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Genetic and Molecular Characterization of a Cryptochrome from the Filamentous Fungus *Neurospora crassa*[▽]

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In plants and animals, cryptochromes function as either photoreceptors or circadian clock components. We have examined the cryptochrome from the filamentous fungus *Neurospora crassa* and demonstrate that *Neurospora cry* encodes a DASH-type cryptochrome that appears capable of binding flavin adenine dinucleotide (FAD) and methenyltetrahydrofolate (MTHF). The *cry* transcript and CRY protein levels are strongly induced by blue light in a *wc-1*-dependent manner, and *cry* transcript is circadianly regulated, with a peak abundance opposite in phase to *frq*. Neither deletion nor overexpression of *cry* appears to perturb the free-running circadian clock. However, *cry* disruption knockout mutants show a small phase delay under circadian entrainment. Using electrophoretic mobility shift assays (EMSA), we show that CRY is capable of binding single- and double-stranded DNA (ssDNA and dsDNA, respectively) and ssRNA and dsRNA. Whole-genome microarray experiments failed to identify substantive transcriptional regulatory activity of *cry* under our laboratory conditions.

Cryptochromes (CRY) are defined as proteins that share sequence similarity with the DNA-repairing enzyme photolyase but lack conventional photolyase activity (45, 58, 59). Phylogenetic analysis of cryptochrome sequences indicates three general classes: plant cryptochromes, animal cryptochromes, and DASH-type cryptochromes (46). Structurally, most cryptochromes have an amino-terminal photolyase-related region (PHR) and a carboxyl-terminal domain. However, the carboxyl-terminal domain is missing in DASH-type cryptochromes (46). In *Arabidopsis thaliana*, *cry1* and *cry2* encode cryptochromes required to mediate the entrainment of the circadian clock, as well as expression of a variety of light-induced genes and developmental processes (31, 48, 62, 72). In contrast, *Arabidopsis cry3* is a DASH-type cryptochrome and appears to have a photolyase activity specific to single-stranded or looped duplex DNA (55, 58, 59). Animal cryptochromes serve functions similar to those of their plant counterparts—acting as either photoreceptors or components of circadian clocks (46). For instance, in *Drosophila*, *cry* is a blue-light photoreceptor (21, 63). Once light activated, CRY facilitates resetting of the clock by mediating the light-dependent degradation of TIM (7). In contrast, mammalian *cry* appears to play no role in circadian photobiology but instead is an integral part of the negative feedback loop of the clock (43). Light-dependent and -independent effects of animal *cry* on the regulation of the circadian clock have been well documented for both

Drosophila and mice (30, 40, 60, 68). In addition to its widespread clock functions, animal *cry* has been shown to mediate light-dependent magnetosensitivity in *Drosophila* (27).

Neurospora crassa has served as a model organism to study the circadian clock and light responses in eukaryotic cells for several decades (13, 19, 34, 35, 56). Two GATA family zinc finger transcription factors, white collar-1 (WC-1) and white collar-2 (WC-2), form an obligate heterodimer (WCC) via their Per-Arnt-Sim (PAS) domains that acts both as the photoreceptor for light responses/clock resetting and, in the dark, as a transcription factor complex that promotes the expression of FRQ, a core clock component analogous to PER/CRY in mammalian clock systems. WC-1 shares extended sequence and functional similarity with one of the heterodimeric activators in the mammalian feedback loop, BMAL1 (brain and muscle Arnt-like protein-1) (44); in the feedback loop, WCC drives expression of FRQ, which then feeds back to repress the activity of its activator, WCC, resulting in closure of the negative feedback loop (19). WC-1 is also a flavin adenine dinucleotide (FAD) binding protein capable of sensing light. For light responses, WCC activates downstream target genes through recognition of the light-responsive elements (LREs) (9, 23) in the promoters of target genes and activation of another light-responsive transcription factor, *sub-1*, to activate late light responses (8). In the absence of WC-1 or WC-2, most early and late light responses are lost, suggesting their dominant roles in mediating light signals in *Neurospora*. Notably, despite extensive efforts in several labs to identify blind strains, only *wc-1* and *wc-2* mutants have been repeatedly isolated (47). However, unexpectedly, the completion of the *Neurospora* genome sequencing revealed the presence of an additional putative blue-light photoreceptor, cryptochrome (*cry*), based on its sequence similarity to known *cry* genes (25). Given the

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regulatory similarities in the molecular basis of the circadian feedback loop and light signaling cascades among different model systems, we were interested in characterizing the clock and light functions of this novel *cry* at the molecular level.

We show that *Neurospora* CRY shares a high level of sequence similarity and domain structure with other DASH-type members, including FAD and MTHF (methenyltetrahydrofolate) binding sites as well as the residues potentially interacting with cyclobutane pyrimidine dimers (CPDs). Spectral analysis with purified CRY verified the interactions with FAD and MTHF. Like other photoreceptors in *Neurospora*, the transcript and protein levels of *cry* are highly induced by light in a *wc-1*-dependent manner. Meanwhile, we found that the transcript of *cry* in the dark is under circadian clock control, with peak expression antiphasic to *frq*, while the protein level becomes quickly dampened after 12 h in darkness. Race tube analysis with the knockout strains suggests that *Neurospora cry* is not a clock component insofar as its loss does not change the free-running period of the circadian rhythm; however, an alteration of the light-entrained phase is noted in *cry* mutant strains. Interestingly, *Neurospora* CRY is capable of binding single- and double-stranded DNA and RNA *in vitro*, as demonstrated by electrophoretic mobility shift assays (EMSA). However, whole-genome microarray analysis indicates that both early and late transcriptional light responses remain unaltered in the absence of *cry*. Thus, although the explicit signal transduction pathway that CRY impacts is unclear, we show that its loss affects the phase of entrainment of the circadian clock by light.

MATERIALS AND METHODS

Strains. The *N. crassa* strains used were 343-25 (*ras-1^{bd} cry*::hph), with 328-4 (*ras-1^{bd}*) as the corresponding *ras-1^{bd} cry^+* strain, and 378-5 (*cry*::hph), with 378-6 as the corresponding wild-type (WT) strain. 378-5 and 378-6 are sibling progeny from a cross of 343-25 with *N. crassa* wild-type FGSC 2489 (OR74A). For circadian entrainment experiments, an independently but almost identically produced knockout strain was used. The 5′ and 3′ untranslated regions (UTRs) of NCU00582.3 (*cryptochrome*) were amplified with 5′CTTGCCTCTTCCAACTT GAG3′ (forward) and 5′CTTGGACAACAGGTAAGG3′ (reverse) and with 5′CCGCTTTCCTTACCAGCCTGCG3′ (forward) and 5′CCGCGCCTCCGTG CTCAAATAC3′ (reverse), respectively. The hygromycin resistance cassette-encoding *hph* was amplified from pCSN44 with GAGGTCGACAGAAGATGA TATTG (forward) and GAGTCTAGAAAGAAGGATTACCTC (reverse). Blunt-end ligations followed by restriction digests and subsequent PCR amplification yielded fragments consisting of 5′UTR-*hph* and *hph*-3′UTR. An additional PCR step using primers within the hygR cassette, hSM-f (5′CGTCGCG GTGAGTTCAGGCTTTTT3′) together with the 3′ UTR reverse primer and hSM-r (5′TCGCCTCGCTCCAGTCAATGACC3′) (both sequences from H. Colot [personal communication]) together with the 5′ UTR forward primer, generated two fragments with a ca. 600-bp overhang within the *hph*-encoded hygR cassette. These were gel purified and cotransformed into *ras-1^{bd}*A (FGSC 1489), where they underwent homologous recombination. Strains selected for hygromycin resistance were verified for correct insertion by PCR, restriction digests, and sequencing. Homokaryons were produced by microconidiation and are called Δ*cry^{CM}* in the text. A post hoc analysis confirmed that the strains created in the two labs performed comparably in all circadian protocols. FGSC 2489 (OR74A) was also used for all microarray expression analyses and reverse transcription-PCR (RT-PCR) follow-up experiments, and for microarray work, the *cry* and *vvd* knockout strains came from the *Neurospora* knockout project (11) and have been deposited in the Fungal Genetics Stock Center, Kansas City, MO (www.fgsc.net). NCU strain numbers are from *Neurospora* annotation (http://www.broad.mit.edu/annotation/genome/neurospora/Home.html).

