Fully Codon-Optimized luciferase Uncovers Novel Temperature Characteristics of the Neurospora Clock

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We report the complete reconstruction of the firefly luciferase gene, fully codon optimized for expression in Neurospora crassa. This reporter enhances light output by approximately 4 log orders over that with previously available versions, now producing light that is visible to the naked eye and sufficient for monitoring the activities of many poorly expressed genes. Time lapse photography of strains growing in race tubes, in which the frq or eas/ccg-2 promoter is used to drive luciferase, shows the highest levels of luciferase activity near the growth front and newly formed conidial bands. Further, we have established a sorbose medium colony assay that will facilitate luciferase-based screens. The signals from sorbose-grown colonies of strains in which the frq promoter drives luciferase exhibit the properties of circadian rhythms and can be tracked for many days to weeks. This reporter now makes it possible to follow the clock in real time, even in strains or under conditions in which the circadian rhythm in conidial banding is not expressed. This property has been used to discover short, ca. 15-h period rhythms at high temperatures, at which banding becomes difficult to observe in race tubes, and to generate a high-resolution temperature phase-response curve.

Circadian rhythmicity is an ancient form of biological regulation. The ability of an organism to determine the time of day in order to regulate metabolic events is close to ubiquitous within the eukaryotes and, additionally, is found in more than one cyanobacterial species (17). Although the ability to tell time from a molecularly based oscillator is thematically conserved across many phyla, the outputs that the circadian clock regulates are organismally dependent, and in many systems, reporters are used to monitor these rhythms. There is a rich history of using luminescence for this purpose in circadian biology. Studies using circadianly regulated endogenous luciferase in the marine dinoflagellate Gonayaulax polyedra to follow the clock (34) were precursors for a host of experiments using luminescent reporters in plants (35), cyanobacteria (28), Drosophila melanogaster (4) and mammalian tissue and tissue culture (21). Recently, Neurospora crassa has become an entrant in this list (36); however, studies with luciferase in Neurospora, until now, have been hampered by low-intensity luminescent signals that precluded routine analysis of clock genes and, in general, of all but the most abundantly expressed genes.

The classical method for assessing rhythms in Neurospora is to observe the periodic changes in asexual development of macroconidiospores (conidia) during growth in glass tubes called race tubes (15, 41). Although this is a robust and invaluable assay, luminescence offers numerous advantages. Foremost, luminescent reporting of circadian rhythms can closely relay molecular events at the level of the core oscillator, and luminescent reporting allows for fine spatiotemporal resolution of clock parameters. When one is monitoring the circadian rhythm by following sporulation, factors affecting the sporulation process per se can mask the underlying status of the core oscillator. Finally, luciferase reporters can simplify and automate the task of tracking multiple strains simultaneously and thereby facilitate high-throughput screens (28, 29). However, transformation of Neurospora with the native firefly (Photinus pyralis) luciferase gene (luc) yields no measurable luciferase activity. Thus, we sought to increase the level of luminescence in Neurospora.

Heterologous gene expression in Neurospora and other organisms has been improved by reengineering an open reading frame (ORF) to optimize codon bias (24, 39), a modification that has been reported to affect translation efficiency in Neurospora (27). Optimization of the first 21 residues of the firefly luciferase ORF allowed detection of luminescence in Neurospora (36), but only at light intensity levels that precluded studies of poorly expressed genes, including the clock gene frequency (frq). In this work, the luc gene was resynthesized such that the entire ORF no longer displayed any negative codon bias for Neurospora (37) (see also Discussion). Here we present the development of this dramatic improvement in this methodology for Neurospora. Strains of Neurospora expressing a completely codon-optimized luciferase exhibit significant levels of luminescence, even at low luciferin concentrations. Using these increased expression levels, we report dynamic spatiotemporal characteristics of clock-controlled gene expression. Moreover, this improved luciferase gene makes it possible to follow, in near-real time and in vivo, the transcrip-
tional activity of the frq gene, a key element in the negative-feedback loop of the Neurospora clock (16). Despite the low activity of the frq promoter, frq rhythms can be clearly tracked for many days and even weeks.

We validated the use of luciferase as a circadian reporter and describe an assay to extend its use that reveals new findings about the Neurospora clock. First, we verify that clock-controlled and core clock gene expression properties, as reported by luciferase, are as expected in the wild type and in a clock mutant. Next, we introduce a colony-based assay for monitoring luciferase activity, and we extend practical options to study the molecular workings of the clock by showing rhythmic luciferase activity in a poorly conidiating mutant. Additionally, we track rhythms at temperatures beyond the range where the conidial banding in race tubes can easily be observed. Finally, a high-resolution temperature pulse phase-response curve is presented along with the identification of a novel temperature pulse singularity.

