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Genetics

Circadian clock locus frequency: Protein encoded by a single open reading frame defines period length and temperature compensation

(*Neurospora*/temperature compensation/mutants)

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ABSTRACT The frequency (*frq*) locus encodes a key component, a state variable, in a cellular oscillator generating circadian rhythmicity. Two transcripts have been mapped to this region, and data presented here are consistent with the existence of a third transcript. Analysis of cDNA clones and clock mutants from this region focuses attention on one transcript encoding a protein. FRQ, which is a central clock component: (*i*) mutations in all of the semidominant *frq* alleles are the result of single amino acid substitutions and map to the open reading frame (ORF) encoding FRQ; (*ii*) deletion of this ORF, or a frameshift mutation within it, results in a strain with a recessive clock phenotype characterized by the loss of rhythm stability and compensation. Single amino acid substitutions within, or disruption of, this single ORF are thus sufficient to drive major alterations in both period length and temperature compensation, two canonical characteristics of circadian systems. The 989-amino acid FRQ protein specifies the circadian function of *frq* in the assembly of the *Neurospora* biological clock.

Circadian rhythms are a nearly ubiquitous feature of eukaryotic organisms. Daily fluctuations in physiological and biochemical parameters driven by the circadian clock include cell division, locomotor activity in animals, and photosynthesis and leaf movement in plants (for review, see ref. 1). All circadian clocks have several defining properties (1): the period length under constant environmental conditions is ≈24 hr; the clock is entrained by environmental signals, usually light; the clock is temperature compensated; the clock is localized intracellularly (2).

In the lower eukaryote *Neurospora crassa*, a vegetative developmental program is under circadian clock control; the commitment to the production of asexual spores known as conidia is made on a daily cycle. The well-studied and easily monitored rhythm of conidiation, the molecular correlates of rhythmicity provided by clock-controlled genes (3, 4), and the organism's small genome size make *Neurospora* an ideal organism for genetic studies. Fourteen *Neurospora* clock mutants have been isolated; eight of these map to a single locus, frequency (*frq*) (2, 5). Mutations at *frq* can result in strains with short period clocks, long period clocks that display a partial loss in temperature compensation (6), or (in the case of the only recessive allele) either conditional arrhythmicity or a complete loss of both temperature and nutritional compensation of clock period length (7).

frq has previously been cloned and shown to encode at least two divergently transcribed polyadenylylated transcripts (8). While only the larger of these transcripts appears to have any protein coding potential (9), DNA spanning both transcripts is required for rescue of the recessive *frq* mutation (8). We have now analyzed cDNAs covering the larger of the *frq* transcripts and report the amino acid sequence of a 989-codon open reading frame (ORF) corresponding to the *frq* product FRQ.§ The molecular identity of the existing *frq* alleles, the finding that point mutations within the ORF can affect both period length and temperature compensation and the characteristics of a definitive null allele are all consistent with the established role of FRQ as a central component, a state variable, of the *Neurospora* circadian oscillator (10).

MATERIALS AND METHODS

***Neurospora* Strains and Growth Conditions.** Vegetative growth conditions, crossing protocols (11), and transformation of spheroplasts (12) were as described. Race tube growth conditions were 0.1% glucose/0.17% arginine/IX Vogel's salts except in the frq^{10} studies where 0.3% glucose/0.5% arginine/IX Vogel's salts was used. The frq^7 plasmid was cotransformed into spheroplasts using the hygromicin B-resistant ($HygB^R$) plasmid pCSN44 (13) as the selectable marker; all other constructs had either benomyl resistance (Bml^R) or $HygB^R$ on the same replicon. $HygB^R$ was selected at 200 μg of Hygromycin B per ml (Calbiochem). Bml^R was scored or selected at 0.25 μg of benomyl per ml (DuPont).