Targeted disruption of *cry* and *cry*-overexpressing strain. Construct pAF67 was generated in which the 5′ and 3′ sequences flanking the CRY open reading frame (ORF) were appended to the hygromycin B phosphotransferase (*hph*) coding region, whose expression was driven by the *Aspergillus nidulans trpC*

promoter, thus allowing selection by hygromycin B resistance. pAF67 was transformed into *N. crassa*, and hygromycin-resistant transformants containing single homologous integrations of the DNA were backcrossed to the wild type (WT) to obtain homokaryotic disruption strains (343-25), as judged by DNA gel blot analysis of genomic DNA (data not shown). Because the annotation of the *N. crassa* genome was incomplete when 343-25 was made, the disruption retained the first exon but spanned the entire second exon, including all chromophore binding sites. The pAF76 construct, similar to those described by Larrondo et al. (42), was used for overexpressing CRY in *Neurospora* and contains the quinic acid-2 (*qa-2*) promoter and 5′ UTR followed by a copy of the genomic region spanning the CRY ORF and then the *Aspergillus nidulans trpC* 3′ UTR in a *his-3* targeting vector. The *qa-2* promoter enables regulated gene induction by the addition of quinic acid. pAF76 was transformed into *N. crassa* strain 87-74 (*ras-1^{bd}*; *his-3*) following standard transformation protocols so that the overexpression of *cry* mRNA, confirmed to be approximately 25-fold (as determined by quantitative RT-PCR, *n* = 3; data not shown), is driven from a transgene at the *his-3* locus.

Culture conditions and light treatment. The general conditions for growth and light manipulations are described elsewhere (8, 16). Liquid culture and race tube experiments were performed as previously described (1, 14, 24) except those experiments with the CRY overexpressor strain, which used a modified growth medium containing 1× Vogel's medium, 0.17% arginine, 50 ng/ml biotin, 1.5% Bacto agar, and 0.015 M quinic acid. For the expression analysis of *cry* and *frq* following light exposure, cultures were irradiated with ~40 μmol photons/m²/s of white light (GE F20T12-CW cool white fluorescent light bulb) for the indicated durations, following of 20 h of dark incubation. Red-, far-red-, and blue-light experiments were performed using an E-30LED growth chamber equipped with red-, far-red-, and blue-emitting diodes (Percival Scientific, Inc., Perry, IA). For circadian entrainment in race tubes, methods were as described previously (64) using 4 μmol photons/m²/s of light and race tube media lacking glucose (or any other obvious carbon source; in this case, OSRAM cool white fluorescent light was used). Data were analyzed using CHRONO and CHRONOSX (57). For microarray experiments, 1×10^7 conidia were inoculated into a 10-cm petri dish with 20 ml Bird medium (50) containing 2% glucose. After 24 h of incubation in darkness at 25°C, a mycelial plug was cut with a no. 4 cork borer (diameter, 8 mm) and transferred into a 125-ml flask with 50 ml Bird medium containing 2% glucose. All procedures were performed under a low-red-light environment to avoid any possible light-stimulating effects. After another 24 h of culture with constant shaking (125 rpm) in darkness (DD) at 25°C, the flasks were moved to a shaker at 25°C with a continuous white-light stimulus (LL) (GE F20T12-CW, 40 to 50 μmol photons/m²/s), harvested at the indicated time points using vacuum filtration, immediately frozen in liquid nitrogen, and stored at −80°C until RNA extraction.

The perithecial beak phototropism assays were performed on cultures grown on Westergaard's synthetic crossing medium. In brief, strains were inoculated onto crossing plates and kept at 25°C in DD for 7 days. Fifty microliters of conidial suspension (strain 343-25, 328-4, or *wc-1^{ER53}*) was pipetted as a thin line along the diameter of the plate, and the plates were returned to the dark. The plates were exposed to a 12-h light–12-h dark cycle (the saturating light was provided by fluorescent lighting), with the plates positioned in a box with a 4-cm-wide opening so that the direction of the light was perpendicular to the line of perithecia. Perithecial beaks were scored 14 days after inoculation, with the orientation of the beaks (toward, neutral, or away) scored relative to the direction of the light. For the dark-grown samples, an arbitrary "direction" was chosen for scoring purposes.

Quantitative RT-PCR (RT-QPCR), Northern blotting, and Western blotting. The RT-PCR analysis was performed as previously described (24). The *cry*-specific primers were ACF160 (5′AAGTGGCATAGATTTCAAGC3′) and ACF161 (5′TTCGGTAGGTGGTGAAGACA3′). Statistical differences between RNA transcript levels were tested by one-factor analysis of variance and a subsequent post hoc Dunnett *t* test, where significance was set at a *P* value of <0.05. Northern blotting and Western blotting were performed as previously described (23).

CRY antibody production and protein analysis. The DNA sequence encoding the full-length CRY cDNA was PCR amplified using primers designed to add an NdeI site proximal to the start codon and an EcoRI site immediately before the stop codon. The product was digested and inserted into pET24a (Novagen), and the resulting C-terminally six-His-tagged expression construct (pAF77) was transformed into BL21-Codon Plus-RIL cells (Stratagene). Transformed cells were grown to logarithmic phase and induced with 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) for 4 h at 37°C. Following sonication of the cells, a majority of the six-His-tagged CRY polypeptide was in the inclusion body fraction, necessitating the addition of 6 M urea for solubilization. This soluble

protein was purified by nickel chelate affinity chromatography using Ni-nitrilo-triacetic acid (Ni-NTA) Superflow agarose (Qiagen) according to the manufacturer's directions. The eluate was dialyzed against phosphate-buffered saline (PBS) buffer (137 mM NaCl, 10 mM KH$_2$PO$_4$, 100 mM Na$_2$HPO$_4$, 27 mM KCl, pH 7.4) and used directly as the antigen for injection into rabbits following standard procedures (Pocono Rabbit Farm & Laboratory, Canadensis, PA).

Electrophoretic mobility shift assays. Using BL21-Codon Plus-RIL cells transformed with pAF77, six-HIS-CRY was induced with 0.75 mM IPTG for 4 h at 37°C, purified over a Ni column, and then dialyzed with 1× PBS. To isolate glutathione *S*-transferase (GST)-tagged CRY, full-length CRY was PCR amplified from pAF77 using primers designed to add an EcoRI site proximal to the start codon and a NotI site immediately before the stop codon. The product was digested and inserted into pET41b (Novagen), and the resulting GST/six-His/CRY/six-His expression construct (pAF88) was transformed into BL21-Codon Plus-RIL cells and then induced with 0.1 M IPTG for 6 h at 30°C before being purified over a Ni column. The CRY-containing fractions were pooled and run over an Amersham GSTrap FF column according to the manufacturer's instructions. For GST protein production, pET41b with no insertion was used under similar induction/purification conditions. Cleaved CRY was generated by the following steps: GST-CRY was bound to a GSTrap FF column (Amersham), and the column was then loaded with thrombin and left at room temperature overnight. An Amersham HiTrap Benzamide FF (high sub) column was placed in line with the GST-CRY-containing column, and cleaved CRY was eluted off the column (thrombin retained in the HiTrap column) using 1 M NaCl and 1× PBS. Eluted fractions were dialyzed against 1× PBS with 20% glycerol. Binding was done in a 20-μl reaction volume for 20 min at room temperature; the reaction mixtures contained 0.5 fmol biotinylated probe, 100 ng bovine serum albumin (BSA), 17.5 mM Tris-HCl, pH 7.5, 52.5 mM NaCl, 0.35 mM EDTA, 5 mM dithiothreitol (DTT), 2.5% glycerol, and the indicated proteins. The following protein amounts were used (see Fig. 6): 2 μg CRY-HIS, 4.5 μg GST-CRY, 3.5 μg GST, and 2 μg cleaved CRY. Reaction mixtures included the following increasing amounts of CRY: 0, 0.0625, 0.125, 0.25, 0.5, 1, and 2 μg. The products of the binding were subjected to electrophoresis on 0.5× Tris-borate-EDTA (TBE)–5% acrylamide gels with 0.5× TBE buffer at 4°C/150V/60 min. The nucleic acids were transferred to nylon membrane (Hybond+), UV cross-linked, and detected using the LightShift chemiluminescent EMSA kit (Pierce) according to the manufacturer's instructions. DNA and RNA probes consisted of 30-mer oligonucleotides that were biotinylated at the 5′ end (IDT). The single-stranded probes were a single oligonucleotide, whereas the double-stranded probes consisted of two complementary oligonucleotides annealed by heating to 100°C and then slowly cooling the mixture to room temperature in 10 mM Tris, pH 8, and 0.04× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate). The sequence of the DNA probe was 5′GGGGGTGGGGTTGGGTTTGGTTTTGTTTTT3′, and that of the RNA probe was 5′GGGGGUGGGGUUGGGUUUGGUUUUGUUUUU3′. The probe sequences were designed to minimize improper annealing or secondary structure.