MATERIALS AND METHODS

Neurospora strains and culture conditions. Neurospora crassa strains used in this study were the his-3; ras-1ΔΔ strain, a long-period mutant (his-3; ras-1ΔΔfrq) and the consolidation-defective flabby mutant (his-3; fla-1ΔΔ) (1). The use of ras-1ΔΔ increases the visibility of asexual development in race tubes (2). Culture conditions and handling of Neurospora were as previously described (11, 15) unless otherwise noted (see below). In some cases, modified race tubes were used; medium was poured into 16-mm by 150-mm Fisher glass tubes, which were inoculated on the end nearest the cap and were capped during the run. Constructs in which luciferase expression was driven by the frq (19) or eas (cpg-2) (3) promoter were targeted to the his-3 locus as described below, yielding (initially as heterokaryons) the his-3::his-3-flaP-luc; ras-1ΔΔ and a his-3::his-3-easP-luc; ras-1ΔΔ strain.

Complete cDNA optimization of luc. The P. pyralis luciferase sequence (GenBank accession no. M15077) was codon optimized according to Neurospora codon usage data from the Kazusa DNA Research Institute (http://www.kazusa.or.jp/codon/cgi-bin/showcodon.cgi?species=15141) (37). For each residue, the codon with the highest expression (o-luc, referred to below simply as luc). A full-length cDNA-optimized cDNA was created using the method of assembly PCR (43). A series of overlapping oligonucleotides encoding Neurospora firefly luciferase codon optimized (Fig. 1) were designed using DNA Builder 3.5.4 for MacOS (see also http://www. sxsystèmes.com/tech/38/361.html). Codon transforming oligonucleotides, 40mers with 20-mer overlaps, were ordered in arrays from Illumina (San Diego, CA) and Integrated DNA Technologies (Corvalle, IA). PCR was performed in masse with subsets of primers in the mixture using Expand High Fidelity Taq (Roche). DNA sections were ligated using a combination of TA cloning and traditional cloning, and all PCR-amplified regions were sequence verified. Since regions of the optimized luc ORF were designed to have high GC content, part of the gene was amplified with a GC-rich Taq (Roche) polymerase designed for GC-rich sequences.

Reporter constructs. Two transcriptional fusions of luc were constructed. A resected eas/cpg-2 promoter (between ∼737 and ∼1, upstream of the ATG) (3) was modified using PCR so that EcoRI and Apal restriction enzyme sites were added to the 5′ and 3′ ends of the resected promoter, respectively. A region of the frq promoter (between ∼4382 and ∼1519, upstream of the long FRO ATG) [8] was similarly modified by PCR. All frq 5′ untranslated-region (UTR) sequences were omitted, because frq is known to have complex posttranscriptional regulation (8), and we sought to simplify subsequent interpretation of the luc signals. To accommodate clustering, an Apal site was engineered at the 5′ end of the luc sequence. At the 3′ end of the luc ORF, we added a stop codon and a BamHI restriction enzyme site; no additional accommodations were made for a 3′ UTR. Constructs were cloned by blunt-end ligation into pBluescript, a plasmid designed for transformation at the his-3 locus of Neurospora (32, 33). In addition to examining the consequences of codon optimization, we also wanted to determine if the presence of an intron might improve reporter gene expression (40). Therefore, we included the first intron of the eas/cpg-2 gene in two of the luciferase constructs at the position where the first intron would naturally occur in the P. pyralis luc gene. Thus, three different constructs with the optimized luciferase gene were initially made: an intronless sequence under the control of the eas/cpg-2 resected promoter (cpg-2-luc) and two constructs in which the luciferase sequences bearing the cpg-2 intron I are under the control of the eas/cpg-2 (cpg-2-luc-I) or the frq (frq-luc-I) resected promoter, respectively. (See Fig. 3 in the supplemental material for the luc-I sequence.) Placement of these constructs in the his-3 targeting vector pB661 (32, 33) yields pCPC2-luc, pCPC-2-luc, and pfrq-luc-I, respectively.