Plasmids and Protein Manipulations. The parent plasmid for most *frq* chimeric plasmids (pCRM101) (8) contains the 8.7-kb *Cla I frq* fragment and the Bml^R marker. All *frq* chimeric plasmids were constructed by exchanging the PCR fragments containing each *frq* allele into pCRM101 by using unique restriction sites. The sole exception was the frq^7 plasmid in which a small segment of wild-type *frq* (from pCRM101) was exchanged into the frq^7-containing plasmid pDSK101. Relevant regions of each complete chimeric construct were sequenced before phenotypic analysis of the chimeras.

Gene Disruption Strategy. *Bgl* II fragments comprising 5.3 kbp of the *frq* genomic clone (containing the N-terminal 893 codons of FRQ, the 5′ untranslated region, >1200 bp of 5′ nontranscribed DNA, and DNA encoding the 5′ half of the small *frq* transcript) were replaced with a 1.5-kbp $HygB^R$ cassette (13) under control of the *Aspergillus trpC* promoter. A *Spe* I fragment containing the entire $HygB^R$-interrupted version of *frq* was then exchanged into the Bml^R construct pCRM101 to yield pCMW1 (see Fig. 3). This final construct thus contains two dominant selectable markers, one of which (Bml^R) is lost upon homologous reciprocal integration of this modified *frq* into the genome in place of the resident copy of *frq* (14). The assay for correct targeting of pCMW1 to yield

Abbreviations: ORF, open reading frame; RT, reverse transcription; NLS, nuclear localization signal.

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§The sequence reported in this paper has been deposited in the GenBank data base (accession no. L14465).

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a *frq* disruption exploited the existence of known sequences at the genomic *frq* locus not present on pCMWI. Only a homologous recombination event can juxtapose the two primer sites and allow for amplification of a DNA fragment of predictable size (14).

Oligodeoxynucleotides, Nucleic Acid Preparation, Manipulation, and Screening of Libraries. Genomic DNA was isolated from *N. crassa* as described (15); ≈1 μg was loaded for Southern analysis and 0.1 μg was used for PCRs. The following oligodeoxynucleotides were used in the PCR-based assay to screen for *frq* disruption events: BDA12, 5′-GAAGCATACTATCGCCAGAC-3′ (annealing site in the *frq* gene distal to the ORF and not present in pCMW1), BDA13, 5′-AGCACTCGTCCGAGGGCAAA-3′ (annealing site in the HygB^R gene). The following oligodeoxynucleotides used for library PCR and reverse transcription (RT) PCR across the intronic region: BDA2, 5′-AACAAGCGGCTTAAGGA-3′; and BDA17, 5′-CTCCTCTGCGATGTCGATTC-3′. The following oligodeoxynucleotides were used for amplification of the *frq* ORF from the mutant strains: BDA1, 5′-ACTGGGAGTGACCGAAT-3′; and BDA9, 5′-GGCAATCACCTTCGTTG-3′.

A cDNA library was constructed by random priming and oligo(dT) priming of poly(A)$^+$ RNA isolated from tissue grown in Horowitz complete medium for 12 hr in the light at 25°C followed by 12 hr in the dark at 25°C (corresponding to circadian time 1). The cDNA library was cloned into the *Eco*RI site of λZAP (Stratagene) and was screened by using genomic DNA probes. Three additional cDNA libraries (16, 17) were also screened by using DNA probes and by PCR. The tissue for these libraries corresponded to germinating conidia or vegetative mycelia (two libraries) grown under uncharacterized light regimes.

To verify the presence of a spliced transcript arising from the region of the large *frq* transcript, RT-PCR was performed according to standard procedures (18). RNA was isolated from mycelia grown vegetatively in the dark and was reverse transcribed by using random hexamers (Pharmacia). The resulting cDNAs were amplified by using oligonucleotides BDA2 and BDA17 (see above), which flank the putative intronic region.

Computer Analyses. The FRQ ORF was used as the query sequence in a search using three algorithms, FASTA (19), BLAZE, and BLAST (20). The data bases used in the searches were Protein Identification Resource and Swiss-Prot. FASTA searches, TESTCODE, GRAIL (21), and secondary structure predictions on the ORF were done using either the Genetics Computer Group (University of Wisconsin) package (22) or programs with the MacProt suite (23). Significant similarity was taken to be alignments >3 SD above the mean in a standard jumble test (RDF2.1 or GAP) at a *ktup* of 1 or 2 (19). Searches for protein motifs were performed with MacPattern (24) and the Prosite data base (release 20); the PEST motifs were found by using an algorithm designed by Rogers *et al.* (25).

