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The Goodwin Model: Simulating the Effect of Light Pulses on the Circadian Sporulation Rhythm of *Neurospora Crassa*

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The Goodwin oscillator is a minimal model that describes the oscillatory negative feedback regulation of a translated protein which inhibits its own transcription. Now, over 30 years later this scheme provides a basic description of the central components in the circadian oscillators of *Neurospora*, *Drosophila*, and mammals. We showed previously that *Neurospora*'s resetting behavior by pulses of temperature, cycloheximide or heat shock can be simulated by this model, in which degradation processes play an important role for determining the clock's period and its temperature-compensation. Another important environmental factor for the synchronization is light. In this work, we show that on the basis of a light-induced transcription of the *frequency* (*frq*) gene phase response curves of light pulses as well as the influence of the light pulse length on phase shifts can be described by the Goodwin oscillator. A relaxation variant of the model predicts that directly after a light pulse inhibition in *frq*-transcription occurs, even when the inhibiting factor Z (FRQ) has not reached inhibitory concentrations. This has so far not been experimentally investigated for *frq* transcription, but it complies with a current model of light-induced transcription of other genes by a phosphorylated white-collar complex. During long light pulses, the relaxational model predicts that the sporulation rhythm is arrested in a steady state of high *frq*-mRNA levels. However, experimental results indicate the possibility of oscillations around this steady state and more in favor of the results by the original Goodwin model. In order to explain the resetting behavior by two light pulses, a biphasic first-order kinetics recovery period of the blue light receptor or of the light signal transduction pathway has to be assumed.

Introduction

In the beginning of the 1960s, Goodwin (1963, 1965) introduced a mathematical model that was able to simulate physiological oscillations on the basis of a negative feedback where a protein inhibits the transcription of its own gene. A description and discussion of the Goodwin model has recently been given by Murray (1993). In the three-dimensional model for circadian clocks that we derived from Goodwin (Fig. 1), X represents clock mRNA, Y the clock protein, while Z represents an inhibitor of transcription. In *Neurospora crassa* the most important clock gene is the *frequency* (*frq*) gene (see below).

The characteristic feature of the Goodwin model is that the production rate of intermediates Y and Z is a (linear) function of the concentrations

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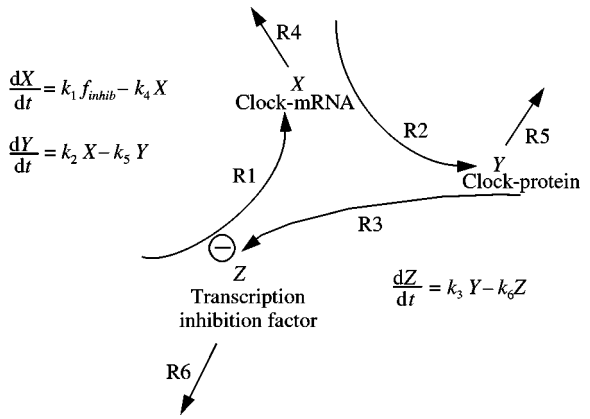

FIG. 1. The Goodwin model. Reaction R1 is the formation of clock mRNA (X); reaction R2 is the synthesis of clock protein (Y) and R3 is the production of a transcription inhibitor (Z). R4, R5 and R6 represent degradation reactions. The inhibition factor is given by $f_{inhib} = 1/(1 + Z^9)$.

of the preceding intermediates (X and Y, respectively), while regular transcription (production of X without inhibition) occurs at a constant rate. However, the production of X is inhibited by increasing concentrations of Z due to the inhibitory factor $f_{inhib} = (1/(1 + Z^9))$. Each intermediate I ($X$, Y or Z) is produced and degraded according to the kinetic scheme S:

$$\xrightarrow{k_{synth}} \mathrm{I} \xrightarrow{k_{degr}} \qquad \text{(S)}$$

depending on synthesis rate constants (k_1, k_2, k_3) and degradation rate constants (k_4, k_5, k_6). During the oscillations the intermediates approach high- or low-steady-state values depending on whether synthesis reactions are turned on or, due to inhibition, are turned off. An interesting consequence of scheme S is that the relaxation time τ (i.e. the time-scale of approach towards the steady state in I) is only dependent on the degradation rate constant k_{degr}, i.e. $\tau = 1/k_{degr}$ (Ruoff *et al.*, 1997). This is the reason why the degradation rate constants (k_4, k_5, k_6) play such an important role in the determination of the oscillator's period length in this model.

Now, more than 30 years later, negative feedback turns out to be an essential part of circadian clocks in various organisms (Aronson *et al.*, 1994; Dunlap, 1998, 1999; Dunlap *et al.*, 1999; Loros, 1998), including *Neurospora*.

Alternative models with negative feedback were developed (Johnsson & Karlsson, 1972), which describe the circadian rhythms of the weta (Lewis, 1994), *Drosophila* (Leloup & Goldbeter, 1997, 1999; Lewis *et al.*, 1997; Hong & Tyson, 1997; Olde Scheper *et al.*, 1999a, b) and *Neurospora* (Gonze *et al.*, 2000).

Our work still focuses on the Goodwin model (Ruoff & Rensing, 1996; Ruoff *et al.*, 1996, 1997, 1999a, b), mainly because of its simplicity, but also because the Goodwin oscillator makes strong predictions concerning the relationship between the degradation (stability) of the clock protein (or clock mRNA) and the oscillator's period length and temperature compensation. For example, the model predicts that a decrease in the degradation rate in the clock-protein FRQ of *Neurospora* would lead to an increase in period length, such that temperature compensation of the rhythm is destroyed, and that the period length decreases with increasing temperature. Some of these predictions have now experimentally been tested and confirmed: Liu *et al.* (2000) mutated several phosphorylation sites in FRQ. At one site (Ser 513) this led to a dramatic reduction in the rate of FRQ degradation and, as predicted by the model, to a very long period ($\approx$31 hr).

Experimental results concerning the phase relationship between frq^+/frq^7-mRNA and $\mathrm{FRQ}^+/\mathrm{FRQ}^7$-protein (Garceau *et al.*, 1997) and the lack of transients after a phase shift (Lakin-Thomas *et al.*, 1990), made us suggest an alternative (threshold) type of inhibition (Ruoff *et al.*, 1999b). In this case, inhibition is assumed to occur whenever the nuclear component of the FRQ-protein exceeds a certain threshold Z_{inhib}. The result of this inhibition is a temporary stop of transcription by setting the "transcription factor" $f_{inhib} = 0$ (Fig. 1). Directly after the start of inhibition, X decreases exponentially, while Y and Z go through phase-delayed maxima (Fig. 2). As soon as Z drops below $Z_{restore}$, transcription starts again and $f_{inhib} = 1$. The factor f_{inhib} is now a step-function, which depends on the state of the transcription inhibitor Z.

