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
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 (methylenetetrahydrofolate) binding sites as well as the residues potentially interacting with cyclolubatan 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 antiphased 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<sup>−</sup> cry<sup>−</sup>), with 328-4 (ras<sup>−</sup> <sup>−</sup>) as the corresponding ras<sup>−</sup> cry<sup>−</sup> strain, and 378-5 (cry<sup>−</sup>), 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 and almost identically produced knockout strain was used. The 5′ and 3′ untranslated regions (UTRs) of NCU005823 (cryptochrome) were amplified with 5′TCGCTCTTCAATCTCAGG3′ (forward) and 5′CCTCGACCAGTTGAAGG3′ (reverse) and with 5′CCGGCGCTCTCGCTAACTAC3′ (forward) and 5′CGGGCGGTCTCATCAGTCAAAATAC3′ (reverse), respectively. The hygromycin resistance coding fragment, encoding hph, was amplified from pCSN44 with GAGGTCGACAGAAGATGACGCTCGCTCGCTCCAGTCAATGACC3′ (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 cry<sup>−</sup> coding region. These were gel purified and cotransformed into ras<sup>−</sup> 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 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.

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protein was purified by nickel chelate affinity chromatography using Ni-nitrito-
triacetic acid (Ni-NTA) Superflow agarose (Qiagen) according to the manufactur-
er's directions. The eluate was dialyzed against phosphate-buffered saline (PBS) buffer (137 mM NaCl, 10 mM KH2PO4, 100 mM Na2HPO4, 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 trans-
formed 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 1X PBS. To isolate glutathione S-transferase (GST)-tagged CRY, full-length CRY was PCR ampli-
ified 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/ CRYHis expression construct (pAF88) was transformed into BL21-Codon Plus-RIL cells and then induced with 0.1 mM IPTG for 6 h at 30°C before being purified over a Ni column. The CRY-containing fractions were pooled and run off an Amersham GSTrap FF column according to the manufacturer's instruc-
tions. 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 over-
night. An Amersham Hitrap Benzamidine 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 1X PBS. Eluted fractions were dialyzed against 1X 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 mM biotinylated probe, 100 ng bovine serum albumin (BSA), 50 μg thrombin, 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, 0.0625, 0.125, 0.25, 0.5, 1, and 2 μg. The products of the binding were subjected to electrophoresis on 0.5X tris-borate-EDTA (TBE)-5% acrylamide gels and 0.5X TBE buffer at 4°C/150V/60 min. The nucleic acids were transferred to nylon membranes (Hybond+) UV cross-linked, and detected using the LightShift chemiluminescent EMSA kit (Pierce) accord-
ing 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 (1X SSC is 0.15 M NaCl plus 0.015 M sodium citrate). The sequence of the DNA probe was 5’GGGGGTTTGTGTTGTTGTTGTTTTT3’, and that of the RNA probe was 5’GGGGGGGUGGGGUUGGGGUUGUUUUU3’. The probe sequences were designed to minimize im-
proper annealing or secondary structure.

Photoreactivation assay. Conidia were harvested, treated, and diluted with 1× M9 medium. Conidial suspensions were irradiated with various levels of UV light using a UV Stratalinker. Appropriate dilutions of the conidia were plated in standard transformation media (15, 46). Given that Arabidopsis thaliana cry3 (Atcry3) 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 (XICRY-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) are conserved in CRY. In general, amino acid residues are conserved in both Neurospora CRY and Atcry3; CRY could function as a DASH-type photolyase/cryptochrome, and might also interact with DNA as a photolyase

RESULTS

Neurospora CRY has conserved chromophore binding sites for FAD and MTHF as well as residues for interacting with CPDs. CRY (encoded by gene NCU000582.4) 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 phylo-
genetic analysis of the chromophore family across different species, CRY is categorized as a DASH-type cryptochrome (46), most similar to those found in bacteria, plants, and ani-
mal. Given that Arabidopsis thaliana cry3 (Atcry3) 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 (XICRY-DASH, NP_001084438) and zebrafish (DrCRY-
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folate (MTHF) (37) are identical across the four species, suggest-
ing 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=H11005S7000004871288104) 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
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), phrRIP, 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 (FADH0, 470 to 472 nm) (3, 37). Notably, the absorption spectrum of CRY is similar to that of flavins, which suggests that CRY binds FAD and MTHF.