RESULTS

An extended ORF Within the Large Transcript of the *frq* Locus. cDNA clones corresponding to the large transcript within the *frq* locus were isolated from λ phage libraries using cloned genomic *frq* DNA (8) as probes. No full-length clones were obtained, but seven independent clones spanning the transcript region were characterized. With the exception of two isolates (considered separately below), the cDNA clones were identical to the genomic sequence and allowed correction of several errors in the published genomic sequence; these cDNAs describe a long ORF of 989 codons (Fig. 1). The predicted initiating methionine codon is in good context for translational start (GAAC<u>ATG</u>GCG) (28). The algorithms TESTCODE (22) and GRAIL (21) predict this 989-aa acid stretch as a coding region. Overall the protein is predicted to be globular, generally hydrophilic (55% polar and uncharged) and quite acidic (net charge −23 at pH 7, including a hyperacidic stretch near the C terminus). Several regions of conspicuous amino acid bias, and other motifs of potential interest, are highlighted. The sequence of the ORF was compared to the protein sequences in the Protein Identifica-

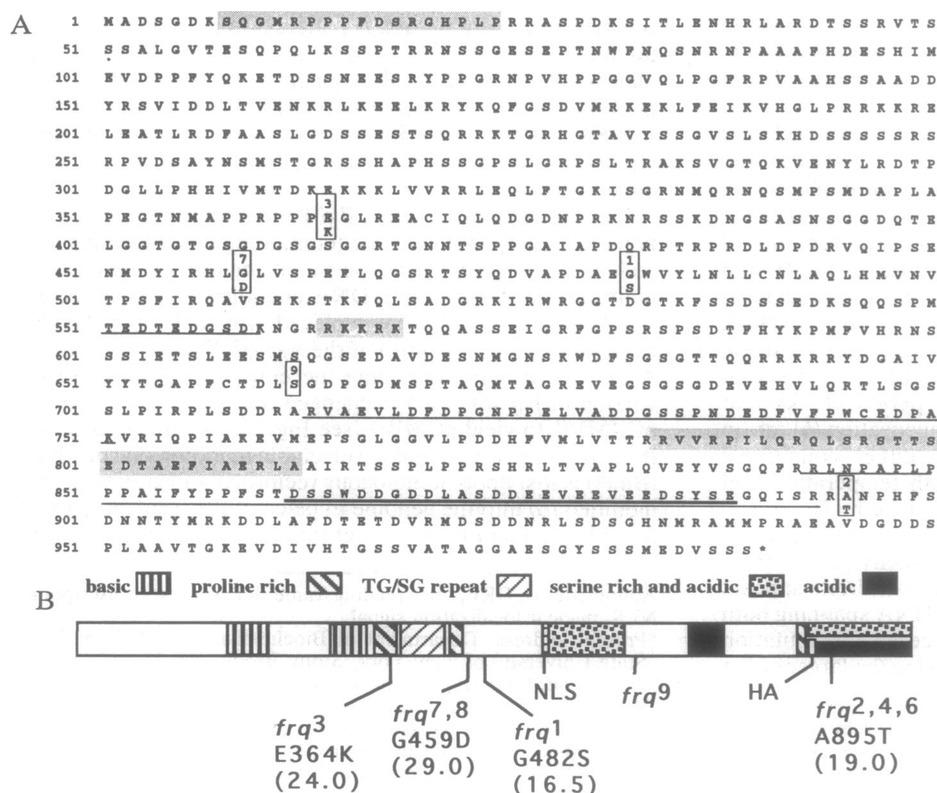

FIG. 1. FRQ protein. (*A*) Sequence of the 989 aa ORF. Boxed amino acids designate sites of amino acid substitutions for each of the *frq* alleles. Three PEST sequences (implicated in rapid protein turnover) (25) are marked by underlines; a potential nuclear localization signal (NLS, codons 564–568) and regions of similarity to rat annexin (codons 784–812; ref. 26) and to a putative nucleic acid binding portion of human small nuclear ribonucleoprotein B/B′ (codons 8–24; ref. 27) are lightly shaded. Hyperacidic region is marked by thick underline. (*B*) Schematic of the *frq* ORF. The positions and aa substitutions associated with the *frq* mutations are noted, along with the resultant period length (in hours) in parentheses. The hyperacidic region (HA), and a NLS are shown; also included are regions of the ORF that show local amino acid content bias.

tion Resource and Swiss-Prot data bases both in full and in overlapping 100-aa windows. Although no extended similarities to other proteins exist, short but significant regions of similarity to a putative nucleic acid binding domain of the human small nuclear ribonucleoprotein SmB'/B (26) (*Z* value, >5) and part of the calcium binding domain of the rat annexin 1 protein (27) (*Z* value, >8) were identified. Two stretches of weak similarity with the *Drosophila* clock gene *per*, previously identified (8, 29) based on an incomplete FRQ sequence, also remain (see *Discussion*). The protein contains several consensus phosphorylation sites but these are not marked in the absence of functional data.