Light is an important environmental factor, which has a strong influence on the *Neurospora* sporulation rhythm. The *Neurospora* light response may therefore serve as an additional tool

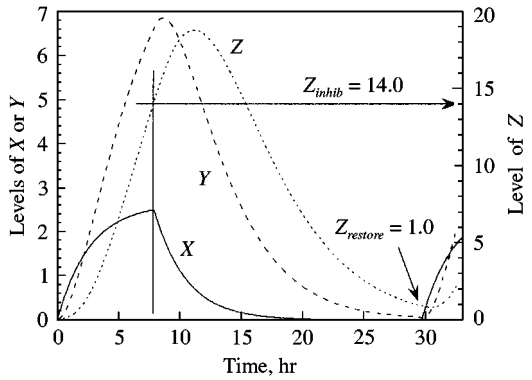

FIG. 2. The relaxation (threshold) model. Z_{inhib} determines the phase at which transcription inhibition starts ($f_{inhib} = 0$, Fig. 1), while $Z_{restore}$ determines the phase at which transcription is restored again ($f_{inhib} = 1$). The parameter set for frq^7 has been used (Table 1).

to test whether the Goodwin model describes the central oscillator adequately.

Experiments have shown that light-treated mycelium increases the level of *frq*-mRNA (Crosthwaite *et al*., 1995, 1997). On the basis of a light-induced transcription of the *frq* gene, we show in this study that phase response curves (PRCs) of light pulses as well as the influence of the light pulse length on phase shifts can be described by the Goodwin oscillator or its relaxation (threshold) variant. Only when two light pulses are given too close together (less than 1 hr apart) they cannot be sensed separately, probably due to the underlying response kinetics of the light receptor or of the light signal transduction pathway.

Materials and Methods

COMPUTATIONS

The differential equations from the models were solved numerically on a Macintosh PowerPC using the double-precision version of the FORTRAN subroutine LSODE (Hindmarsh, 1980). The rate constant values for the *Neurospora crassa* wild type (frq^+) and the long period mutant frq^7 are given in Table 1. It should be mentioned that the values of frq^+ and frq^7 parameters of the Goodwin model given in Table 1 give rise to (slightly) damped oscillations, i.e. for these parametrizations the Goodwin model is not a true limit-cycle oscillator.

DETERMINATION OF *FRQ*-MRNA LEVELS BY RT-PCR

Left and right primers *frqfor1* (agaagaagctggttgtccga) and *frqrev1* (tccgaccattcttatccgag) were constructed using the primer construction program PRIMER3 (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3.cgi). The *frq* PCR product size is 747 bp.

Total RNA was extracted from 10 mg mycelium using a commercial extraction kit (Purescript®, Gentra Systems, www.gentra.com). The

TABLE 1
*frq^+ and frq^7 parametrizations**

Type	k_4	k_5	k_6	$Z_{restore}$	Z_{inhib}	Period	Start values ($t = 0$)
Goodwin model $f_{inhib} = 1/(1 + Z^9)$							
frq^+	0.2	0.2	0.1	—	—	22.3	$X_0 = 3.931 \times 10^{-2}$ $Y_0 = 1.811 \times 10^{-1}$ $Z_0 = 1.712$
frq^7	0.2	0.1	0.1	—	—	28.2	$X_0 = 2.214 \times 10^{-2}$ $Y_0 = 1.918 \times 10^{-1}$ $Z_0 = 1.826$
Goodwin relaxation (threshold) model							
frq^+	0.455	0.455	0.455	0.1	2.0	21.5	$X_0 = 9.994 \times 10^{-6}$ $Y_0 = 2.174 \times 10^{-2}$ $Z_0 = 9.624 \times 10^{-2}$
frq^7	0.38	0.29	0.29	1.0	14.0	30.0	Same as for frq^+

*$k_1 = k_2 = k_3 = 1.0$ in all cases. All rate constants in hr^{-1}.

RNA was treated with DNase I (10 min at 37°C and 5 min at 65°C). The final RNA solution was stored at − 30°C.

rTth DNA polymerase from PE Biosystems (www.pebiosystems.com) was used in the RT-PCR according to the manufacturer's instructions. Two hundred and fifty nanogram total RNA was added to the RT reaction. The RT-PCR temperature profile was: {65°C (15 min)}–{95°C (1 min)}–{95°C (15 s), 65°C (30 s)}$_{35\ \text{cycles}}$–{60°C (7 min)}–{4°C (storage)}. PCR products were fluorimetrically detected by electrophoresis and UV-illumination of gels which contained ethidium bromide. The gels consisted of 1.2% ultrapure agarose (Gibco, www.lifetech.com) and 10 μg ethidium bromide in 100 ml gel.

Photographs of gels were taken and fluorescence intensities were measured by using Geldoc 2000 (Bio-Rad, www.bio-rad.com) and the program Quantity One (Bio-Rad).

Phase Resetting with Single Light Pulses

PHASE RESPONSE CURVES

Crosthwaite *et al.* (1995) have shown that *frq* is rapidly induced by short pulses of light and that light overrides the inhibition of *frq*-transcription by FRQ (or its phosphorylated forms). We assume that this is most likely due to an increase in the rate of transcription (i.e. k_1) and not to an inhibition of mRNA degradation (R4). As there is no experimental study so far that has quantitatively investigated the kinetics of the *frq*-mRNA increase during light treatment, we somewhat arbitrarily assume that the value of k_1 is doubled during a light pulse. For the Goodwin model and its relaxational variant (Figs 1 and 2) we investigated what might occur directly after a light pulse. In the Goodwin model, it was assumed that k_1 resets to unity when transcription is resumed in darkness. Also in the relaxational model transcription could continue in darkness as long as Z is below Z_{inhib}. However, a perfect fit between calculations and the Dharmananda (1980) phase response curves was found for the relaxation model only when transcription was inhibited directly after the light treatment. In order to resume transcription in darkness, the oscillator has first to pass through $Z_{restore}$.

The typical light single pulse PRC in *Neurospora* (frq^+) shows a slight phase advance at the beginning of the period (ct 0–ct 4), while phase delays are observed from ct 4 to ct 16 up to ct 22 with the breakpoint occurring between ct 16 and ct 22 (Sargent & Briggs, 1967; Dharmananda, 1980; Perlman *et al.*, 1981; Nakashima, 1985; Schulz *et al.*, 1985; Rensing & Schill, 1987). Figure 3(a) shows the experimental frq^+ light PRC by Perlman *et al.* (1981) together with the calculated PRC using the Goodwin model. The figure also shows experimentally determined frq^+-mRNA levels and the calculated X levels.