Photoreactivation assay. Conidia were harvested, treated, and diluted with 1 M sorbitol. Conidial suspensions were irradiated with various levels of UV light using a UV Stratalinker. Appropriate dilutions of the conidia were plated in duplicate in standard transformation media (1× Vogel's, 1× fructose-inositol-glucose-sorbose medium, 1.5% agar cooled to ~45°C) (11), one plate from each pair was exposed to fluorescent light at 40 to 60 μmol photons/m^2/s for 60 min (photoreactivation), and then all plates were kept in constant darkness until colonies formed. All subsequent work was done under red safe lights. The colonies on each plate were counted, and the numbers were plotted as percent survival relative to non-UV-treated conidia. The strains used for the photoreactivation assay were 378-6 (WT), 378-5 (*cry::hph*), and FGSC 8929 (*phr^{RIP}*).

Absorption spectrum and fluorescence spectrum analysis. Bacterially expressed and purified His-CRY (375 μg) was mixed 1:1 with chloroform-isoamyl alcohol (24:1), vortexed for 5 min, and centrifuged for 10 min at 16,000 × *g* at 4°C. The upper aqueous phase, containing the released chromophore, was transferred to a new tube, and the extraction was repeated. Fluorescence emission between 470 nm and 600 nm was measured for the aqueous chromophore-containing fraction using an ISS PC1 photon-counting spectrofluorometer and excitation at 450 nm/4.0-mm slit. Fluorescence measurements were also performed on FAD and flavin mononucleotide (FMN) standards under the same conditions at pH 2 and pH 7.4. Protein absorbance spectra were measured between 300 and 600 nm using a Beckman DU 600 spectrophotometer.

TLC analysis. Thin-layer chromatography (TLC) was performed essentially as described previously (10). His-tagged CRY was purified as described above. Then, 200 μg of CRY was combined with ethanol to a final concentration of 70% ethanol, boiled for 2 min, chilled on ice, and centrifuged at 16,000 × *g* for 10 min at 4°C. The supernatant was snap-frozen, lyophilized in a speed vacuum, and

then resuspended in 10 μl of 35% ethanol. FAD, FMN, and riboflavin standards were dissolved in 35% ethanol. The TLC plate was prewarmed, and 10 μl of the standards and CRY chromophore was loaded. The plate was placed in a TLC chamber saturated with 150 ml *n*-butanol–acetic acid–H$_2$O (3:1:1) and incubated for 3 h.

Microarray sample preparation, hybridization, image, and data analysis. The detailed protocols for microarray sample preparation, hybridization, image, and data analysis have been described by Chen et al. (8).

Significance analysis of microarrays (SAM). Microarray data from the wild-type strain were used as class I (*n* = 3) in contrast to class II, the data from the cryptochrome knockout strain. A two-class unpaired test was performed with a *t*-statistic method. Missing data were imputed via a *k*-nearest neighbor algorithm (*k* = 10) before permutations (*n* = 100) for estimating the false discovery rate (FDR).

Microarray data accession numbers. Complete data sets are available on Gene Expression Omnibus (data accession numbers GSE8932 and GSE14909; http://www.ncbi.nlm.nih.gov/projects/geo).

RESULTS

***Neurospora* CRY has conserved chromophore binding sites for FAD and MTHF as well as residues for interacting with CPDs.** CRY (encoded by gene NCU00582.4) is predicted to be a 722-amino-acid protein expressed from a 2,956-nucleotide (nt) transcript having one intron (Fig. 1A). Based on the phylogenetic analysis of the cryptochrome family across different species, CRY is categorized as a DASH-type cryptochrome (46), most similar to those found in bacteria, plants, and animals (15, 46). Given that *Arabidopsis thaliana cry3* (At*cry3*) is one of the best-characterized members in this family, we aligned the amino acid sequence of *Neurospora* CRY (XP_965722) with that of AtCRY3 (NP_568461), together with those of two other DASH-type members from *Xenopus* (XlCRY-DASH, NP_001084438) and zebrafish (DrCRY-DASH, NP_991249), to check the integrity of its functional regions. Overall amino acid similarity between *Neurospora* CRY and AtCRY3 is about 44%, with a very low E value (4e-53, NCBI BLASTP). Of 16 amino acids which act in binding FAD in AtCRY3 (marked by black asterisks in Fig. 1) (5), 15 are conserved in CRY. In addition, four of the five residues (E129, E130, E459, and Y465, marked by green asterisks in Fig. 1) essential for hydrogen bonds with methenyltetrahydrofolate (MTHF) (37) are identical across the four species, suggesting that *Neurospora* CRY might bind FAD and MTHF in a manner similar to that of other members in the DASH-type family. The crystal structure study of AtCRY3 predicts six residues to directly interact with CPD-containing DNA (37); all six residues (R281, E342, W345, N433, R434 and Q437, marked by red asterisks in Fig. 1) are conserved in CRY, suggesting that *Neurospora* CRY might also interact with DNA (55, 59) in a manner similar to that of AtCRY3. In addition to the DNA/FAD binding domains which constitute the N-terminal two-thirds of the protein, CRY contains an ~200-amino-acid C-terminal region rich in glycines (Fig. 1A) not found in any other predicted members of the photolyase/cryptochrome family.

To find out if the *Neurospora* CRY could function as a photolyase *in vivo*, a disruption mutant strain of *cry* (343-25) was tested for photoreactivation. In the WT strain, increasing exposure to UV irradiation results in decreased survival, while light treatment following UV exposure increases survival of the WT due to the activation of DNA-repairing photolyase (Fig. 1C). With light treatment following UV irradiation, the sur-