Neurospora transformation and generation of homokaryotic strains. Strain 87-74 (his-3; ras-1ΔΔ a) was transformed by electroporation with a linear plasmid as previously described (32). Plasmids pCPC-2-luc and pCPC-2-luc-I were transformed into 87-74, and primary transformants were tested for luminescence by the addition of firefly luciferin (BioSynt L-8200) o-luciferin firefly [synthetic] potassium salt; 10 to 125 μM). Approximate levels of 50% of his protoporphyrin transfectants displayed light emission and a number of these light-emitting clones, all producing similar levels of light, were chosen. A representative heterokaryon was backcrossed to ras-1ΔΔ a to obtain homokaryotic derivatives. Homokaryons of luc strains with other relevant genotypes in the background (frq and fla) were produced by mating against his-3; ras-1ΔΔfra-1 and his-3; fla, respectively. Individual ascospores were picked and germinated, and luciferase activity was confirmed by measuring luminescence on a Turner TD-20e lumimeter (Turner Designs).

Assessment of strain light intensities. Strains of Neurospora were inoculated into 100-mm-diameter petri dishes containing approximately 25 ml liquid culture medium (11) (1 × Vogel’s medium, 2% glucose, 0.5% arginine, 50 μg/liter biotin, 10 μM luciferin [added after autoclaving]) and were grown for 24 h in constant light until they formed a mycelial mat with a ring of conidia around the edge of the dish. Viable cultures were then synchronized by placing them overnight in the dark at 4°C, followed by removal to constant darkness at 25°C. After 21 h, a sample of conidia was collected with sterile wooden sticks from the edge of the dish, and the spores were weighed by placing them in a preweighed luminometer tube. One milliliter of a 10 μM luciferin solution was added, the mixture was vortexed, and the light intensity was measured in an LKB 1251 luminometer over 15 s. The relative intensity per milligram of conidial tissue was recorded.

Sorbore colony assays. Sorbose medium containing 1% FGS (0.05% fructose, 0.05% glucose, 2% sorbose), 1× Vogel’s medium (46), 50 μg/liter biotin, and 1.8% agar was autoclaved for 20 min and was used to induce colonial growth of Neurospora (13). We find that in order to see dependable rhythmity in colonial Neurospora, rather than autoclaving the FGS components separately (5), we need to autoclave all of the components together, which, incidentally, produces a slight caramelization of the medium. This partially caramelized medium is referred to as AFV (autoclaved FGS-Vogel’s) medium. Firefly luciferin (10 to 125 μM) was added to the medium after autoclaving; conidial suspensions in 0.5% glucose were plated on AFV and grown in constant light (LL) for 3 to 4 days until conidial eruptions were seen. These cultures were either transferred from 4°C or from LL to DD at the relevant temperature, and luminescence was then recorded in real time.

Luciferin and oxygen dependence. An ice-cold 10-μl conidial suspension (cpg-2-luc-I-bearing strain; 9.0 × 105 conidia/ml) was added to make a 100-μl solution at the indicated luciferin concentrations. Immediately after mixing, luminescence readings were taken using an LKB 1251 luminometer. The same strain of Neurospora was grown into mycelial mats in petri plates on liquid culture medium (2% glucose, 0.5% arginine, 1× Vogel’s medium, 50 μg/liter biotin). Five 8-mm plugs were placed in 2.5 ml of 200 μM luciferin (air-saturated solution) in a sealed transparent cuvette with an oxygen electrode (Rank Brothers, Cambridge, CB2 9DA, United Kingdom). Luminescence (versArray 700 B/LN camera; Roper Scientific) and oxygen were simultaneously measured as oxygen was depleted over 12 min. The system was then opened for reoxygenation to normal levels, resealed, and the procedure repeated. The oxygen concentration was normalized to an air-saturated solution.

Real-time charge-coupled device (CCD) recording. Liquid nitrogen or electronically cooled cameras from Roper Scientific (VersArray 700 B/LN and VersArray 1300 B/LN) and Hamamatsu (model C4742-98 ERGB) were used to follow luminescence. Quantification was performed on regions of interest from the camera’s field of view. The field of view can be calibrated to include an entire race tube or a narrow region within the tube. The cultures were monitored in environmentally controlled Pericel incubators or Hotpack rooms that were modified to accommodate the cameras. Luciferase signals were quantified with WInspec32 or WinView32 software (Roper Scientific) and processed in Microsoft Excel or Matlab. Typically, luminescent signals were accumulated either for 10 min every 1 h in DD or for 30 min at a time, over the time course, in DD.
Temperature resetting. Forty petri plates (60 by 20 mm) containing 30 ml of AFV medium and 1.8% agar were each inoculated with six 20-μl dots of a 1:250 solution of *Neurospora* spores (frq-luc-I; 1.2 × 10^7 conidia/ml of stock) in 2.5 mM luciferin. The cells were then entrained to a 24-h cycle of 12 h of light (0.13 μmol s^{-1} m^{-2}) alternating with 12 h of dark for 4 days at 25°C. At the end of the fourth 12-h light cycle, the plates were moved into constant darkness at 25°C. A VersArray 700 B/LN CCD camera was used to measure the intensity of luminescence (30 min per frame). Data were smoothed using Loess smoothing (6), and peak and trough times were objectively determined from a second-order polynomial equation using the three Loess-smoothed points around the peak or trough.
TABLE 1. Relative luminescence intensities of light-producing strains