In the experiments with frq^+ by Dharmananda (1980), the breakpoint occurs at a later phase of perturbation [Fig. 3(b)] compared with the data by Perlman *et al.* (1981) [Fig. 3(a)]. However, when testing the relaxation variant of the model, it was astonishing to see that the PRC data agreed best with the Dharmananda results [Fig. 3(b)]. In order to achieve this agreement for light perturbations before ct 4, one has to assume that directly after the light pulse an inhibition of transcription occurs (even if the Z variable is still below Z_{inhib}). If this assumption is not made, i.e. if the transcription resumes after the light pulse, the advance phase shifts are very small. For perturbations after ct 4 it is not necessary to assume an immediate inhibition, because above ct 4 [at which the maximum in frq^+-mRNA occurs, Fig. 3(b)] the transcription in frq^+ is inhibited. The experimental light phase response curve for frq^7 [Fig. 3(c)] is also described closely by the relaxational model. Figure 3(b) and (c) also show, as already observed by Crosthwaite *et al.* (1995), that zero phase shifts occur at the experimentally determined maximum levels of *frq*-mRNA. The reason for this is that the *Neurospora* sporulation rhythm appears to behave like a simple geometrical model (Fig. A1): light pulses change (increase) almost instantaneously the *frq*-mRNA levels, which, due to the oscillator's topology in the phase space, leads to a zero phase shift, whenever the phase of perturbation coincides with the phase of maximum *frq*-mRNA level.

INFLUENCE OF LIGHT PULSE LENGTH AND PULSE STRENGTH ON PHASE SHIFTING

Dharmananda (1980) studied the *Neurospora* phase resetting by varying light pulse lengths

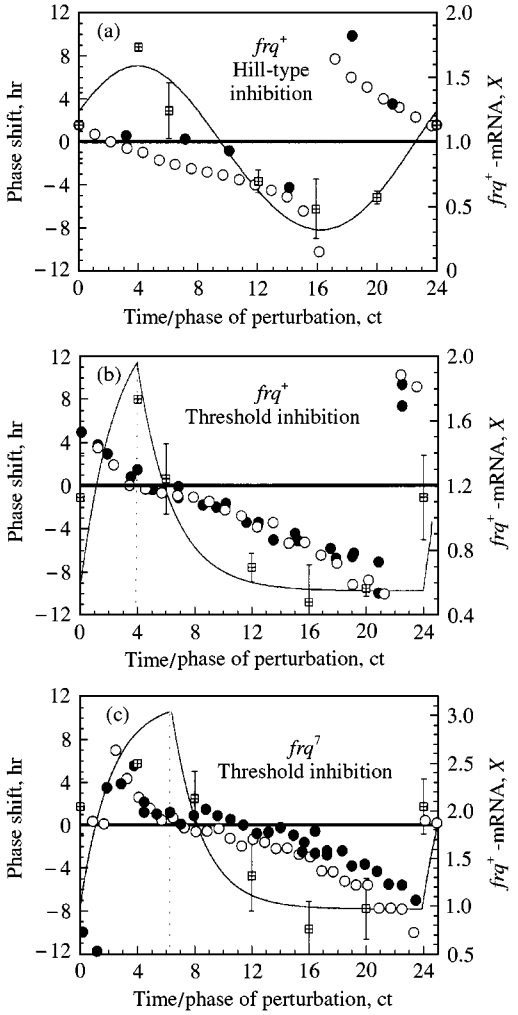

FIG. 3. Single light pulse phase response curves (PRCs). Experimental phase shifts are shown by solid circles and calculated phase shifts by open circles. Squares with crosses show relative levels of experimentally determined *frq*-mRNA levels with standard deviation [$n = 3$ for frq^+ in (a) and (b) and $n = 4$ for frq^7 in (c). Note different scales in (a) and (b)]. Solid lines show calculated *frq*-mRNA concentrations X. Calculated X-levels are scaled to compare with experimental *frq*-mRNA values. In all PRC calculations, during the pulse, k_1 is increased from 1 to 2 hr^{-1}. (a) Calculations performed with Goodwin model ($f_{inhib} = 1/(1 + Z^9)$), frq^+ parametrization (Table 1), and 1 hr pulse length. After the light pulse the original rate constant value 1 hr^{-1} is restored. Experimental PRC by Perlman *et al.* (1981). Calculated X-maximum is set as ct 4. No match between calculated zero phase shift and maximum X level has been observed. (b) Calculated PRC using threshold (relaxation) model with frq^+ parametrization (Table 1). Pulse length 0.2 hr. After the pulse, inhibition in frq^+-transcription occurs ($k_1 = 0$). Transcription is restored once Z goes below $Z_{restore}$. Experimental PRC by Dharmananda (1980). (c) Calculated PRC using threshold model with frq^7 parametrization (Table 1). Pulse length 0.2 hr. After the pulse, inhibition in frq^7-transcription occurs ($k_1 = 0$). Transcription is restored once Z goes below $Z_{restrore}$. Experimental PRC by Dharmananda (1980).