FIG. 1. *Neurospora* CRY has conserved chromophore binding sites for FAD and MTHF as well as residues for interacting with CPDs and does not have photolyase activity. (A) The sequence for *Neurospora* CRY encoded by NCU00582.4 (http://www.broadinstitute.org/annotation/genome/neurospora/GeneDetails.html?sp = S7000004871288104) was aligned with AtCRY3 (NP_568461), and two other DASH-type cryptochromes from *Xenopus* (XlCRY-DASH, NP_001084438) and zebrafish (DrCRY-DASH, NP_991249), using MUSCLE software (20). Asterisks point to amino acids essential for FAD (black) and MTHF (green) binding. Conserved CPD-interacting residues are marked by red asterisks. The highlighted (red) box indicates the unique RGG repeats in *Neurospora* CRY. (B) Phylogenetic analysis of the photolyase/cryptochrome family. Protein
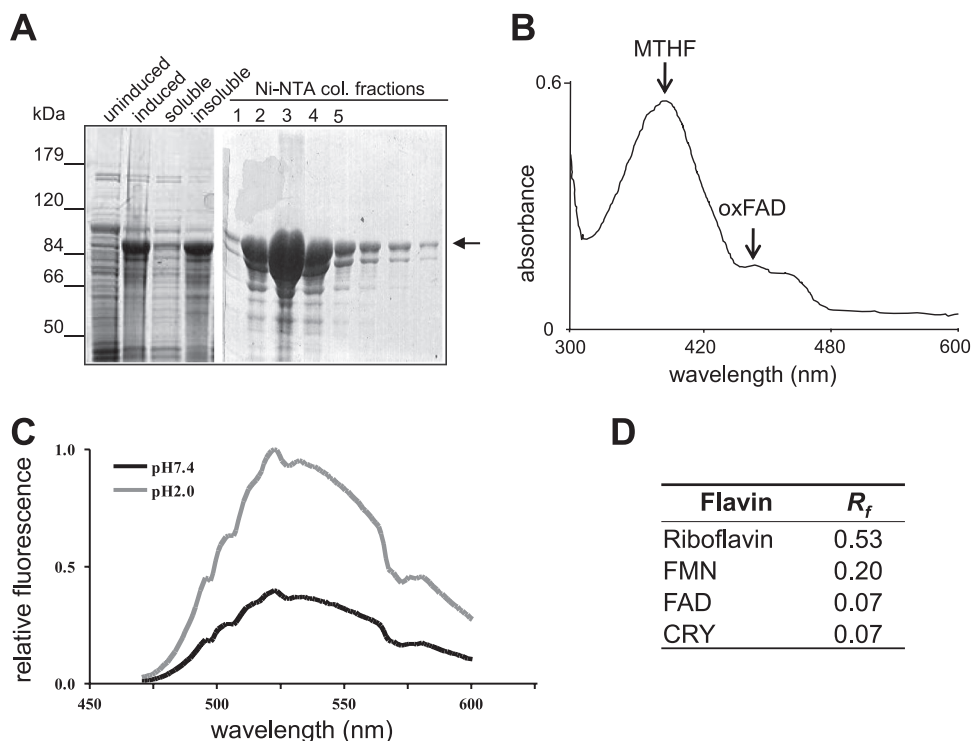
FIG. 2. Bacterially expressed *Neurospora* CRY is a FAD and MTHF binding protein. (A) Expression of *Neurospora* CRY in *E. coli* cells. A Coomassie blue-stained SDS-polyacrylamide (6.5%) gel is shown for the total protein from uninduced and induced cells expressing a His-tagged CRY. Also shown are the proteins of soluble and pelleted (insoluble) fractions, as well as CRY-containing fractions eluted from a Ni-NTA column as indicated by the arrow. The faster-migrating proteins in the Ni-column fractions are predominately CRY degradation products. Approximate molecular sizes are indicated on the left. (B) Absorption spectra of the purified CRY protein. Similar results were observed after incubation in the dark and light treatments. (C) Comparison of fluorescence emission spectra at pH 2.0 and pH 7.4 for the chromophore released from CRY. (D) TLC assay for the chromophore released from CRY. The mobility of the chromophore released from the purified CRY, as well as riboflavin, FAD, and FMN standards, is indicated relative to the solvent front (Rf).

vival of 343-25 was restored to the same level as in the wild-type strain, suggesting that the photolyase activity is undamaged in the absence of CRY. In contrast, as an internal control for the assay, a loss-of-function mutant strain of the previously characterized *Neurospora* CPD photolyase (61), *phr^RIP^*, showed no sign of increased survival with light treatment, suggesting a total lack of photoreactivation in this strain. The *phr* gene appears to encode the only photolyase in *Neurospora*, and CRY, lacking any detectable photoreactivation activity at least under these assay conditions, would be by definition a cryptochrome.

Bacterially expressed *Neurospora* CRY is a FAD and MTHF binding protein. To empirically test whether *Neurospora* CRY binds FAD and MTHF, CRY was expressed and purified in *Escherichia coli* using a six-His tag and Ni-NTA affinity chromatography. A small proportion of the expressed protein was soluble, allowing purification of CRY under nondenaturing conditions with its associated cofactors as shown in Fig. 2A. Fractions containing CRY appeared yellow, supporting that CRY binds a flavin (59). The CRY absorbance spectrum exhibited a major peak at 375 nm, two minor peaks at 445 and 470 to 472 nm, and a tail extending out to 600 nm (Fig. 2B). The dominant peak at 375 nm suggests the presence of MTHF (49); the two smaller peaks indicate the presence of fully oxidized FAD (445 nm) and flavin neutral radical (FADH[0], 470 to 472 nm) (3, 37). Notably, the absorption spectrum of CRY is
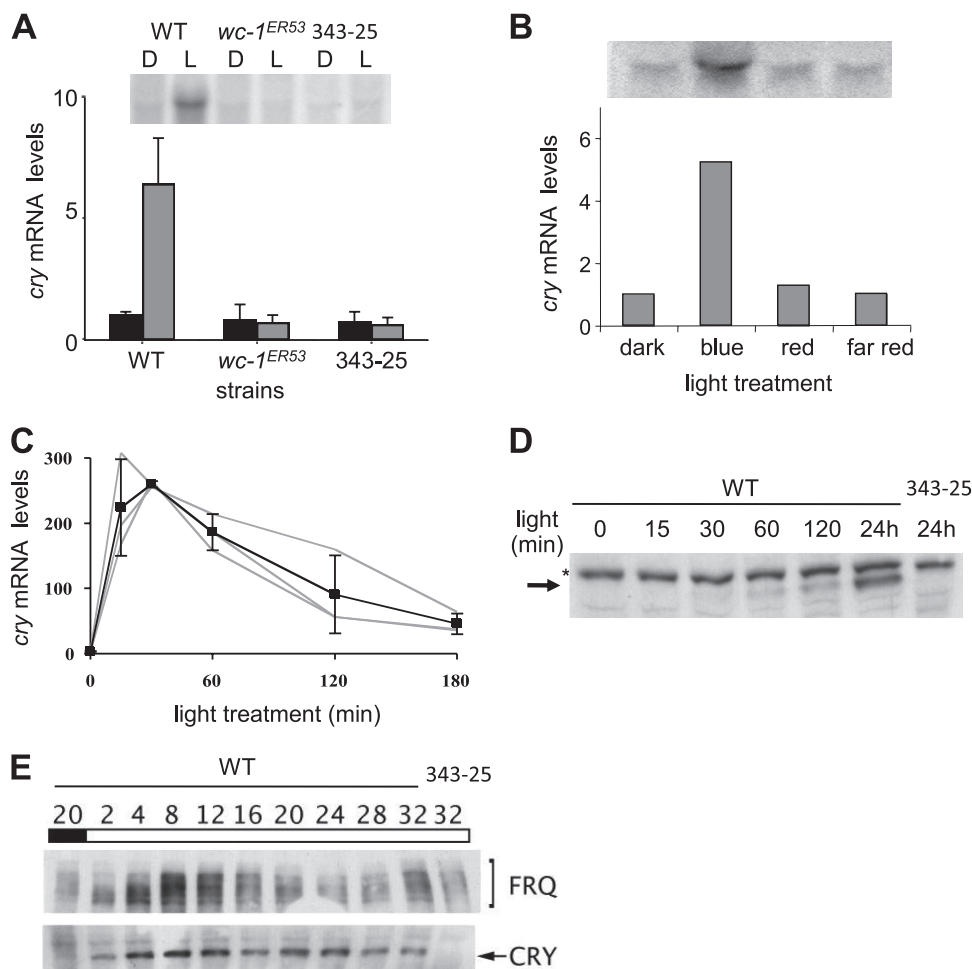
FIG. 3. *Neurospora cry* transcript and protein levels are both strongly induced by light in a *wc-1*-dependent manner. (A) Light induction of *cry* transcript is *wc-1* dependent, as shown by Northern blot analysis. Cultures were grown in the dark for 20 h with and without a subsequent 30-min fluorescent light treatment. The average of results of three Northern blot experiments determined from densitometric analysis is plotted with standaard error of the mean (SEM), with the level of *cry* expression in the dark set to 1. (B) *cry* transcript is induced by blue light, but not red or far-red light, as shown by Northern blot analysis; the average of results of two experiments is plotted. (C) *cry* transcript levels were determined by RT-QPCR analysis following various durations of fluorescent light treatment (~40 μmol photons/m²/s). Gray lines represent three separate experiments, and the black line shows the average of results of the three. The error bars indicate the SEM. (D) CRY protein levels are induced by light, as shown by Western blot analysis. The arrow indicates the position of CRY, and the asterisk indicates a nonspecific band. These data are representative of results of two separate experiments. (E) Light induction of CRY protein levels over a 32-h period as shown by Western blot analysis.

