, open squares, and dashed line) shows a peak in sensitivity at ~455 to 470 nm and no response to wavelengths above 500 nm. Bands corresponding to shifted complexes from three independent experiments were analyzed, and the average is reported ± SEM. A representative gel is shown. The original in vivo action spectrum for inhibition of circadian banding by continuous light, to which in vitro response is quite similar, replotted (right axis, log scale, and grey line) from (*25*).

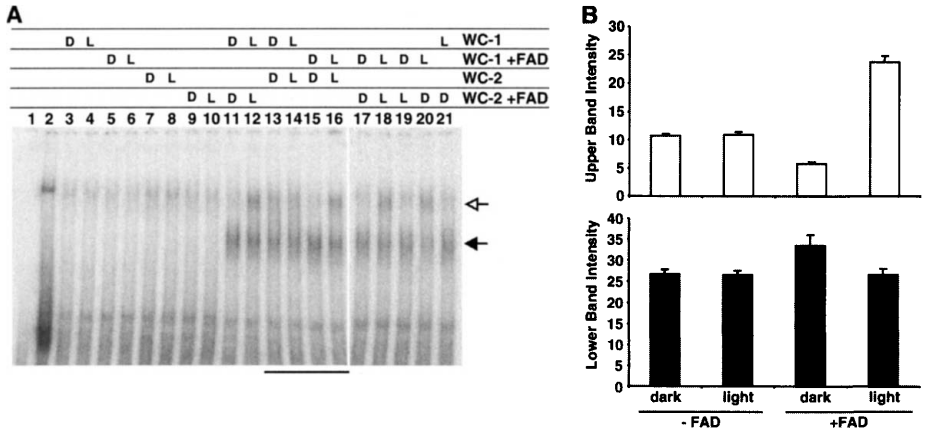

Fig. 5. In vitro–expressed WC-1 in the presence of FAD is light sensitive. (**A**) WC-1 and WC-2 were separately produced using an in vitro transcription/translation reticulocyte lysate system with FAD added to some reactions, as indicated, and then used in a series of binding reactions with distal LRE probe. Addition of WC-1 or WC-2 to a reaction is indicated above lane; "D" indicates protein added not exposed to white light, and "L" indicates protein added exposed to white light. The arrows highlight WC-1/WC-2 complexes bound to the distal LRE probe. Lane 1, no protein or lysate. Lane 2, unprogrammed lysate. Lanes 3 to 10, WC-1 or WC-2. Lanes 11 to 18, WC-1 and WC-2 incubated together before indicated light treatment and before addition to binding reactions. Lanes 19 to 21, WC-1 and WC-2 given light treatments individually, as indicated, and then incubated together in the dark before being added to binding reaction. (**B**) The reactions in the lanes indicated by the line at the bottom of the gel in (A) (lanes 13 to 16) were repeated in triplicate (fig. S1), and WC-1/WC-2 complexes were densitometrically quantified. The white bars are upper/slower migrating complex, and the black bars are lower/faster migrating complex. Error bars are ± SEM.

approximate size of a WC-1/WC-2 dimer (*17*).

No difference in binding appeared between WC proteins (translated under dim red lights) treated with or without light [(Fig. 5A), compare lanes 13 and14] (Fig. 5B), suggesting that the expressed proteins were potentially missing a necessary cofactor. However, our own and other data noted above suggested the need for a flavin cofactor for the absorption of light. We repeated the in vitro translation experiments and subsequent binding reactions, this time adding FMN or flavin adenine dinucleotide (FAD) to the WC-1 translation reaction. [FAD is the flavin cofactor for both the blue light–sensing cryptochromes (*30*) and the *Euglena* photoactivated adenyl cyclase (*31*) and most recently was shown to copurify with *Neurospora* WC-1/WC-2 complex (*32*).]

We found no light regulation of binding when FMN was added (*17*). However, addition of FAD conferred light sensitivity to the in vitro–translated proteins, indicating that all components necessary and sufficient for a light response were there. When FAD was present with WC-1 and WC-2, light caused a marked increase in the amount of slower migrating complex (Fig. 5A, lanes 12 and 16) relative to the reactions not treated with light (Fig. 5A, lanes 11 and 15), as well as causing a marked increase in the slower migrating complex, as compared to reactions lacking FAD treated with or without light (Fig. 5A, lanes 13 and 14, and Fig. 5B). Additionally, FAD resulted in more of the faster migrating complex in the dark- relative to the light-treated/FAD reactions or the reactions minus FAD, with or without light (Fig. 5A, lanes 11 to 16, and Fig. 5B). Taken together, these results suggest that, in the absence of FAD, the WCC can form both the faster/dark complex and the slower/light complex, but that light has no effect on the amount of either complex formed. The addition of FAD confers light responsiveness to the WCC, similar to that seen with the nuclear extracts; in the dark, a faster migrating WC-1/WC-2 dimeric complex dominates, yielding in the presence of light to a larger/slower migrating WC-1/WC-2 multimeric complex. In Fig. 5A, lanes 19 to 21 show that WC-1 with FAD, exposed to light in the absence of WC-2, can initiate the mobility shift when subsequently combined in the dark with WC-2. [WC-1 can be reconstituted with free FAD after in vitro translation (Fig. 5A, compare lanes 11 and 12)]. Thus, it is WC-1 that is the initial active protein partner in mediating this photoresponse.

Discussion. Light provides essential phase information for all circadian systems, and it has been asserted that rhythms evolved from PAS/LOV domain-mediated light responses (*15*). A bacteriophytochrome mediates light input in cyanobacteia (*33*), while phytochromes and cryptochromes play this role in plants (*34*). Insect clocks use cryptochrome with additional input from opsin-based pigments in the compound eye (*34*); in mammals, cryptochromes may sense light (*35*) but recent work has focused on melanopsin as the mammalian circadian photoreceptor (*36–38*). WC-1 can now be confirmed within this list of circadian photoreceptors. Located directly on DNA in the dark in a dimeric complex with WC-2, it is poised to absorb blue light using its bound FAD chromophore, an action that may trigger multimerization of WCC perhaps aiding its role in transcriptional activation.

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