Although most of the cDNAs analyzed showed a direct correspondence between the genomic and transcript sequences, cDNA clones arising from two different libraries (9, 16) contained a sequence that was not collinear with the genomic sequence. Both clones lacked an identical 76 bases corresponding to codons 169–194 (Fig. 1). This suggested either that there was another previously unidentified transcript lying across this part of *frq* (to which these cDNAs corresponded), or that intronic sequences had been differentially spliced out of an initially unprocessed *frq* transcript to produce different versions of the final transcript. The existence of an alternative transcript arising from this region was confirmed by RT-PCR using oligonucleotides flanking this region (see *Materials and Methods*), which yielded fragments of ≈180 bp (from the predicted unspliced sequence) and ≈100 bp (corresponding to a spliced transcript). The sequence of the 76-bp putative intron strongly suggests that the spliced transcript is distinct from the ORF-containing *frq* transcript and is transcribed in the opposite direction: consensus 5' splice, lariat formation sequences, and 3' intron splice sites among *N. crassa* genes [5'-GUAAGUNNYCNYY . . . (A/U)RCURAC(A/C) . . . (A/U)ACAG-3; where Y is pyrimidine and R is purine] (35) match the 76 bp of an opposite polarity transcript well (5'-GUAAGCCAUGUAC . . . AACUGCUU . . . UUCAG-3'; 16/23 matches) but do not conform to splicing of these 76 bp from the ORF-containing transcript (5'-CUGAAGAGGUAUA . . . AAGGUACA . . . CUUAC-3'; 4/23 matches including nonconservative bases at two absolutely conserved sites). Although cDNA and RT-PCR evidence support the existence of this reverse polarity transcript, no RNAs from this region, other than the transcript encoding the ORF, have been detected by Northern analysis; thus, this transcript is apparently quite nonabundant. In any case, extensive computer analysis (9, 21) predicts that no ORF of >81 aa encoded by a transcript of polarity opposite the *frq* ORF has a high likelihood of being used, suggesting that the transcript does not encode a protein important for FRQ's role in the clock. This is further supported by data elucidating the molecular nature of the mutant alleles of *frq*.