| Strain genotype   | Luminescence intensity* |
|-------------------|-------------------------|
|                   | Heterokaryon | Homokaryon |
| eas (ccg-2) driven, partially optimizeda | 0.007, 0.001 | ND |
| eas (ccg-2)-luc-Ic | 53, 37       | 137, 38    |
| eas (ccg-2)-lucId | 87           | 63         |
| frq-luc-Ia        | 0.6, 0.26    | 1.0, 0.58  |

*a Expressed as relative light units per milligram of conidia. Independent measures from different transformants are shown. ND, not done.

b Partially optimized luciferase (first 21 codons) under eas (ccg-2) promoter control. See reference 36.
c Fully optimized luciferase with an eas (ccg-2) intron under the control of a resected eas (ccg-2) promoter.
d Like eas (ccg-2)-luc-I above but without the intron.

trough. The temperature of the agar was rapidly (~5 min) increased or decreased by placing plates on an ice block or on a warm plate. The temperature of the plates was monitored with a calibrated thermistor embedded in the agar of a control plate. In the case of the dose-response curve, the length of the circadian period of the control was 22.2 ± 0.8 h. The indicated pulse of temperature was administered for 1 h at 32 h (a time of maximal phase shifting) after the start of DD, corresponding to circadian time 22.6 (15).

RESULTS

Codon-optimized luciferase under eas/ccg-2 control produces abundant light. A schematic of the codon-optimized luciferase gene construction is provided in Fig. 1A, and the final sequence of the optimized luciferase (luc-I) gene is shown in Fig. S1 in the supplemental material. Expression of the optimized luciferase gene produces high levels of light. We estimate that eas/ccg-2-driven optimized-luciferase generates approximately 4 to 5 orders of magnitude more light than a nonoptimized luciferase driven by the same promoter (Table 1). Light from eas/ccg-2-driven luciferase can be seen with the naked eye, dark adapted for at least 5 min. We also tested both intron-containing and intronless constructs (luc-I and luc), because we thought intron-containing constructs might show higher levels of light; however, we saw no substantial difference in intensity (Table 1).

In ccg-2-luc-I-bearing strains, light can be imaged in individual growing hyphae and newly formed conidia, as seen in Fig. 1B (bottom left). Importantly, under race tube assay conditions with the levels of luc gene expression and luciferin used here, it is clear that the amount of light produced is less than the amount that would noticeably influence the Neurospora circadian system, since transformed strains grown on luciferin-containing race tubes show wild-type period lengths of the banding rhythm (Fig. 1C, lower panel). The actively growing cells at the growth front show the highest levels of light production, although mycelia throughout the race tube rhythmically produce light; a time lapse side view of the race tube (see movies S1 to S3 in the supplemental material) also reveals extensive luminescence within cells beneath the agar surface (see movie S1 in the supplemental material). Furthermore, a leading projection of mycelia, on and below the agar surface, precedes the bulk of the growth front (Fig. 1B, bottom left; see also movie S1 in the supplemental material). Individual cells of hyphae emit light as they grow along the agar surface (see movie S2 in the supplemental material). The time lapse video of this ccg-2-luc-I strain shows an overall cyclic pattern of luminescence (see movie S3 in the supplemental material). Moreover, there is a daily surge in the expression of light at the growing tips, and this is accompanied by the previously documented daily cycle of the growth rate at the actively growing front (22). Together these phenomena result in a daily increase in light as Neurospora grows down the race tube (see also movie S3 in the supplemental material).