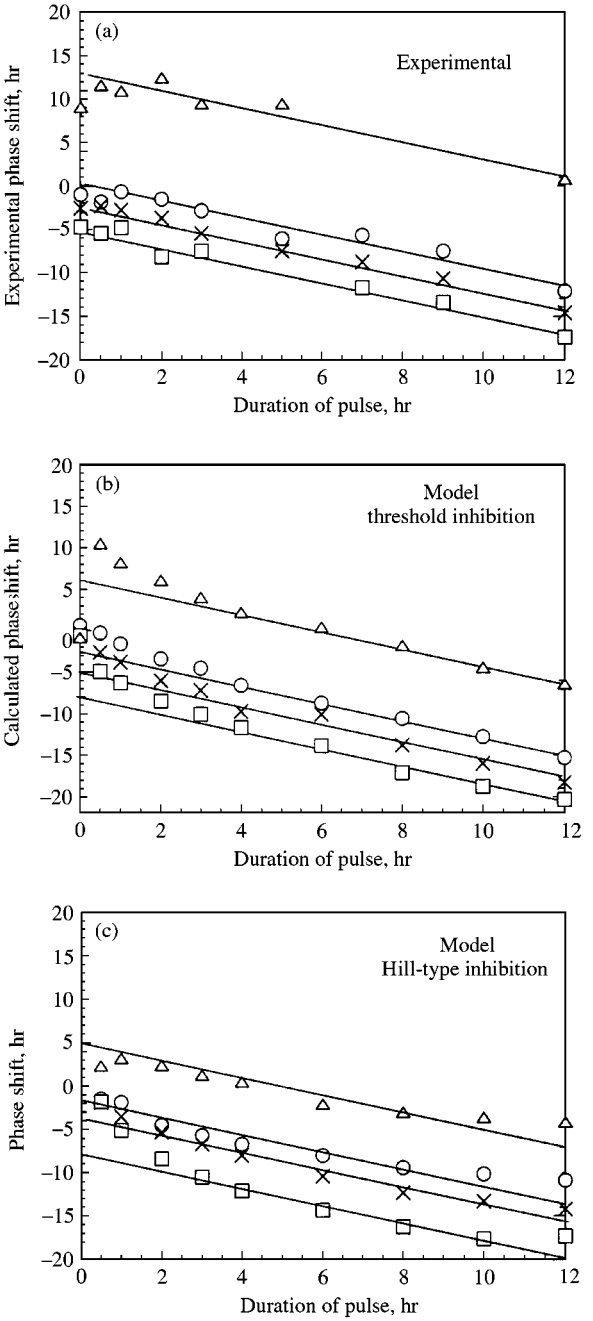

FIG. 4. Experiments [(a) Dharmananda, 1980] and calculations [(b), relaxation model, frq^+ parametrization; (c), Goodwin model, frq^+ parametrization] showing the effect of increased pulse length on phase shift. Solid lines indicate the case when each additional hour of illumination gives an additional hour of delay. Phase of perturbations (from above) (a) ct 19, ct 9, ct 12, ct 15; (b) ct 18, ct 3.5, ct 7, ct 10; (c) ct 18, ct 3.5, ct 7, ct 10.

from 5 min up to 12 hr. He found that, independent of the phase of perturbation, after 2 hr of illumination all phase shifts showed a 1 hr delay for each additional illuminated hour [Fig. 4(a)].

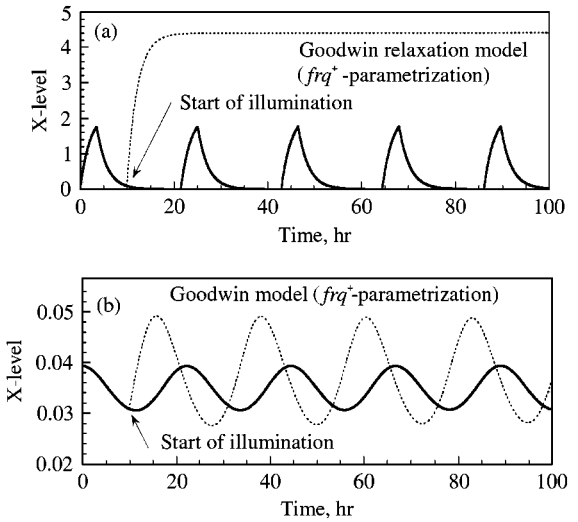

FIG. 5. Response of Goodwin relaxation model (a) and Goodwin model (b) at constant illumination ($k_1 = 2.0\,\mathrm{hr}^{-1}$). Note that the relaxation model predicts a nonoscillatory high *frq*-mRNA (X) steady state, while the Goodwin oscillator shows large amplitude *frq*-mRNA (X) oscillations. Experimental results (Gooch, 1985; Crosthwaite *et al.*, 1997) indicate the presence of a steady state with superimposed small-amplitude oscillations.

This result indicates that after 2 hr of light the oscillator is driven into a steady state, where it stays as long as the light treatment persists. The relaxation model also shows this behavior [Fig. 4(b)], i.e. light treatment drives the oscillator into a steady state with a high *frq*-mRNA (X) level [Fig. 5(a)]. Quite differently is the behavior of the Goodwin oscillator, which shows large amplitude oscillations in *frq*-mRNA during continuous illumination [Fig. 5(b)].

Experiments performed by Gooch (1985) indicate that there are small amplitude circadian oscillations under light conditions in *Neurospora*, which appear to be damped. Similar results have also been found for the flesh-fly *Sarcophaga argyrostoma* (Peterson & Saunders, 1980) and in the mosquito *Culex pipiens quinquefasciatus* (Peterson, 1980). Northern blot analyses of frq^+-mRNA show high but variable levels in light-grown cultures (Crosthwaite *et al.*, 1995). However, the experimental uncertainties in the Crosthwaite *et al.* data are still too large in order to allow firm conclusions about whether *frq*-mRNA levels are oscillating or in a steady state.

Once the light period is over, the relaxation model resets, the *frq*-mRNA level decreases and oscillations restart as soon as the transcription inhibition is removed. Since the relaxation time from such a steady state (or small-amplitude oscillatory state) is independent of the length of time the system has been in the steady state, the phase delay is simply the sum of the time the system has been in the steady state plus the relaxation time to reach the phase mark in the next cycle [Fig. 4(b)]. On the other hand, calculations with the Goodwin model show a marked variability of the phase shift dependent on the phase of the oscillatory X (*frq*-mRNA) level during light conditions [Fig. 4(c)]. In agreement with the experimental results [Fig. 4(a)], this indicates that if there are small-amplitude *frq*-mRNA oscillations at light conditions, they should have only little influence on the phase shift after a transition to darkness.

We also investigated the effect of an increased light pulse strength by increasing k_1 during

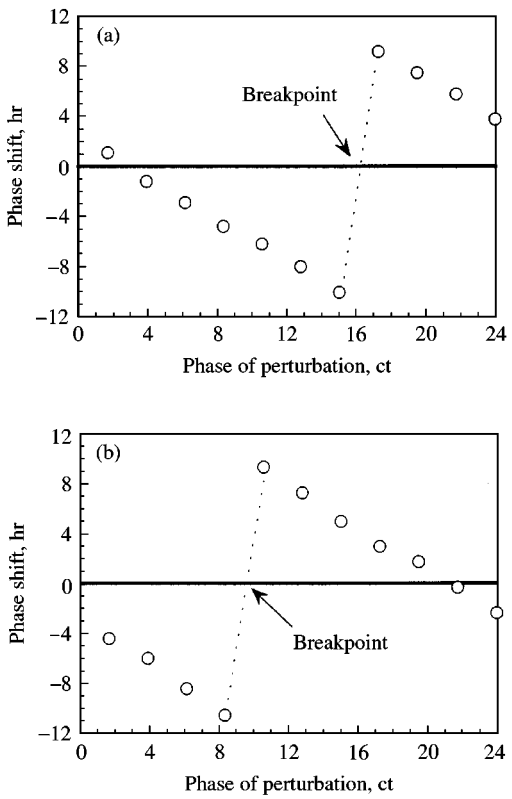

FIG. 6. PRCs of relaxation model with increased transcription rate k_1 during the light pulse (pulse length 1 hr). (a) $k_1 = 2.0\,\mathrm{hr}^{-1}$; (b) $k_1 = 16.0\,\mathrm{hr}^{-1}$. Note that the breakpoint is moved to earlier ct values as the intensity of the pulse is increased.

the perturbation. For this purpose, the relaxation model was used with pulse lengths of 1 hr. Figure 6(a) shows the PRC under the same conditions as in Fig. 3(b), except that light pulse length is changed from 0.2 to 1.0 hr. By increasing k_1 during the 1 hr light pulse, the breakpoint in the PRC is moved to lower ct values (Fig. 6b). This has not yet shown experimentally and might be difficult to prove, because the light receptor in *Neurospora* is extremely sensitive and easily saturated. For example, Dharmananda (1980, Table II in his thesis) was not able to observe any phase shifts due to increased light intensities (which varied in the range between 50 and 950 fc). Phase shifts only occurred due to changes in the duration of the light pulse.