Most of the *frq* Mutations Lie Within a Confined Region of the Protein. Eight independently isolated mutant alleles of *frq* have been reported (5). Assignment of the molecular basis for each mutation was made by sequencing DNA containing the region of the long ORF from strains bearing each of the alleles. For *frq*^{7} DNA was subcloned into pBluescript (Stratagene) and sequenced, revealing a single G to A nucleotide substitution resulting in a missense mutation within the ORF. For each of the remaining alleles, PCR fragments from the region containing the ORF were subcloned and sequenced. In each case, a single G to A nucleotide alteration was detected compared to the wild-type sequence (except for *frq*^{9}, in which a base was deleted). Surprisingly, alleles with identical phenotypes (*frq*^{2,4,6} and *frq*^{7,8}) contained the same single nucleotide change. Direct sequencing of PCR fragments was performed on genomic DNA from independent quiescent stocks of *frq*^{2,4,6} and *frq*^{7,8} that had not previously been propagated in this laboratory. The same nucleotide substitutions were detected, eliminating the possibility that either cross-contamination or PCR-induced replication errors could account for the sequence discrepancies.

To verify that the base pair changes determined by sequence analysis could confer the appropriate phenotypes for each class of mutant, a small portion of the wild-type gene was replaced with the corresponding region of the mutant allele that contained the sequence discrepancy (Fig. 2). Because the entire *frq*^{7} locus had already been cloned, the reverse experiment was carried out, in which a small portion of the wild-type gene was subcloned into *frq*^{7}. All of the mutant/wild-type chimeric constructs were transformed into the recessive *frq*^{9} null strain, and the period length of the resulting transformants was determined by the race tube assay at 25°C (Fig. 2). All transformants have the period length predicted from the genotype and confirm the assignment of the period altering changes. Although banding in the *frq*^{1} chimera was too indistinct to allow precise estimates of period, transformation of the *frq*^{1} chimera into *frq*$^{+}$ or *frq*^{7} resulted in period shortening (data not shown) as expected based on the codominance of these alleles (2, 5) and consistent with this assignment.

In addition to period length, the second canonical property of circadian rhythms altered in the *frq* mutants is that of temperature compensation, referring to the homeostatic capabilities of the clock that keep the period length relatively unaffected by growth temperatures (6). Period lengths for strains transformed with chimeric constructs (Fig. 2) were determined at temperatures ranging between 20°C and 30°C (Fig. 2). The temperature compensation properties of all strains correlated with the genotype of the transformant; the single nucleotide differences in the *frq* mutants give rise to the appropriate period length as well as the appropriate temperature compensation pattern.

The amino acid changes responsible for the period changes of each allele are shown (Fig. 1). Most of the *frq* mutations are clustered within one part of the protein, *frq*^{1} (16 hr), *frq*^{7} (29 hr), and *frq*^{3} (24 hr) lying within just 124 codons of one another, with the most extreme mutations just 24 codons apart. Moreover, this region is highly conserved among several diverse fungal species (30), suggesting that this region

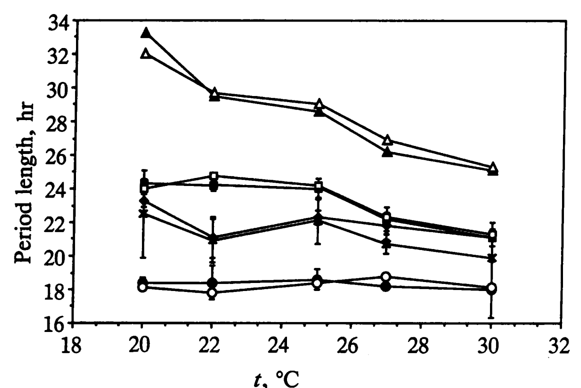

FIG. 2. Period length and temperature compensation properties of *frq* chimeric genes. Small DNA fragments of the *frq* mutant alleles bearing the relevant nucleotide alterations were exchanged with the corresponding wild-type sequences, and these constructs were transformed into the recessive *frq*^{9} allele. Period length values for strains carrying transformed chimeric DNA (±2 SEM) are averages of at least three race tubes and 5 separate days from a minimum of three independent ectopic transformants; for these calculations, *n* = no. of independent ectopic transformants analyzed. Relevant genotypes of experimental samples are shown by solid symbols and are as follows [recipient (transforming DNA)]: ●, *frq*^{9} (*frq*^{2}) (*n* = 3); ■, *frq*^{9} (*frq*^{3}) (*n* = 3); ◆, *frq*^{9} (*frq*$^{+}$) (*frq*$^{+}$ chimeric of *frq*^{7}, plasmid; *n* = 4); ▲, *frq*^{9} (*frq*^{7}) (*n* = 1). Control strains are as follows: ×, *frq*^{9} (*frq*$^{+}$) (*n* = 2); ○, *frq*^{2}; □, *frq*^{3}; △, *frq*^{7}.