The frq promoter drives rhythmic luminescence. While eas/ccg-2 is a highly expressed gene, frq, a gene encoding a key component in the negative arm of the core circadian clock, is expressed at much lower levels. Therefore, a true test of reporter improvement was to reliably observe frq-driven rhythms in luminescence. Thus, we transformed a genotype his-3; ras-1(bd) a strain with pfrq-luc-I and selected light-emitting his-3 prototrophs in which frq-luc-I was inserted at his-3. Light-emitting primary transformants were run on race tubes containing 10 to 125 μM luciferin. While frq promoter-driven luminescence is significantly lower than eas/ccg-2-driven luminescence (10 to 100-fold [Table 1]), it is still ~100 times brighter than the partially optimized luciferase driven by the much stronger eas/ccg-2 promoter (Table 1) (36). Strains bearing this new reporter also exhibited robust 22-h wild-type rhythms (Fig. 2A and B, see also movie S4 in the supplemental material).

Colonies grown on sorbose show luminescence. A useful property of Neurospora is that addition of sorbose to the medium results in dramatically increased hyphal branching. Consequently, when cultures are grown on sorbose, they grow as colonies (13). We asked if colonial Neurospora bearing frq-luc-I might also show luminescence. Conidia or conidial suspensions were inoculated onto a medium containing 2% sorbose (AFV medium; 10 μM luciferin). Colonies were grown in the light until conidial eruptions or puffs were seen (Fig. 3A, left panel). Such colonies emit light, as detected by a CCD camera (Fig. 3A, right panel).

The colonial assay facilitated examination of specific properties of the system. Luciferin is long-lasting in the medium: we have seen signals lasting more than 4 weeks (data not shown). Even at these time points, we do not believe that luciferin is exhausted, since a light stimulus results in high levels of luciferase expression via induction of the frq promoter. In addition, other parameters can influence the amount of the luciferase signal. More luciferin in the medium produces more light (Fig. 3B); the light reaction appears very sensitive to oxygen (Fig. 3C); and increased glucose levels in the medium also facilitate light production when driven by this promoter (data not shown). Finally, luciferin is quickly (within seconds) incorporated into Neurospora and can be sprayed directly onto colonies, as is done with Arabidopsis thaliana (35), leading to rapid observation of luminescence (data not shown).

Luminescence on sorbose and in race tubes shows circadian properties. When the frq-luc-I strain is used, strong peak-to-trough oscillations of light emission can be seen on pulled conidiating colonies growing on sorbose-containing plates (AFV medium) (Fig. 4A, top panel), and the period of light emission is within the expected circadian range (22.7 ± 0.2 h) for the frq<sup>−</sup> strain. This is also seen in race tubes bearing
light-emitting strains (Fig. 4A, second panel from the top). These cycles are sustained for many days. Whenever *frq-luc-I* cells are transferred from light to dark, the curve is not quite sinusoidal, since the rising phase is always faster than the decaying phase. In cultures transferred from light to dark, we always observe an initial high level of luminescence that decays over the next 36 h, a phenomenon consistent with Northern blot studies showing that *frq* RNA is strongly induced in the presence of light (10). Light and temperature entrain the luminescent rhythms in this *frq-luc-I* luminescent system, consistent with previous work on *Neurospora*. Although light induces a dramatic increase in luminescence, temperature does not have a similar effect (data not shown), consistent with previous work (31). Also, this new luminescent system shows period lengths that are consistent with previously published reports (Fig. 4A, bottom two panels [replotted with data from references 22 and 7, respectively]). It should be noted that each of the traces shown in Fig. 4A is measuring periods under differing conditions; thus, one must be cautious in making phase comparisons between clocks observed on diverse media.

To further confirm that luminescence was faithfully reporting clock state parameters, we backcrossed the reporter to an *frq*-long-period mutant (16). As expected, the period length of light emission for this strain increased to 30.9 ± 0.9 h, a period within the normal range of variability observed for *ras-1bd; frq*-a strains in race tubes (representative traces are shown in Fig. 4B).

**Rhythms can be seen in nonbanding strains and under nonbanding conditions.** To date, we had been able to assess rhythms in nonbanding strains only by labor-intensive Northern or Western blot analysis for *frq* or an appropriate clock-controlled gene. Using our *luciferase* reporter, we can now more easily determine rhythms in nonbanding strains. The *fluffy (fl)* strain is a *Neurospora* mutant that produces very few conidia (1) and, in our hands, bands poorly. While a representative race tube shows no discernible rhythm in

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**FIG. 2.** Strains bearing *frq-luc-I* show rhythmic luminescence. (A) The same race tube is shown as an optical (top) or luminescent (bottom) image. Growth proceeds from the left. Bands marked A to C are individually tracked in panel B. (B) Luminescence as a function of time. In the top panel, the trace represents quantification of the luminescence of the entire race tube in panel A. Luminescence is shown as a function of position. In subsequent panels, signals (black traces) were collected from individual bands A thru C, respectively (as indicated in panel A). The superimposed blue traces represent one-quarter of the signal from the entire tube. Older bands show reduced intensity and damping rhythms.
bANDING (Fig. 5A, top), a clear rhythm in luciferase activity (21.6 ± 0.1 h) can be observed for this fl strain bearing frq-luc-I (Fig. 5A, bottom) on standard race tube medium (11).