Phase Resetting with Two Light Pulses

Dharmananda (1980) studied the overall phase shift of two successive light pulses occurring at phases Φ_1 and Φ_2 as a function of the time interval $L = \Phi_2 - \Phi_1$ between pulses. He found that when L was larger than approximately 60 min the additional phase shift (delay) $\Delta\Delta\Phi_{12}$ of the second pulse can be determined from a corresponding single-pulse PRC. In other words, for large L values, the two pulses behave independently and have uncorrelated effects on the oscillator. For L values smaller than 60 min, however, Dharmananda found that $\Delta\Delta\Phi_{12}$ decreases with decreasing L in a certain biphasic manner [Fig. 7(a)].

We determined $\Delta\Delta\Phi_{12}$ by assuming that transcription is inhibited and X decreases due to process R4 at the end of each light pulse (Fig. B1, Appendix B):

$$\Delta\Delta\Phi_{12} = -L + \frac{1}{k_4}\ln\left(\frac{X(\Phi_1)}{\Delta + X(\Phi_1)\exp(-k_4 L)}\right), \tag{1}$$

where Δ is the (instantaneous) increase in X due to the light pulse, and $X(\Phi_1)$ is the concentration of X at phase Φ_1 directly after the light pulse. The dashed line in Fig. 7(b) shows the calculated $\Delta\Delta\Phi_{12}$ values [eqn (1)] as a function of L.

How can a biphasic response for small L values be understood? We suggest that the *Neurospora* light-signal transduction pathway

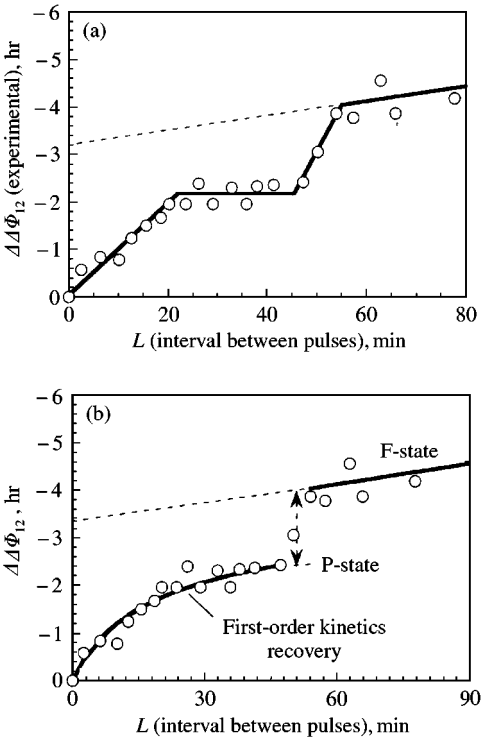

FIG. 7. Additional phase shift (delay) $\Delta\Delta\Phi_{12}$ due to a second pulse in a two-pulse perturbation as a function of interval L between pulses. (a) Experimental results by Dharmananda (1980) including original interpretation as piecewise linear biphasic response. First light pulse is given at ct 18. (b) Same data as in (a), but now interpreted as a biphasic transition between a first-order recovery of the receptor/light signal transduction pathway (partially recovered state, P-state) and a fully recovered state (F-state). For discussion see text.

for *frq*-induction can exist in three states: (i) a fully responsive state (F-state), which occurs whenever *Neurospora* has stayed sufficiently long in darkness (large L values), (ii) an unresponsive state (U-state), which is observed directly after light treatment ($L = 0$), and (iii) a partially responsive state (P-state) for intermediate L values ($0 < L < 50$ min). We assume further that light drives *Neurospora's* light-signal transduction pathway very rapidly from the F-state (via the P-state) into the unresponsive U-state. Once in the U-state, the recovery of the P- (and F)- state in darkness occurs only slowly (process LD). We assume that the recovery of the P-state is a first-order process:

$$\mathrm{F} \underset{\text{darkness (slow)}}{\overset{\text{light (rapid)}}{\rightleftharpoons}} \mathrm{P} \underset{\text{darkness (slow)}}{\overset{\text{light (rapid)}}{\rightleftharpoons}} \mathrm{U}. \qquad \text{(LD)}$$

In pace with the recovery of the P-state in darkness, the light-signal transduction system also regains its ability to induce *frq*-transcription by light. If $\mathbf{P}_0$ represents a quantitative measure of a fully recovered P-state, recovery of P by first-order kinetics is described by the relationship

$$\mathbf{P} = \mathbf{P}_0\,(1 - \exp(-t/\tau_{rp})), \tag{2}$$

where τ_{rp} is the relaxation time for the recovery of the P-state in darkness. We may further assume that the amount of produced *frq*-mRNA by a light pulse is proportional to the recovered **P**, i.e.

$$\Delta = \boldsymbol{\kappa}\mathbf{P} = \boldsymbol{\kappa}\mathbf{P}_0(1 - \exp(-t/\tau_{rp}))$$

$$= \Delta_0(1 - \exp(-t/\tau_{rp})). \tag{3}$$

Δ_0 represents the amount of *frq*-mRNA produced by a light pulse which was transduced by a fully recovered P-state. If eqn (3) is substituted into eqn (1), we get a theoretical description of how $\Delta\Delta\Phi_{12}$ depends on L when the light-signal transduction pathway only slowly recovers in darkness. Figure 7(b) shows that the experimental data by Dharmananda (1980) are well described by first-order recovery kinetics of the P-state. When the P-state is recovered in darkness to approximately one-third, it is rapidly converted to the fully responsive F-state [Fig. 7(b)]. However, experimental evidence for this rapid transition between P and F states is not yet available.