![Figure 2](https://eukaryot.cell.org/content/2/2/742/F0000-1.png)

**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 (RI).

sequences of members of the photolyase/cryptochrome family were retrieved from the NCBI database. A multiple sequence alignment was constructed with Lasergene MegAlign software using the Clustal method with the Identity residue weight table. Similar phylogenetic trees were generated using either the Structural or PAM100 residue weight tables (not shown). Shown as abbreviation, organism, and accession number are Bf MTHF, *Bacillus firmus*, Q04449; Ec MTHF, *Escherichia coli*, P00914; St MTHF, *Salmonella typhimurium*, P25078; Sc MTHF, *Saccharomyces cerevisiae*, P05066; Nc MTHF, *Neurospora crassa*, Q04449; Hh 8-HDF, *Halobacterium halobium*, P20377; Sg 8-HDF, *Streptomyces griseus*, P12768; Mt type II, *Methanosarcina thermooautotrophicum*, P12769; Mx type II, *Mycobacterium xanthus*, U44437; Dm type II, *Drosophila melanogaster*, S52047; At 6-4, *Arabidopsis thaliana*, AB003687; Dm 6-4, *Drosophila melanogaster*, D83701; Dm CRY, *Drosophila melanogaster*, AF099734; Mm CRY1, *Mus musculus*, AB000777; Mm CRY2, AB003433; AtCRY1, *Arabidopsis thaliana*, S66907; AtCRY2, U43397; Sa CRY, *Sinapis alba*, P40115; and Cr CRY, *Chlamydomonas reinhardtii*, S57795. (C) Photoreactivation analysis of the WT (378-6), cry-disruptant (378-5), and phrRIP strains. Each strain was irradiated by UV light at the indicated doses and plated onto two petri plates. One plate was exposed to fluorescent light for 60 min (photoreactivation [PR]) and the other received no light treatment.
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-1<sup>ER53</sup>), 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
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 antisera against the full-length CRY protein. Western blot analysis of extracts from the WT and Δcry 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 ($F_{2.27} = 7.3; P = 0.0001$), as shown in Fig. 4A (1). The cry transcript appeared to be circadianly regulated ($F_{2.27} = 2.27; P = 0.05$), 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
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, ΔcryCM 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 in entrainment. When we replaced dawn and dusk by 2-h light pulses (LD 2:10:2:10), the phase of entrainment of the ΔcryCM 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 fromGST 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 DNA 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, Δcry, and Δvvd strains (data accession number GSE 8932). As 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 Δcry strain. There was no sign of photoadaptation defects in the Δcry strain in contrast to the Δvvd strain, which is a strain defective in regulating phototropism 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 Δcry 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<sup>del</sup> 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 entry 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 perithecal 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 perithecal phototropism by inoculating crossing plates with one of the three strains (ras-1<sup>del</sup>, 343-25, or ras-1<sup>del</sup> wc-1<sup>ER53</sup>) 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 perithecal necks relative to the direction of the light. The WT perithecal necks displayed random growth directions when grown in the dark, whereas 82% of the WT perithecal necks pointed toward the light, as shown in Fig. 7C. The wc-1 mutant displayed a random distribution of perithecal necks both in the dark and with directional lighting. In contrast, 343-25 exhibited WT perithecal phototropism, with 74% of perithecal 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 (Vcry1 and Vcry2) have no photolyase activity *in vivo* or *in vitro* (71). In *Arabidopsis thaliana*, the only DASH-type member, Atcry3, 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 Atcry3 *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 *Neurospera 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), *Neurospera* 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 *Neurospera* 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 *Sc* *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 *Sc* *cry1* exhibit a slight decrease in scerotial mass and increased numbers of pigmented hyphal projections on apothecial stipes under UV-A treatment but are otherwise developmentally normal. The study concludes that *Sc* *cry1* may have a function during UV exposure but is not essential for 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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