The traditional method of race tube analysis has a temperature limit on conidial band detection. Banding is not easily observed above 30°C and has not been reported beyond 34°C (20) even when specially engineered strains are used (38). This is likely due to direct effects of temperature on asexual development that override or mask clock control of banding. We asked if frq rhythmicity persisted beyond the range previously reported for banding in order to confirm the upper limits of wild-type clock periodicity and temperature compensation. When we ran the sorbose colony assay at 36°C, we saw rhythms of approximately 15 h (14.6 h in the detrended example shown in Fig. 5B). The rhythms appear to be of lower amplitude, show variability in acrophase and period (we see periods ranging from 13.5 to 15 h), and are generally not as robust as those at lower temperatures. Interestingly, although clearly out of the temperature-compensated range, the oscillator appears to function reasonably well at the level of frq transcription.

Luciferase allows high-resolution mapping of the temperature phase-resetting response. Circadian rhythms can be reset by changes in either light or temperature, and the degree of that resetting is known to depend on the magnitude of the change as well as the time in the cycle at which the change is applied (18, 47). The luminescent colonial system offers us the opportunity to do such experiments with more accuracy, using more data points, and with greater ease. To determine the characteristics of such resetting with temperature, we ran a series of experiments using frq-luc-I-bearing colonies growing on AFV sorbose plates at 25°C and then applied 1-h pulses of higher temperatures at different times of the circadian cycle. We then compared the subsequent phase of peaks of luminescence.
pulses (Fig. 6B) at hour 32 after DD shows a sharp sigmoidal curve. The dose-response curve using different 1-h temperature levels of luminescent output (see Table 1). This work, as well as exclusion of an intron appeared to have no major effect on the oscillation of the optimized naked eye. This increase is presumably due to increased trans-crecence to the phases of nonpulsed controls. We found that 1-h temperature increases to 29°C gave weak resetting responses (known as “type 1” resetting [47]) (Fig. 6A, top left). Pulses at temperatures greater than 32°C gave a strong phase-resetting response (“type 0”) (Fig. 6A, bottom right). A 31°C pulse given at hour 32 after transfer to DD resulted in arrhythmicity (Fig. 6A, top left). Rhythmic expression driven by the frq promoter at elevated temperatures signals the presence of an uncompensated short period rhythm in the colonized form on AFV medium. In the trace shown as an example, raw luminescence data have been replotted by linear detrending on Matlab, revealing an oscillation of approximately 15 h.

FIG. 5. Luminescence rhythms are observed in nonbanding mutants and at very high temperatures. (A) The fluffy genotype shows a robust luminescent rhythm. The various blue traces depict the luminescence rhythms in race tubes for three fluffy isolates bearing luciferase. The race tube at the top, with growth from left to right, shows the lack of a sporulation rhythm in this strain. (B) Rhythmic expression approximately follows previously measured protein levels of FRQ (7), peaking at a time when asexual development occurs where there are asexual conidia and at the actively growing region of the growth front. This is not inconsistent with previously described developmental roles for this gene (3). The frq-luc-I rhythms are also most intense at the growth front, although there is a clear, but dampening, rhythm in older conidial bands. Interestingly, the presence of highest expression in new conidia may be a general property; for colonial Neurospora grown on AFV medium also, it appears that significant luminescence is not seen until conidia begin to form. We caution that luciferase activity may not accurately report certain properties (e.g., nuclear/cytoplasmic localization) of gene expression patterns.

Importantly, for our purposes, luciferase correctly reports circadian activity. Four lines of evidence support this conclusion. First, the frq and eas/ccg-2 promoters drive oscillatory luminescence levels with appropriate period lengths. In the case of frq, under free running conditions in DD, the period length of the rhythm is 22.7 ± 0.2 h, with the first peak close to 19 h after transfer to DD. Moreover, in race tubes, the luminescence approximately follows previously measured protein levels of FRQ (7), peaking at a time when asexual development is maximally repressed by the circadian oscillator. By contrast, eas/ccg-2-driven rhythms dampen more quickly, perhaps reflecting developmental effects on the resected eas/ccg-2 promoter (3). Importantly, this difference between frq and eas/ ccg-2 indicates the second line of evidence; the observed circadian activity is indeed due to the promoter activity and not to other oscillatory cellular components merely affecting the luciferin-luciferase chemical reaction. Third, circadian resetting properties are recapitulated with our reporter. When using the frq promoter, we see that longer durations of light proportionately induce increased levels of luminescence (V.D.G., unpublished data), a pattern consistent with previous reports showing that light induces more frq production through the induction of the frq promoter (10). Finally, using the frq long-period mutant, we get an expected increase in the period, which shows that frq-luc-I is indeed reporting the clock.