Discussion

SINGLE LIGHT-PULSE RESETTING AND MOLECULAR TRANSCRIPTIONAL EVENTS

The primary goal of this work was to investigate how the presently known facts on the influence of light on *frq*-transcription are in accord with the assumed negative feedback loop within *Neurospora*'s circadian clock mechanism, and to what extent a minimum model like the Goodwin oscillator or its relaxational variant is capable of simulating the influence of light on the clock. The results described by Fig. 3 show quite convincingly that the response of the sporulation rhythm towards light is in agreement with an increased level of the *frq*-transcript and with a negative feedback in the *frq*-transcription–translation cycle. Furthermore, in context with the Goodwin model agreements between experimental and computed PRCs were obtained (Fig. 3) when light-induced transcription of the *frq*-gene is approximately doubled. For this condition, the relaxation variant of the Goodwin model (Fig. 2) shows that zero phase shifts occur at the maximum level of *frq*-mRNA as observed experimentally (Crosthwaite *et al*., 1995; Figs 3b, c). When assuming higher light-induced levels of *frq*-transcription ($k_1 > 3$ during light pulse), the correlation between zero phase shift and *frq*-mRNA maximum vanished and the form of the PRC was changed. While the unperturbed *frq*-mRNA maximum remains the same, the breakpoint is moved to earlier ct times as k_1 increases during the pulse (Fig. 6). Because of a saturation of the light receptor at low light intensities, it seems to be experimentally difficult to verify a shift of the breakpoint as a function of light intensity (Dharmananda, 1980).

A prediction of the relaxation model is the apparent inhibition of transcription directly after a light pulse (for ct values 0–4, Figs 3b, c), even if the level of the inhibitory factor Z is below inhibiting conditions after the pulse. This inhibition directly after a pulse may be related to the molecular events responsible for light-increased transcription of *frq*, which were recently uncovered. There is now experimental evidence that the white collar-1 (*wc-1*) and white collar-2 (*wc-2*) genes and their proteins WC-1 and WC-2 play a central role in the light-signal transduction pathway in *Neurospora*. Sequence analysis of WC-1 and WC-2 (Ballario *et al*., 1996; Linden & Macino, 1997) indicates the presence of GATA-like zinc finger DNA-binding domains in both proteins (Orkin, 1992), suggesting that they function as transcription factors. In addition, WC-1 and WC-2 sequences indicate that both proteins have multifunctional PAS domains, which are mainly involved in protein–protein interactions (Huang *et al*., 1995; Ballario & Macino, 1997). PAS domains are widely distributed in proteins able to sense environmental changes (Lindebro *et al*., 1995; Zhulin *et al*., 1997).

Talora *et al.* (1999) have demonstrated that WC-1 and WC-2 form a white-collar complex (WCC) *in vivo*, which is considered to be important for the light-induced transcription of genes. Light has been found to increase the transcription of *wc-1*, but light also increases the degradation of WC-1 protein by hyperphosphorylation (Talora *et al.*, 1999). In contrast to WC-1, WC-2 is unmodified and stable during and after light treatment. Crosthwaite *et al.* (1997) found that both WC-1 and WC-2 are essential for the operation of the circadian rhythm in *Neurospora*. WC-1 is needed for the light resetting of the sporulation rhythm by inducing *frq*-transcription. WC-2 is apparently not needed for the light resetting of the rhythm, but is necessary as a component of an operative WCC transcription factor to maintain circadian cycling.

On the basis of these findings the models by Crosthwaite *et al.* (1997) and Talora *et al.* (1999) can be combined to a molecular model of light resetting in *Neurospora* (Fig. 8). In the dark the WCC contains a minimal phosphorylated form of WC-1 allowing the WCC to act as a positive element in the transcription of *frq*. Under light conditions the phosphorylation of WC-1 is increased which leads, according to Talora *et al.* (1999), to an increase of its own transcription (and of other light regulated genes). Phosphorylation of WC-1 in the WCC also leads to an