is critical to FRQ function and cannot tolerate change without adversely affecting the clock. Long period mutants have amino acid substitutions that are nonconservative; both the *frq*[3] (Glu-Lys) and *frq*[7,8] (Gly-Asp) mutations result in a net charge difference as well as a significant difference in amino acid side-chain bulk. In contrast, the short period mutations (*frq*[1], Gly-Ser; *frq*[2,4,6], Ala-Thr) are conservative substitutions. The defect in the recessive *frq*[9] allele is a single base pair deletion yielding a frame shift and premature termination of the protein at 674 amino acids. *frq*[9] could thus be interpreted either as a null allele or as an extreme hypomorph.

Construction of an Unambiguous *frq* null allele by gene disruption. Because some rhythmic behavior can still be observed in *frq*[9] under some conditions (7), and because the *frq*[9] allele still encodes a truncated and potentially partially functional protein, the possibility existed that the *frq*[9] mutation did not result in a complete loss of function of *frq*. To confirm the phenotype of a *frq* null mutation, we adapted a previously used strategy to develop a protocol for targeted gene replacement in *Neurospora* (Fig. 3) and used this protocol to disrupt *frq*.

Screening of 300 *frq* disruption candidates led to identification of one appropriately constructed gene-replacement strain in which a HygB[R]-disrupted *frq* has replaced the normal resident copy. When examined on race tubes at 25°C this primary transformant showed a wild-type rhythm (data not shown), presumably due to the heterokaryotic state of the strain. After rendering the strain homokaryotic by conidial plating, it was seen to be phenotypically similar to *frq*[9], displaying sparse and sporadic production of conidia and only a rudimentary rhythm. Genetic mapping of HygB[R] and the mutant clock phenotype in this homokaryotic isolate (both lie 10 map units from *un-10* on LGVIIR) as well as Southern (loss of *frq* genomic DNA) and Northern (absence of the ORF containing transcript) analysis provided verification of the gene disruption event (data not shown). This *frq* disruption strain has been designated *frq*[10].

The circadian clock phenotype of *frq*[10] was analyzed in parallel with isogenic *frq*[+] and *frq*[9] strains (Fig. 4). In both mutant strains, evidence of even weak rhythmicity can be seen only on certain media and on some but not all replicate tubes, and when rhythms are seen the SD of the period length is quite large (Fig. 4*A*). Both strains occasionally exhibited short period banding (≈12-hr period length, not included in Fig. 4*A*) during the first 3–4 days of growth, as previously reported for *frq*[9] (7). *frq*[10] can be complemented by transformation with *frq*[+] at frequencies comparable to *frq*[9] and rescued transformants give rise to wild-type period lengths (Fig. 2). Temperature compensation properties were determined and compared for *frq*[+], *frq*[9], and *frq*[10] (Fig. 4*B*). A characteristic of *frq*[9] is a drastic loss of temperature compensation (7) resulting in a Q_{10} of the rhythm approaching 2 (Q_{10}, calculated as $\tau_{20°C}/\tau_{30°C}$ where τ = period length, here refers to the change in the "rate" of the clock as the temperature increases). The Q_{10} values of the *frq*[10] and *frq*[9] strains are ≈1.9, indistinguishable from one another and clearly different from the wild-type value of 1.1. Thus, by the criteria of altered and sporadic rhythmicity, loss of temperature compensation, and conidial growth habit, *frq*[9] and *frq*[10] are indistinguishable, demonstrating that pleiotropic developmental effects and loss of reliable overt and compensated rhythmicity are the characteristics of null mutations at the *frq* locus that disable FRQ.