almost identical to the spectra of Atcry3 shown to bind FAD and MTHF (37). To confirm the flavin moiety, the bound cofactors were released from CRY by a chloroform extraction method and the fluorescence spectrum was recorded. Measurement of the excitation at 450 nm resulted in fluorescence emission with a maximum at 522 nm that was 3.5-fold higher at pH 2.0 than at pH 7.4 (Fig. 2C). This emission peak is indicative of a flavin moiety, and the increase in fluorescence intensity with decreased pH identifies FAD as the specific flavin derivative (70). A thin-layer chromatography (TLC) assay further confirmed one of the released cofactors to be FAD (Fig. 2D). Overall, our spectral analysis data support that the purified *Neurospora* CRY was loaded with FAD and MTHF, as predicted by the sequence analysis.

Neurospora *cry* transcript and protein levels are both strongly induced by light in a *wc-1*-dependent manner. To

determine if the *cry* gene is regulated by light, Northern blot analysis was performed on RNA isolated from fungal mycelia. A 30-min white-light treatment resulted in a dramatic increase in *cry* transcript levels relative to a dark-grown sample (Fig. 3A). The light-induced *cry* transcript ran at approximately the same position as the smallest *Neurospora* rRNA species, indicating that the *cry* transcript is approximately 2 kb, in agreement with the cDNA analysis. Light failed to induce *cry* transcript levels in a WC-1 mutant (*wc-1ER53*), suggesting that WC-1 is the photoreceptor/transcription factor responsible for the light regulation of *cry* transcription. Using blue-, red-, or far-red-light-emitting diodes (LEDs) as the light source, only blue-light treatment resulted in an increase in *Neurospora cry* transcript levels (Fig. 3B). WC-1 is responsive to blue-light wavelengths but not to red or far-red light, consistent with WC-1 being the photoreceptor directly responsible for the light
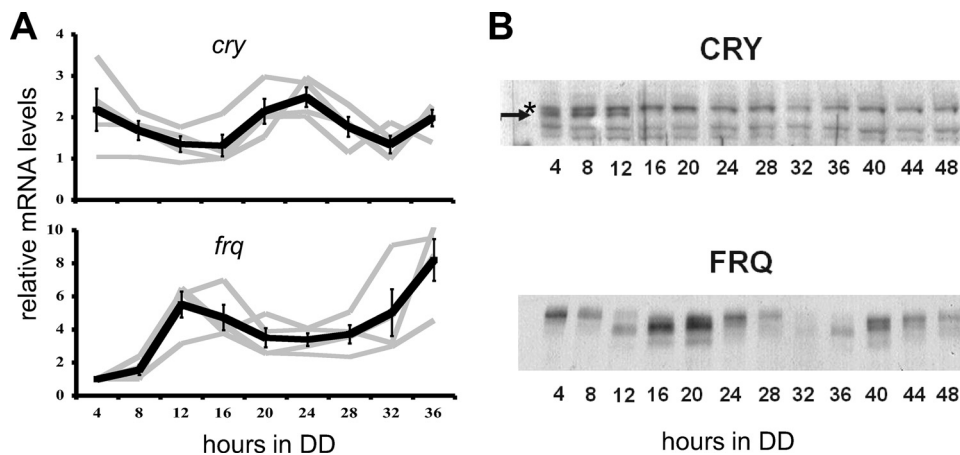
FIG. 4. *Neurospora cry* transcript is rhythmically expressed and antiphasic to *frq*. (A) RT-QPCR analysis of *cry* and *frq* transcript levels in constant darkness throughout 1.5 circadian cycles. Gray lines represent results of four separate experiments, and the black line shows the average of the four. The error bars indicate the SEM. (B) Western blot analysis of CRY and FRQ in constant darkness over a 48-h period. The arrows indicate the position of CRY, and the asterisk indicates a nonspecific band. These data are representative of results of two separate experiments.

induction of *cry*. Further quantification was performed using RT-QPCR. A wild-type strain was subjected to increasing doses of white light for 15 min to 24 h. The *cry* transcript was rapidly and highly induced, reaching levels 200- to 300-fold higher than levels found under dark conditions within 15 to 30 min (Fig. 3C). The *cry* transcript levels declined following the initial peak due to photoadaptation but remained elevated approximately 50-fold higher than dark levels after 24 h in constant light.

To detect CRY protein levels and study its regulation, we generated antiserum against the full-length CRY protein. Western blot analysis of extracts from the WT and Δ*cry* strains demonstrated the specificity of the antiserum (Fig. 3D). The antibody recognized two proteins: one was nonspecific, and a single specific protein band was detected in the WT extracts, but not in the disruption mutant (343-25) extracts, at the approximate predicted molecular mass of CRY (80 kDa). CRY was present at very low to undetectable levels in the dark but was detectable following as little as 30 min of light treatment (Fig. 3D). The CRY protein levels continued to increase, reaching a peak between 4 and 8 h, and then remained elevated under constant light exposure (Fig. 3D and E). Although both the transcript and protein levels remain elevated under constant light, the profiles differ slightly, with transcript levels rapidly peaking at 15 to 30 min and protein levels lagging by several hours.

***Neurospora cry* transcript is rhythmically expressed and antiphasic to *frq* but dispensable for the circadian clock.** The transcript levels of *cry* genes from organisms, including *Arabidopsis*, *Drosophila*, and mouse, are circadianly regulated, with amplitudes of less than 3-fold (21, 33, 41, 51, 66). To test for circadian regulation of the *cry* transcript, mycelial tissue of approximately the same developmental age was harvested at 4-h intervals following a light-to-dark transfer. A light-to-dark transfer resets the *Neurospora* clock to subjective dusk, after which the clock continues to run in constant darkness (19). The circadian regulation of the central clock component frequency (*frq*) was apparent under these conditions, with a peak in mRNA levels occurring ~12 to 16 h in constant darkness

($F_{8,27} = 7.3$; $P = 0.0001$), as shown in Fig. 4A (1). The *cry* transcript appeared to be circadianly regulated ($F_{8,27} = 2.27$; $P = 0.05$), with levels decreasing following the light-to-dark transfer, reaching a trough after ~12 to 16 h followed by an increase reaching a peak after a total of ~20 to 24 h in the dark. The *cry* transcript oscillated with an ~2-fold amplitude but at levels well below those seen in the light (i.e., time zero in Fig. 3C is equal to 20 h in Fig. 4A). Notably, in *Neurospora*, where most clock-regulated genes are morning specific (12), including *frq*, *cry* appears to be an evening-specific gene. Meanwhile, CRY protein levels remained elevated for the first 12 h but then decreased to low/undetectable levels by 16 h with no subsequent increase detected, as shown in Fig. 4B. In this regard, CRY is similar to the *Neurospora* photoreceptor VVD, which regulates photoadaptation and thereby acts to modify the primary biological response elicited by the WCC. As a positive control, FRQ abundance and phosphorylation patterns were seen to oscillate in a circadian manner as previously described (26). Although CRY does not appear to be circadianly regulated, it is possible that CRY is fluctuating at very low levels, similar to what is found in the *cry* transcript.

To determine whether *cry* plays any role in the *Neurospora* circadian system, the WT strain, a *cry* disruption strain (343-25), and a strain overexpressing *cry* (*cry*^OX^; see Materials and Methods) were grown in race tubes as described previously (19). Strains 343-25 and *cry*^OX^ had periods (22.15 ± 0.07 h and 22.77 ± 0.11 h, respectively) similar to that of the WT (22.44 ± 0.11 h; data not shown) in constant darkness, indicating that the *cry* gene is not a core clock component. In addition, light induction of *frq*, a mechanism by which light input resets the clock (14), was also unaltered in strain 343-25, suggesting that *cry* is not involved in light signaling to the clock (data not shown). Using a temperature step-up from 4°C to 25°C (in constant darkness) to reset the race tube cultures also resulted in a WT period for strains 343-25 and *cry*^OX^ (data not shown).