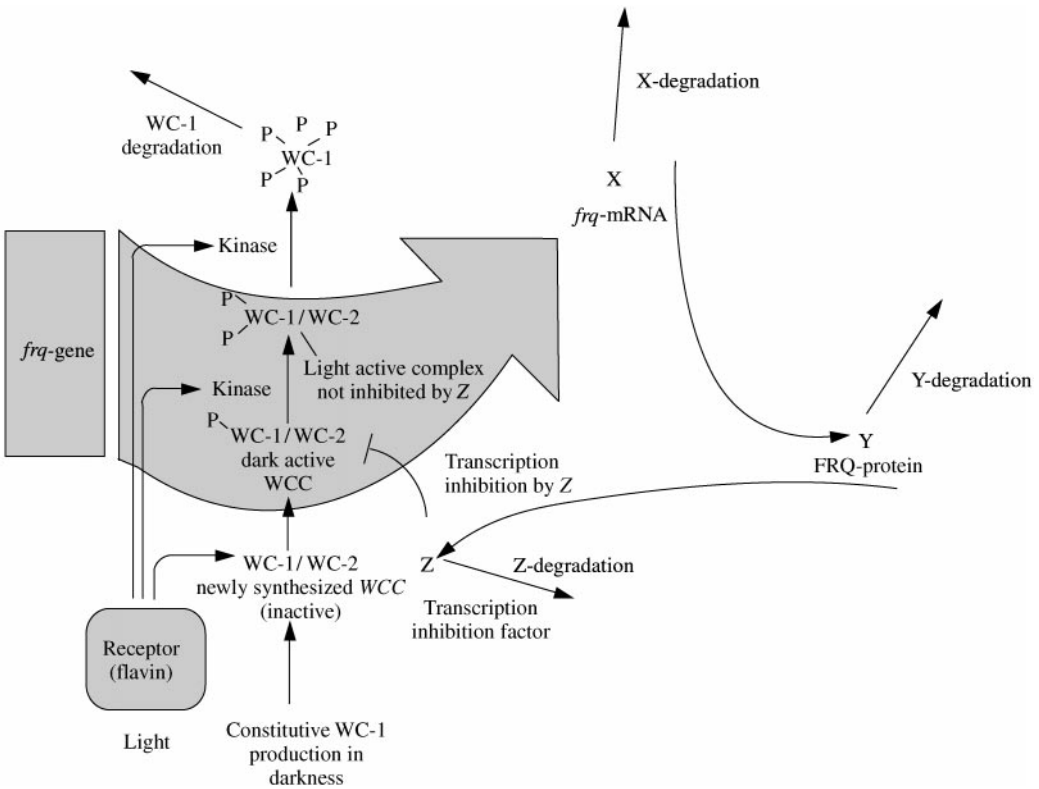

FIG. 8. Model describing circadian *frq*-mRNA/FRQ-protein rhythmicity in darkness and increased *frq* transcription in light. In darkness, WC-1 is constitutively expressed and forms a white-collar complex (WCC) with WC-2. In the presence of light, the *wc-1* gene (but not *wc-2*) is further activated leading to increased WC-1 levels. Newly synthesized WCC is inactive both in darkness and light conditions. A minimal phosphorylated form of WCC, occurs in darkness and is active as a transcription factor for *frq*, but can be inhibited by *Z*, probably by interacting with the complex (Crosthwaite *et al.*, 1995, 1997; Dunlap, 1999). It has been suggested that WC-1 could directly participate in the first step of light perception (Ballario *et al.*, 1998). A light-activated kinase increases the phosphorylation state of WC-1 leading to a WC-1/WC-2 complex with increased *frq*-transcription activity (Talora *et al.*, 1999). In this activated state, *Z* is no longer able to inhibit *frq*-transcription. Hyperphosphorylated WC-1 is degraded and replaced in WCC by newly synthesized WC-1 leading to an inactive WCC. The model-predicted inhibition of *frq*-transcription at the end of the light period may hypothetically be related to a delayed decay of the kinase activity in darkness: the (light) production of WC-1 protein is stopped, and the still active kinase may "empty" the active WC-1 component due to hyperphosphorylation, i.e. hyperphosphorylated WC-1 is degraded and replaced by newly synthesized WC-1, which may lead to the predicted inactive WCC.

increased transcription rate of *frq*. However, light-induced phosphorylation also causes hyperphosphorylation and degradation of WC-1. In WCC, hyperphosphorylated WC-1 is degraded and replaced by newly synthesized and unphosphorylated WC-1. However, the WCC which contains newly synthesized WC-1 has been found to be inactive. As long as light conditions persist newly synthesized WC-1 will enter the WCC, is then phosphorylated, and thus leads to a dynamic equilibrium (steady state) in the active form of WCC and to an increased transcriptional activity of *frq*. At this stage, FRQ or phosphorylated FRQ (which is able to inhibit the *frq*-transcriptional activity of WCC in the dark) is not able to inhibit the light-induced *frq*-transcription.

At the end of the light period (transition to darkness) transcription of *wc*-1 and activation of kinases will stop, but phosphorylation activity may still persist as long as kinases are present in darkness. Thus, hyperphosphorylation and degradation of WC-1 may still persist in darkness and may lead to an inactive WCC which contains newly synthesized WC-1. During further darkness, WC-1 in the WCC will eventually reach a minimally phosphorylated state and thus cause the WCC to regain activity. Now, FRQ or phosphorylated FRQ is again able to act as inhibitor of its own transcription.

The Goodwin model and its relaxation variant show different behavior in response to extended light exposure. The original Goodwin model shows large-amplitude oscillations in *frq*-mRNA, while the relaxation variant reaches a non-oscillatory steady state at high *frq*-mRNA levels. The experiments by Gooch (1985) and Crosthwaite (1995) indicate possible (low-amplitude) oscillations around this steady state. More experimental data are needed to decide between the two possibilities.

PHASE RESETTING BY TWO LIGHT PULSES

Schrott (1981) investigated the light response of *Neurospora* conidiation. By applying two light pulses he found a biphasic response, i.e. a recovery period of approximately 2 hr was necessary after the first illumination to obtain maximum response to a second light treatment. Corrochano *et al.* (1995) also reported a two-phase stimulus–response curve for the light inducible *con-10* gene. For the *al-3*-mRNA induction by light Macino *et al.* (1993) found that a dark period of at least 60 min seems necessary to recover the sensitivity of the photosensory system between two light pulses. The data by Dharmananda (1980, Fig. 7) fit into this biphasic response, but it is still not clear what processes are the rate determining steps in the recovery of the light-signal transduction pathway.

Conclusion

The negative feedback loop of the Goodwin model applied to the basic circadian clock mechanism is able to describe single light pulse phase response curves quite well. The relaxation variant of this model predicts that directly after a light pulse *frq*-transcription should be inhibited, even if the concentration of the inhibiting factor Z is below the inhibiting threshold. This has so far not been investigated experimentally, but complies with recent considerations on light-induced gene transcription by a phosphorylated WCC and an inactive WCC containing newly synthesized WC-1 (Talora *et al.*, 1999). For elongated light pulses, the relaxation model predicts that the sporulation rhythm is arrested in a steady state of high *frq*-mRNA concentrations. However, experiments by Gooch (1985) and Crosthwaite *et al.* (1997) indicate that small-amplitude oscillations may still exist during longer light exposure, which is more in favor of the results by the original Goodwin model. In order to explain the resetting behavior by two light pulses, a first-order kinetics recovery has been assumed (either in the *Neurospora* receptor or in the light transduction pathway), but which has not been observed at the molecular level so far.

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REFERENCES

ARONSON, B. D., JOHNSON, K. A., LOROS, J. J. & DUNLAP, J. C. (1994). Negative feedback defining a circadian clock: autoregulation of the clock gene *frequency*. *Science* **263,** 1578–1584.

BALLARIO, P. & MACINO, G. (1997). *White collar* proteins: PASsing the light signal in *Neurospora crassa*. *Trends Microbiol.* **5,** 458–462.

BALLARIO, P., VITTORIOSO, P., MAGRELLI, A., TALORA, C., CABIBBO, A. & MACINO, G. (1996). White collar-1, a central regulator of blue light responses in *Neurospora*, is a zinc finger protein. *EMBO J.* **15,** 1650–1657.

BALLARIO, P., TALORA, C., GALLI, D., LINDEN, H. & MACINO, G. (1998). Roles in dimerization and blue light photoresponse of the PAS and LOV domains of *Neurospora crassa* white collar proteins. *Mol. Microbiol.* **29,** 719–729.

CORROCHANO, L. M., LAUTER, F. R., EBBOLE, D. J. & YANOFSKY, C. (1995). Light and development regulation of the gene *con-10* of *Neurospora crassa*. *Dev. Biol.* **167,** 190–200.

CROSTHWAITE, S. K., LOROS, J. J. & DUNLAP, J. C. (1995). Light-induced resetting of a circadian clock is mediated by a rapid increase in frequency transcript. *Cell* **81,** 1003–1012.

CROSTHWAITE, S. K., DUNLAP, J. C. & LOROS, J. J. (1997). *Neurospora wc-1* and *wc-2:* transcription, photoresponses, and the origins of circadian rhythmicity [see comments]. *Science* **276,** 763–769.

DHARMANANDA, S. (1980). Studies of the circadian clock of *Neurospora crassa*: light-induced phase shifting. Ph.D. Thesis, University of California, Santa Cruz.

DUNLAP, J. C. (1998). An end in the beginning. *Science* **280,** 1548–1549.

DUNLAP, J. C. (1999). Molecular bases for circadian clocks. *Cell* **96,** 271–290.