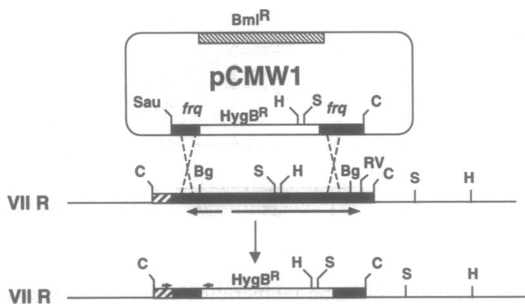

FIG. 3. Targeted disruption of *frq*. Plasmid pCMW1 has a deletion at the *frq* locus into which the HygB[R] cassette (*hph*) has been inserted. A total of 2.9 kbp of *frq* homology is available for recombination with the genomic site (1.5 kbp of homology upstream and 1.4 kbp downstream of the ORF). A 400-bp sequenced segment of the *frq* locus (▨) is absent from pCMW1. Large divergent arrows position the large and small transcript regions within *frq* (2), and small arrows designate primer sites for the PCR-based screen used to detect homologous recombinants; one primer site lies within the *hph* gene and the other lies within the aforementioned 400-bp segment. Bg, *Bgl* II; C, *Cla* I; H, *Hin*dIII; RV, *Eco*RV; S, *Sal* I; Sau, *Sau*3AI. Drawing is not to scale.

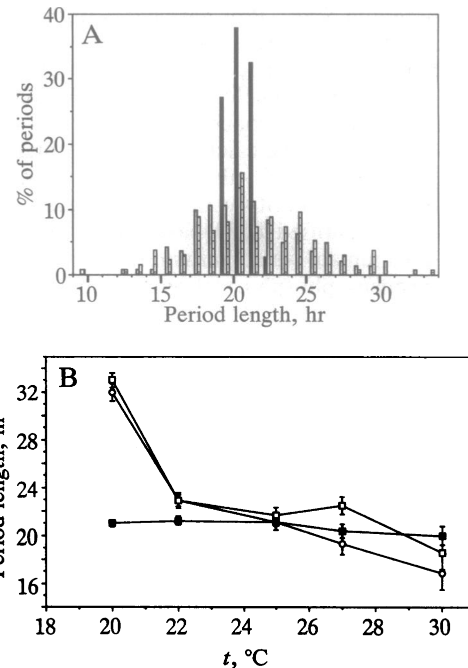

FIG. 4. *frq*[10] defines the *frq* null phenotype. (*A*) Strains bearing *frq* null alleles show large variability in period lengths. Histogram of period lengths observed for *frq*[+], *frq*[9], and *frq*[10] strains at 25°C. Period length data for each strain were pooled and means ± SD were calculated. SDs at 25°C are as follows: ■, *frq*[+] (SD = 0.9); □, *frq*[10] (SD = 4.0); ⊟, *frq*[9] (SD = 3.8). (*B*) Temperature compensation properties of the *frq*[10] allele. Period lengths were determined between 20°C and 30°C. □, *frq*[10]; ○, *frq*[9]; ■ *frq*[+]. Error bars reflect ± 2 SEM.

DISCUSSION

We report here the predicted sequence of a 989-aa protein encoded by one of the larger transcripts of the most salient clock locus in *Neurospora*, *frq*, a locus known to encode a central component of the circadian clock. This is the only extended ORF detected within the region required for complementation of *frq* null alleles (ref. 8; this report). Thus, despite a complex transcript map within the *frq* region, this work emphasizes the importance of the product encoded by this ORF, the FRQ protein, as a key component of the *Neurospora* circadian clock.