We then investigated synchronization of the circadian clock to zeitgeber cycles, a protocol that yields a strain-specific phase relationship between endogenous (circadian) and exogenous cycles, an active process called entrainment. (Zeitgebers [from
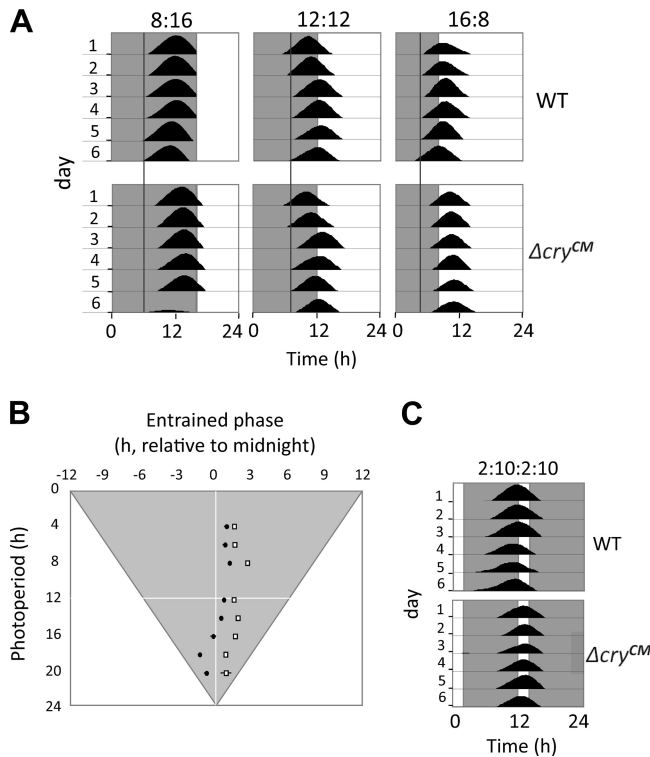
FIG. 5. Entrained phase of Δcry^{CM} is delayed in light cycles. Race tubes were subjected to 24 h cycles with various amounts of light (L) and darkness (D). The tubes were scanned, and the locations of the conidial bands within the light/dark cycle were graphed as increased pixel density. (A) Six days of conidiation are shown (black tracings), averaged from results for three (LD 8:16) to six (LD 12:12 and LD 16:8) race tubes. The relative positions of conidiation and the LD cycle are graphed, with the gray rectangles representing darkness and the open rectangles representing light. The black vertical lines are supplied as an aid to visualize relative onsets of conidiation. (B) The phase information (onset of conidiation) for a series of photoperiod experiments is graphed. Filled circles are wild-type results; open squares are the Δcry^{CM} results with error bars (frequently smaller than the symbol) representing SEM. Phase estimates are based on analysis of between 5 and 14 race tubes per entrainment condition. (C) Race tubes were incubated in a skeleton photoperiod of LD 2:10:2:10, shown as open rectangles (light) and gray rectangles (darkness). The position of conidial bands (black peaks) is later in the mutant strain.

the German for "time-givers"] are environmental stimuli that alter the phase of a biological clock; light and temperature are common examples.) In temperature cycles of 22°C to 27°C, Δcry^{CM} resembled the wild-type strain (data not shown). That is, conidiation occurred at the same phase of the temperature cycle for both the mutant and the wild type. However, in light cycles (with incubations in light followed by darkness), a delay in conidiation (interpreted as a delay in entrained phase) was observed in the absence of CRY (Fig. 5). In general, when white light was used, longer photoperiods yielded larger phase delays (e.g., 50 min at a light/darkness cycle [LD] of 12 h of light/12 h of darkness [12:12] versus 2 h at LD 18:6; Fig. 5A and 5B). An additional protocol for entrainment of circadian rhythms is that of skeleton photoperiods, which calls for delivering a light pulse at either end of a dark incubation. This procedure often mimics full photoperiod entrainment, suggesting that the lights-on and lights-off signals play a dominant role
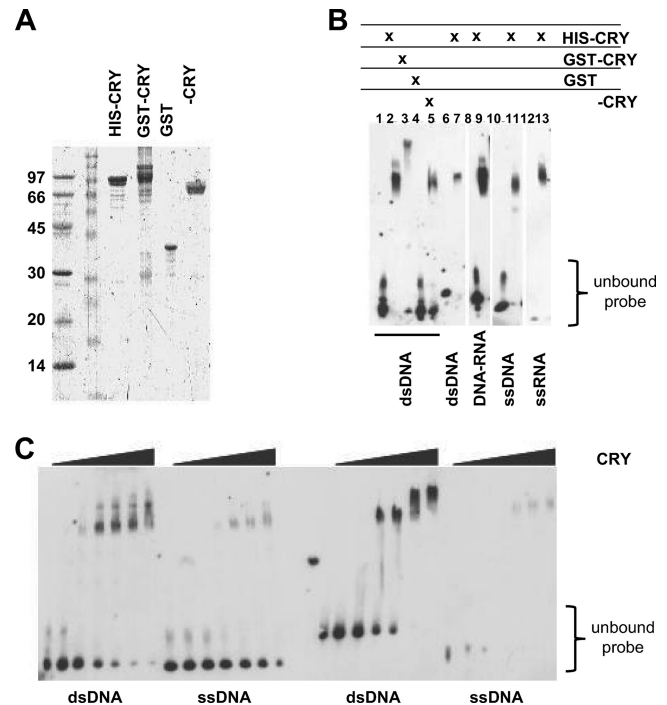

FIG. 6. *Neurospora* CRY binds to DNA and RNA, both single and double stranded. (A) Coomassie blue-stained gel showing proteins used for nucleic acid binding reactions. Amounts loaded on the gel were as follows: 4 μg HIS-CRY, 14 μg GST-CRY, 3.5 μg GST, and 4 μg cleaved CRY (-CRY). (B) Electrophoretic mobility shift assays (EMSA) using bacterially expressed proteins and the nucleic acid probes indicated. The following protein amounts were used: 2 μg CRY-HIS, 4.5 μg GST-CRY, 3.5 μg GST, and 2 μg cleaved CRY. (C) EMSA using increasing amounts of HIS-CRY (from 0 to 2 μg) and one of four different nucleic acid probes. Reaction mixtures included the following increasing amounts of CRY: 0, 0.0625, 0.125, 0.25, 0.5, 1, and 2 μg.

in entrainment. When we replaced dawn and dusk by 2-h light pulses (LD 2:10:2:10), the phase of entrainment of the Δcry^{CM} strain was delayed by 3 h compared to that of the wild-type strain (Fig. 5C).

***Neurospora* CRY binds to DNA and RNA.** In addition to the interaction with the CPD-containing DNA, DASH-type members from *Arabidopsis thaliana* (39) and *Synechocystis* sp. strain PCC6803 (5) have been shown to bind to double-stranded DNA nonspecifically, although the functional significance of this binding is not yet understood. We tested CRY's ability to bind to DNA using bacterially expressed CRY in electrophoretic mobility shift assays (EMSA).