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**DISCUSSION**

Optimization of the entire firefly luciferase sequence increases luminescence to levels detectable by the dark-adapted naked eye. This increase is presumably due to increased translation of the optimized luciferase mRNA, and inclusion or exclusion of an intron appeared to have no major effect on the level of luminescent output (see Table 1). This work, as well as work by others (9, 39), suggests that complete codon optimization is an important consideration for heterologous reporters in Neurospora. Moreover, though luc gene expression is increased, relative differences in gene activity are still observed; we can see a clear difference between highly expressed genes, e.g., eas/ccg-2, and poorly expressed genes, e.g., frq. Thus, our new luciferase construct might act as a suitable reporter for a wide variety of gene expression studies of Neurospora and various microbial and nonmicrobial systems that share a GC-rich codon bias, including gram-positive bacteria (e.g., Arthrobacter, Streptomyces, Mycobacterium, and Pseudomonas spp.), trypanosomes, and perhaps selected vertebrates. Moreover, while we have used luciferase in the context of the clock, given that many fungal species (e.g., Aspergillus, Phytophora, Alternaria, and Magnaporthe spp.) have a propensity for G or C in the third position of their codons (http://www.kazusa.or.jp/codon/cgi-bin/showcodon.cgi?species =5141), we foresee widespread use of this luciferase in the fungal community for a variety of biological investigations.

In addition to being a reporter of gene activity, optimized luc may also serve as a gross spatiotemporal developmental reporter. In race tube cultures, the highest expression of ccg2-luc occurs where there are asexual conidia and at the actively growing region of the growth front. This is not inconsistent with previously described developmental roles for this gene (3). The frq-luc-I rhythms are also most intense at the growth front, although there is a clear, but dampening, rhythm in older conidial bands. Interestingly, the presence of highest expression in new conidia may be a general property; for colonial Neurospora grown on AFV medium also, it appears that significant luminescence is not seen until conidia begin to form. We caution that luciferase activity may not accurately report certain properties (e.g., nuclear/cytoplasmic localization) of gene expression patterns.

Importantly, for our purposes, luciferase correctly reports circadian activity. Four lines of evidence support this conclusion. First, the frq and eas/ccg-2 promoters drive oscillatory luminescence levels with appropriate period lengths. In the case of frq, under free running conditions in DD, the period length of the rhythm is 22.7 ± 0.2 h, with the first peak close to 19 h after transfer to DD. Moreover, in race tubes, the luminescence approximately follows previously measured protein levels of FRQ (7), peaking at a time when asexual development is maximally repressed by the circadian oscillator. By contrast, eas/ccg-2-driven rhythms dampen more quickly, perhaps reflecting developmental effects on the resected eas/ccg-2 promoter (3). Importantly, this difference between frq and eas/ ccg-2 indicates the second line of evidence; the observed circadian activity is indeed due to the promoter activity and not to other oscillatory cellular components merely affecting the luciferin-luciferase chemical reaction. Third, circadian resetting properties are recapitulated with our reporter. When using the frq promoter, we see that longer durations of light proportionately induce increased levels of luminescence (V.D.G., unpublished data), a pattern consistent with previous reports showing that light induces more frq production through the induction of the frq promoter (10). Finally, using the frq long-period mutant, we get an expected increase in the period, which shows that frq-luc-I is indeed reporting the clock.

Because our robust reporter extends the conditions under
which we can study rhythmicity, we have now seen oscillations in \textit{frq} where rhythmicity had not been seen previously. For example, we show clear rhythms in the \textit{fl} strain, which shows no conidial banding phenotype. This result is consistent with the observation that some clock-controlled genes cycle in this strain (9). Of course, \textit{frq} cycling might have been observed by traditional Northern blot analysis, but our approach obviates such tedious procedures while easily providing a much higher time resolution and a larger dynamic range. Analysis of banding using race tubes is not feasible beyond 34°C, because temperature effects on conidiation mask underlying rhythmicity; in this regime we now show evidence of rapid, low-amplitude \textit{frq} oscillations. Moreover, traditional methods of monitoring \textit{FRQ} using liquid cultures at temperatures approaching even 30°C appear to show high levels of \textit{FRQ}. We suspect that under these conditions there are environmental inputs that obscure rhythmicity. However, luciferase has allowed us to see low-amplitude rhythms at temperatures above these limits, and the period of these rhythms is consistent with a decreasing rhythm as a function of temperature beyond 30°C (20). A detailed analysis of temperature effects on clock gene expression has recently been done with zebrafish (30), and such comparative analyses should rapidly increase our understanding of temperature interactions in all biological clocks.