The biochemical activities associated with FRQ that are involved in the operation of the circadian oscillator remain mysterious, and few clues are offered by the predicted sequence of this clock protein. FRQ is expected to be a highly acidic globular protein. It bears several potential NLSs,

including a strong consensus signal preceded by a casein kinase II site, an arrangement similar to that seen in the simian virus 40 large tumor antigen where the rate of nuclear translocation is enhanced by phosphorylation of the casein kinase II site (31). The presence of a consensus NLS and a hyperacidic region within FRQ is reminiscent of transcriptional activator proteins such as GAL4 and GCN4, which bear acidic activator domains. Using predictive algorithms (22), this region of FRQ shows a strong tendency to form an amphipathic α-helix; the length of this helix is predicted to be reduced as a result of the *frq*^{2} mutation. Caution must be exercised, however, in interpreting hyperacidic regions as activation domains; thus, it will be of interest to test whether FRQ is localized to the nucleus and whether this hyperacidic region can direct activation of transcription.

FRQ shows two regions of weak similarity (8, 29) to another putative clock component, the *Drosophila* PER protein. Within one of these regions there resides a short mixed repeat of alternating threonine/serine and glycine residues. This repeat motif, quite conspicuous in PER, is not necessary for circadian rhythms in *Drosophila* (32), nor is this motif highly conserved among Drosophilids (33). The second region of similarity lies just N-terminal to the TG/SG repeat (residues 254–375) and spans one of the *frq* alleles, *frq*^{3}. The site of the *frq*^{3} mutation, however, is not conserved and parts of this region that are conserved between *D. melanogaster* and *N. crassa* are not preferentially conserved between *N. crassa* and another fungal *frq* gene from *Sordaria fimicola* (30). Thus, whether this similarity has any functional relevance is unknown. Because only weak similarities exist between FRQ and PER, it remains unclear whether the two clock proteins have any evolutionary or functional relationship.

Sequence analysis of the *frq* alleles is consistent with FRQ's role as a central component of the feedback loop comprising the biological clock and thus complements functional data (10) that have established the same point. All alleles, including the recessive *frq*^{9} allele, are due to single base pair alterations in the *frq* ORF; disruption of the ORF gives rise to the same phenotype as the *frq*^{9} frameshift mutation. It is tempting to speculate that the *frq*^{3} and *frq*^{7} mutations result in diminished function of FRQ, since an allelic series can be built with respect to increasing defects in temperature compensation. *frq*$^{+}$, *frq*^{1}, and *frq*^{2,4,6} all show similar compensation patterns; temperature compensation defects, however, increase from *frq*^{3} to *frq*^{7} to *frq*^{9} and *frq*^{10}. Since *frq*^{10} and *frq*^{9} represent loss of function as well as the worst case of temperature compensation, it would be consistent to consider *frq*^{3} and *frq*^{7} as having diminished function, with *frq*^{7} being more impaired than *frq*^{3}. It is also noted that the *frq*^{3} and *frq*^{7} mutations, which affect temperature compensation, flank the TG/SG repeat region.

Compensation and stability are the characteristics that distinguish a biological clock from a biological oscillator. Here we have shown that null mutations in *frq* virtually eliminate both rhythm stability and temperature compensation; even rhythmicity itself is not seen among all replicate samples on tubes. The existence of residual and sporadic rhythmicity in such strains suggests that other remaining clock components may still be interconnected so that their activities fluctuate over time, albeit in an unstable, uncompensated manner. It has been stated (34) that the function of the circadian clock is to measure elapsed time precisely and recognize local time reliably. *frq* null strains have lost the ability to do either task well, so in this sense clock function can be considered to have been abolished. The finding that single amino acid substitutions in one clock component, FRQ, grossly affect both period length and temperature compensation strongly suggests that temperature compensation, and compensation in general, is likely to be a characteristic that derives directly from the components, here FRQ, within the pacemaker itself, or from the manner in which components are interconnected, rather than being a separate property that can be appended to a preexisting feedback loop.

The molecular characterization of the *frq* locus has revealed great complexity: divergent transcription of small and large *frq* transcripts, a small transcript that shows little protein coding potential (9), and the apparent presence of a third transcript. Among the complexity, however, this work illustrates the central role of the FRQ protein in the generation of circadian rhythms and in stabilizing these rhythms against gradually changing environmental conditions. Determining the functions of FRQ that define period length and compensation characteristics of the clock will be instrumental in dissection of the *Neurospora* circadian clock.

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