We purified *Neurospora* CRY with either a six-His C-terminal (CRY-HIS) or a GST (glutathione *S*-transferase) N-terminal (GST-CRY, Fig. 6A) tag. The CRY-HIS protein was used in binding reactions with a 30-bp double-stranded DNA probe. CRY-HIS clearly bound to the double-stranded DNA probe, as shown by the significant decrease in migration of the DNA (Fig. 6B, compare lanes 1 and 2). To ensure that the slower migration of the DNA was due specifically to binding by CRY and not by a contaminant from the purification, we repeated binding reactions using CRY purified with an N-terminal GST tag. The use of GST-CRY in a binding reaction also resulted in

a decreased migration of the DNA (Fig. 6B, lane 3), and more importantly, the GST-CRY reaction had a greater decrease in mobility of the DNA (compare lanes 2 and 3) due to the mass increase of the GST tag. GST alone did not bind to the DNA (lane 4). As an additional control, we cleaved CRY from GST and used this cleaved CRY in a binding reaction which resulted in a decrease in migration of the DNA (lane 5) similar to the HIS-CRY reaction. Taken together, these results indicate that *Neurospora* CRY is capable of binding to double-stranded DNA *in vitro*. Meanwhile, CRY appears to bind to DNA in a non-sequence-specific fashion, because binding reactions using DNA probes of different sequences produced similar results and addition of unlabeled DNA of unrelated sequence to the binding reactions resulted in decreased binding of CRY (data not shown).

To further elucidate the nature of the CRY/DNA interaction, we tested CRY's ability to bind to single-stranded DNA and single-/double-stranded RNA. Our rationale for these additional experiments came from several observations. First, AtCRY3 is capable of binding to single-stranded DNA as efficiently as to double-stranded DNA (39). Second, RNA fragments were found to interact with *Vibrio cholerae* Cry1, another DASH-type member, when it was expressed and purified from *E. coli* (71). Third, the C terminus of *Neurospora* CRY contains multiple arginine-glycine-glycine (RGG) repeats, which usually participate in protein-RNA or protein-protein interactions (29). We found that *Neurospora* CRY was capable of binding double-stranded RNA (lane 7), single-stranded DNA (lane 11), single-stranded RNA (lane 13), and even an RNA-DNA hybrid molecule (lane 9). To begin to determine the relative affinity that CRY has for each type of nucleic acid, we performed a series of binding reactions using increasing amounts of CRY with a fixed amount of each nucleic acid species (dsDNA, ssDNA, dsRNA, or ssRNA) (Fig. 6C). CRY appears to exhibit similar affinities for dsDNA and dsRNA and a lower affinity for ssDNA. The low intensity of the ssRNA probe (bound or unbound) makes it difficult to quantitatively compare binding relative to that of the other three probes. Notably, multiple slower-migrating bands were seen in the binding reactions with dsDNA and dsRNA (Fig. 6C). The slower-migrating bands become more predominant as the CRY concentration increases, presumably due to multiple CRYs bound to each probe, supporting the notion that *Neurospora* CRY binds to dsDNA/dsRNA in a sequence-independent manner.

***Neurospora* CRY appears not to affect major regulation of either early or late light responses.** Given that *Neurospora* CRY is a putative photoreceptor capable of interacting with DNA directly, we hypothesized that *Neurospora* CRY might have some light-related functions regarding gene regulation epistatic to WCC and decided to test it using microarrays with full-genome coverage (18, 65). It has been suggested that the DASH-type CRY in *Synechocystis* sp. PCC6803 functions as a transcriptional repressor (5). Identification of bona fide light-responsive genes in our previous study (8) provides a list of good targets in which to explore the transcriptional regulatory activity of *Neurospora* CRY in response to a light stimulus. Five sequential time points, from 0 to 15, 30, 60, and 120 min after the onset of light, were monitored for three strains, the WT, Δ*cry*, and Δ*vvd* strains (data accession number GSE 8932). As
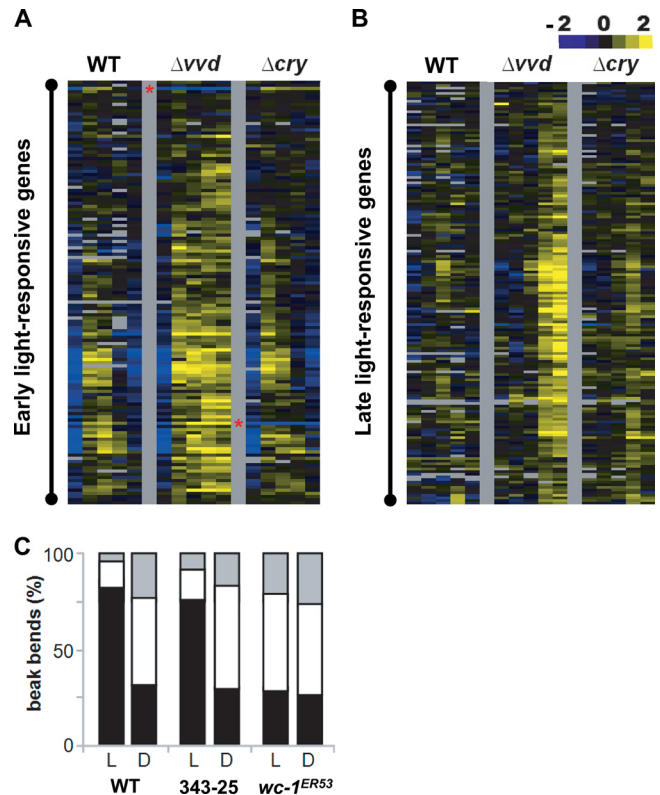

FIG. 7. *Neurospora* CRY is not involved in regulating early or late light responses. (A and B) Comparison of early and late light-responsive genes as defined previously for *N. crassa* (8). For each lane, from left to right, the individual columns correspond to light treatment for 0, 15, 30, 60, and 120 min, respectively. For each row, the data were centered across different columns before clustering. The red asterisk to the left indicates the row corresponding to the knocked-out gene in the respective knockout strain. Yellow squares indicate transcripts with increased expression, blue squares indicate transcripts with decreased expression, and gray squares represent missing data. (C) Phototropism analysis of the perithecial beak. Protoperithecia were induced in WT, 343-25, and *wc-1ER53* strains grown on petri plates. Duplicate plates for each strain were used in a cross and then placed in directional lighting (L) or in the dark (D). The orientation of the resulting perithecial beaks (black, toward; white, neutral; gray, away) was scored relative to the direction of the light and plotted as a percentage of total perithecial beaks.

shown in Fig. 7A and 7B, the regulation of most early light-responsive genes (ELRGs) and late light-responsive genes (LLRGs) appeared unchanged in the Δ*cry* strain. There was no sign of photoadaptation defects in the Δ*cry* strain in contrast to the Δ*vvd* strain, which is a strain defective in regulating photoadaptation for all light responses (8). To add a statistical verification to our conclusion, we focused on two specific time points (DD and LL60) and repeated the microarray experiments each with three independent biological replicates (data accession number GSE14909). The data were analyzed with the SAM (significance analysis of microarrays) package using a 5% false discovery rate (FDR) as a cutoff (67). SAM identified only four genes, including the *cry* transcript, which were significantly different between the WT and Δ*cry* strains. However, with the exception of *cry* (as an intrinsic positive control for our approach), none of the other genes could be further validated

by RT-QPCR (data not shown). In separate experiments, quantitative RT-PCR analyses in a *ras-1^bd^ Δcry* strain failed to find statistically significant increases in light induction of *frq*, *al-1*, or *con-6* expression (22a). Although small effects on the expression of *al-1* or *con-6* have been recently reported (53), we expect the differences in results are due to genetic background effects rather than major undiscovered influences of CRY, and indeed the reproducible lack of major effects seen in our microarray analyses, which might well not report the results shown in reference 53 as statistically significant, supports this interpretation. Given cryptochrome's central role in the photobiology of many other organisms and the delay in entrained phase in the *cry* mutant, it is possible that *Neurospora* CRY shows more substantive transcriptional regulatory activity under nonlaboratory conditions or at other developmental stages in the *Neurospora* life cycle.