We suggest that our reporter is, to a first approximation, reading out the level of de novo transcription. It is thought that the half-life of luciferase in cells is fairly short, with estimates ranging between 15 min for the effective biological half-life in \textit{Petunia} spp. to 3 h in mammalian cells (12, 44, 45). Moreover, we do not suspect that after transcription of \textit{luc} there will be regulated translation of the \textit{LUC} protein, and in this study we excluded the long 5’ UTR of \textit{frq}, known to control translation.
expressly for this reason. Thus, we propose that luciferase translation largely tracks \textit{frq-luc-I} transcription and that enzymatic activity is likely a direct reflection of this circuit.

However, we have identified other parameters of the system that can affect readout levels, and these should be considered when one is using this tool. Luminescence is linearly proportional to the concentration of luciferin added, a finding consistent with other reports describing the use of the firefly luciferase system (e.g., reference \textit{45}); this might aid in the examination of extremely low-level gene expression. However, we have seen an inhibitory effect of the potassium salt of luciferin on \textit{Neurospora} conidial formation and growth at very high luciferin concentrations (200 \textmu M and above) (data not shown). The luminescent reaction rate is approximately proportional to oxygen concentration, as previously observed (\textit{45}), although in AFV medium on petri plates, oxygen depletion is not a significant issue. Finally, in using sorbose-colonized \textit{Neurospora}, though many samples can be monitored simultaneously, the potential for cross-contamination, of the sample or the signal, between samples may be an important experimental design consideration.

We have combined this ability to assess many samples and to monitor their near-continuous luciferase activity in order to experimentally address theoretical properties of the clock. We report high-resolution data on the effects of temperature pulses on phase resetting. For the phase-resetting experiments, a large number of data points were gathered to generate a high degree of confidence in measurements that would have been much more labor-intensive with conventional race tube assays. We see strong phase-resetting effects ("type 0") with 1-h pulses of temperatures higher than 31°C, and the transition from "type 1" to "type 0" resetting seems to occur at about 31°C, consistent with previous temperature data on \textit{Neurospora} (23, 25).

In summary, in the context of rhythms, this luminescent reporter offers a variety of advantages over a conventional race tube assay. First, the luminescent system can use promoters that directly monitor the molecular clockwork, whereas asexual spore formation is several genetic steps downstream. Because the expression of clock components can now potentially be followed in real time, \textit{luc} in \textit{Neurospora} will allow analysis of the dynamics of the circadian oscillator at a level previously unattainable with this organism. Second, large numbers of samples can be measured simultaneously, allowing for more experimental variables and/or greater experimental accuracy. Additionally, sorbose-colonized \textit{Neurospora} bearing the \textit{frq} promoter yields robust oscillatory activity for several weeks. Together, these advantages will facilitate large-scale genetic screening for mutants, as it has done in a number of other systems (for examples of recent work, see references \textit{26} and \textit{42}). Third, rhythms in localized areas or cell types can be monitored. We have demonstrated this by showing spatiotemporal resolution in \textit{eas/ccg-2}-driven rhythms. We foresee that this will benefit circadian and developmental biologists alike.

Fourth, luminescence can be measured under conditions that do not permit sporulation. We have demonstrated this by showing an unequivocal rhythm in an \textit{fl} strain in the absence of conidiation. In particular, sporulation is directly affected, independently of circadian activity, by light, high temperatures, and low temperatures. Questions about \textit{Neurospora} circadian activity under different lighting conditions or at temperatures far from 25°C can now be more easily approached. Additionally, continuous quantitative measurements of luminescence allow more-accurate amplitude and waveform data to be collected. Predictions of modeling can now be tested more easily, as we have demonstrated for temperature phase-resetting and singularity determinations.

Finally, although we have developed and used this tool in the context of chronobiology, we emphasize that it should find widespread utility for gene expression in a variety of experimental contexts in \textit{Neurospora} and in many additional GC-rich organisms.

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