Analysis of long-term light phenotypes in the mutant *cry* strain. Given that CRY protein levels remain elevated under constant light exposure (Fig. 3D and E), we tested if there is any detectable long-term light-dependent developmental phenotype in the absence of *cry*. Constant light would trigger the carotenoid biosynthesis pathway, which can be seen in the accumulation of orange pigmentation. However, no gross defects in light-induced carotenoid biosynthesis can be detected (data not shown). Another light-regulated process, the development of *Neurospora*'s sexual spores in the perithecial organ, is also regulated by light (17). Positive phototropism of the perithecial beaks (beak bending, a maternal effect) is induced by blue light, resulting in the sexual spores being ejected toward the direction of light (32). We tested for defects in perithecial phototropism by inoculating crossing plates with one of the three strains (*ras-1^bd^*, 343-25, or *ras-1^bd^ wc-1^ER53^*) as the female parent and fertilizing these crosses with a WT strain. The plates were placed in directional lighting and then scored for the direction of the perithecial necks relative to the direction of the light. The WT perithecial necks displayed random growth directions when grown in the dark, whereas 82% of the WT perithecial necks pointed toward the light, as shown in Fig. 7C. The *wc-1* mutant displayed a random distribution of perithecial necks both in the dark and with directional lighting. In contrast, 343-25 exhibited WT perithecial phototropism, with 74% of perithecial necks pointing toward the light. Having found no differences in 343-25 in the developmental processes known to be regulated by light nor in general growth rate (data not shown), we looked for novel phototropism during *Neurospora*'s asexual life stage. Different assays for phototropism were tested using a variety of culturing methods in combination with various directional lighting configurations (24). Culturing systems included standard shaking and static liquid cultures, solid media in petri plates, and race tube assays, including modified race tubes which enabled inoculation in the middle of the tubes to allow analysis of fluence responses. In order to study the fine branching structure of mycelia, we used thin vertical gels consisting of standard media poured between two glass plates similar to those used to electrophorese protein samples. These various culturing setups were combined with lighting configurations consisting of standard fluorescent lighting, sunlight, and custom-fabricated light-emitting diode arrays (blue, red, and white). The light sources and intensities were tested in a variety of configurations (e.g., high-intensity blue

light above the vertical gels and low-intensity red light below). Under none of the conditions tested did we find any signs of phototropism in the WT or mutant *cry* strains (data not shown).

DISCUSSION

DASH-type CRY members have been identified from various organisms, ranging from bacteria and plants to fungi and animals. In *Synechocystis* sp. PCC 6803, CRY-DASH has been shown to have no photolyase activity *in vitro* but weak photolyase activity *in vivo* (36). The same protein has nonspecific DNA binding ability and possibly acts as a transcriptional repressor for a few genes (5). In *Vibrio cholerae*, two DASH-type members (Vc*cry1* and Vc*cry2*) have no photolyase activity *in vivo* or *in vitro* (71). In *Arabidopsis thaliana*, the only DASH-type member, At*cry3*, is capable of binding to single- and double-stranded DNA (nonspecifically) *in vitro* but without any conventional photolyase activity *in vivo* (39); however, biochemical and structural studies suggest that At*cry3 in vitro* can act as a CPD photolyase with specificity for either single-stranded (37, 59) or looped double-stranded (55) DNA. Among animals, DASH-type members have been identified only in *Xenopus laevis* and *Danio rerio* (15); both have a weak photolyase activity when expressed in *E. coli*. No nonspecific DNA binding activity could be detected *in vitro*, and only DrCRY-DASH displays CPD binding activity on double-stranded DNA (15). Overall, although weak photolyase activity and DNA binding ability have been demonstrated for several DASH-type members, the definitive biological function of these DASH-type cryptochromes is still unclear: no strong phenotype could be observed in any case, and they might be involved in other biological processes not necessarily requiring DNA repair functions (5, 69). Because of this, we have focused on exploring clock and light phenotypes in the knockout strain of *Neurospora crassa* CRY, focusing on characteristics that have not yet been extensively examined in other DASH-type members. To be clear, the CryA recently reported in *Aspergillus nidulans* is actually a class I CPD photolyase based on both enzymatic and phylogenetic analyses (2).

Since it was first identified in 2003 (25), *Neurospora* CRY has been referred to as a cryptochrome ortholog based on sequence similarity. Later, it was categorized as a DASH-type member (5, 15, 19), which is the only type of cryptochrome existing in the fungal kingdom; no animal or plant-type cryptochromes have been detected in the sequenced fungal genomes. However, it was surprising to us that free-running rhythms and a catalog of other light-regulated phenotypes are not perturbed in the absence of *cry*. Only entrainment in light cycles gave a mutant phenotype. The data clearly suggest that *Neurospora* CRY does not participate in the transcription-translation feedback loop as does its counterpart in other organisms, but this is in fact consistent with findings for other DASH-type members. For instance, in a transient transfection assay using an E-box-driven luciferase that is responsive to CLOCK: BMAL1, the DASH-type members in *Xenopus laevis* and *Danio rerio* effect no inhibition on the luciferase activity. In contrast, in the same assay, animal-type CRYs from the same organism (DrCRY1a) inhibit the luciferase activity completely (15), suggesting that DASH-type and animal-type CRYs are

functionally distinct from each other. Surprisingly, although *Neurospora* CRY is not a clock component and is dispensable for most light responses and various light-regulated developmental processes, the *cry* transcript is controlled by the circadian clock with peaks antiphasic to *frq*. Interestingly, the gene expression of another DASH-type member from tomato (*Solanum lycopersicum*) has been shown to be under the control of the circadian clock as well (22). The physiological significance of the circadian regulation of DASH-type CRYs, however, remains unclear.

Here, we showed that the transcript and protein levels of *Neurospora* CRY are both strongly and rapidly induced by light in a *wc-1*-dependent manner. However, whole-genome microarray analyses of mycelium samples collected under different light conditions indicate that there is no significant difference between WT and *cry* knockout strains at the transcriptional level. Given this, perhaps the major biological function of *Neurospora* CRY might be related to posttranslational regulation (54), or perhaps it becomes evident only at specific developmental stages or under particular growth conditions. For instance, in *Neurospora* the regulatory function of the opsin photoreceptor gene *nop-1* could be observed only in late developmental stages (4), and a recent report suggests that CRY can modulate the primary response elicited by WC-1 and WC-2 (53); this would perhaps provide an explanation for the small effects we report on the entrained phase of the conidiation rhythm in strains lacking CRY. That report (53) also provides confirmation of the light induction of *cry* reported here. Additionally, some supporting evidence comes from the study of *cry1* from *Sclerotinia sclerotiorum* (69), which is the only other fungal DASH-type member characterized so far and has orthologs for all the *Neurospora crassa* light-sensing components (i.e., WC-1, WC-2, and VVD). The transcript level of Ss*cry1* is strongly induced by UV-A and appears to follow induction kinetics similar to those of the *cry* transcript in *Neurospora*. Knockout strains of Ss*cry1* exhibit a slight decrease in sclerotial mass and increased numbers of pigmented hyphal projections on apothecial stipes under UV-A treatment but are otherwise developmentally normal. The study concludes that Ss*cry1* may have a function during UV exposure but is not essential for completing the developmental life cycle under laboratory conditions. Therefore, these principles may hold true for revealing additional biological functions of *Neurospora* CRY and other DASH-type members in the future.

Similar to most DASH-type cryptochromes, *Neurospora* CRY has no detectable photolyase activity *in vivo* and appears to bind FAD and MTHF when expressed in *E. coli*. In addition to its nonspecific DNA binding activity, which has been shown for other DASH-type members, we discovered that *Neurospora* CRY also has the capability to bind to single- and double-stranded RNA, as well as to a DNA-RNA hybrid. Although these properties might be shared among DASH-type family members, we speculate that this feature might be unique to *Neurospora* CRY due to the existence of multiple arginine-glycine-glycine (RGG) repeats at the C terminus, which are missing for all other DASH-type cryptochromes, including the *cry1* found in *S. sclerotiorum*. RGG repeats are commonly found in proteins involved in regulation and coupling of RNA maturation events (29). RGG repeats are generally found at the C terminus of the protein and contribute to RNA binding

nonspecifically (6, 28, 38, 52), both features of *Neurospora* CRY. Given the expression of *cry* RNA and protein late in the circadian cycle relative to most clock-regulated transcription, CRY may act via regulation of RNAs to fine-tune the phase of entrainment. Conversely, it may be worth reevaluating whether the capability of binding to both DNA and RNA is restricted to *Neurospora* CRY or widespread among other CRY-DASH members and if this function is tied to clock phenotypes in these other model organisms.

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