DUNLAP, J. C., LOROS, J. J., LIU, Y. & CROSTHWAITE, S. K. (1999). Eukaryotic circadian systems: cycles in common. *Genes Cells* **4,** 1–10.

GARCEAU, N. Y., LIU, Y., LOROS, J. J. & DUNLAP, J. C. (1997). Alternative initiation of translation and time-specific phosphorylation yield multiple forms of the essential clock protein FREQUENCY. *Cell* **89,** 469–476.

GONZE, D., LELOUP, J.-C. & GOLDBETER, A. (2000). Theoretical models for circadian rhythms in *Neurospora* and *Drosophila*. *C. R. Acad. Sci.* (*Paris*), *Sciences de la vie* **323,** 56–67.

GOOCH, V. D. (1985). Effects of light and temperature steps on circadian rhythms of *Neurospora* and *Gonyaulax*. In *Temporal Order* (Rensing, L. & Jaeger, N. I., eds), pp. 232–237. Berlin: Springer-Verlag.

GOODWIN, B. C. (1963). *Temporal Organization in Cells. A Dynamic Theory of Cellular Control Processes*. New York: Academic Press.

GOODWIN, B. C. (1965). Oscillatory behavior in enzymatic control processes. In: *Advances in Enzyme Regulation* (Weber, G., ed.), Vol. 3, pp. 425–438. Oxford: Pergamon Press.

HINDMARSH, A. C. (1980). LSODE and LSODI, two new initial value ordinary differential equation solvers. *ACM-SIGNUM Newslett.* **15,** 10–11.

HONG, C. I. & TYSON, J. J. (1997). A proposal for temperature compensation of the circadian rhythm in *Drosophila* based on dimerization of the PER protein. *Chronobiol. Int.* **14,** 521–530.

HUANG, Z. J., CURTIN, K. & ROSBASH, M. (1995). PER protein interactions and temperature compensation of a circadian clock in *Drosophila*. *Science* **267,** 1169–1172.

JOHNSSON, A. & KARLSSON, H. G. (1972). A feedback model for biological rhythms. Mathematical description and basic properties of the model. *J. theor. Biol.* **36,** 153–174.

LAKIN-THOMAS, P. L., COTÉ, G. G. & BRODY, S. (1990). Circadian rhythms in *Neurospora crassa*: biochemistry and genetics. *Crit. Rev. Microbiol.* **17,** 365–416.

LELOUP, J.-C. & GOLDBETER, A. (1997). Temperature compensation of circadian rhythms: control of the period in a model for circadian oscillations of the PER protein in *Drosophila*. *Chronobiol. Int.* **14,** 511–520.

LELOUP, J.-C. & GOLDBETER, A. (1999). A model for circadian rhythms in *Drosophila* incorporating the formation of a complex between PER and TIM proteins. *J. Biol. Rhythms* **13,** 70–87.

LEWIS, R. D. (1994). Modelling the circadian system of the weta, *Hemideina thoracica* (Orthoptera: Stenopelmatidae). *J. Roy. Soc. New Zealand* **24,** 395–421.

LEWIS, R. D., WARMAN, G. R. & SAUNDERS, D. S. (1997). Simulations of free-running rhythms, light entrainment and the light-pulse phase response curves for the locomotor activity rhythm in *period* mutant of *Drosophila melanogaster*. *J. theor. Biol.* **185,** 503–510.

LINDEBRO, M. C., POELLINGER, L., & WHITELAW, M. L. (1995). Protein–protein interaction via PAS domains: role of the PAS domain in positive and negative regulation of the bHLH/PAS dioxin receptor-Amt transcription factor complex. *EMBO J.* **14,** 3528–3539.

LINDEN, H. & MACINO, G. (1997). White collar 2, a partner in blue-light signal transduction, controlling expression of light-regulated genes in *Neurospora crassa*. *EMBO J.* **16,** 98–109.

LIU, Y., LOROS, J. & DUNLAP, J. C. (2000). Phosphorylation of the *Neurospora* clock protein FREQUENCY determines its degradation rate and strongly influences the period length of the circadian clock. *Proc. Natl Acad. Sci. U.S.A.* **97,** 234–239.

LOROS, J. J. (1998). Time at the end of the millenium: the *Neurospora* clock. *Curr. Opin. Microbiol.* **1,** 698–706.

MACINO, G., BAIMA, S., CARATTOLI, A., MORELLI, G. & VALLE, E. M. (1993). Blue light-regulated expression of geranylgeranyl pyrophosphate synthetase (*albino-3*) gene in *Neurospora crassa*. In: *Molecular Biology and its Applications to Medical Mycology* (Meresca, B., Kobayashi, G. S. & Yamaguchi, H., eds), pp. 117–124. NATO ASI Series, Vol. H 69, Berlin: Springer-Verlag.

MURRAY, J. D. (1993). *Mathematical Biology*, p. 143. Berlin: Springer-Verlag.

NAKASHIMA, H. (1985). Biochemical and genetic aspects of the conidiation rhythm in *Neurospora crassa*: phase shifting by metabolite inhibitors. In: *Circadian Clocks and Zeitgebers* (Hiroshige, T. & Honma, K., eds), pp. 35–44. Sapporo: Hokkaido University Press.

OLDE SCHEPER, T., KLINKENBERG, D., PENNARTZ, C. & VAN PELT, J. (1999a). A mathematical model for the intracellular circadian rhythm generator. *J. Neurosci.* **19,** 40–47.

OLDE SCHEPER, T., KLINKENBERG, D., VAN PELT, J. & PENNARTZ, C. (1999b). A model of molecular circadian clocks: multiple mechanisms for phase shifting and a requirement for strong nonlinear interactions. *J. Biol. Rhythms* **14,** 213–220.

ORKIN, S. H. (1992). GATA-binding transcription factors in hematopoietic cells. *Blood* **80,** 575–581.

PERLMAN, J., NAKASHIMA, H. & FELDMAN, J. F. (1981). Assay and characteristics of circadian rhythmicity in liquid cultures of *Neurospora crassa*. *Plant Physiol.* **67,** 404–407.

PETERSON, E. L. (1980). A limit cycle interpretation of a mosquito circadian oscillator. *J. theor. Biol.* **84,** 281–310.

PETERSON, E. L. & SAUNDERS, D. S. (1980). The circadian eclosion rhythm in *Sarcophaga argyrostoma*: a limit cycle representation of the pacemaker. *J. theor. Biol.* **86,** 265–277.
RENSING, L. &. SCHILL, W. (1987). Perturbations of cellular circadian rhythms by light and temperature. In: *Temporal Disorder in Human Oscillatory Systems* (Rensing, L., an der Heiden, U. & Mackey, M., eds), pp. 233–245. Heidelberg: Springer-Verlag.
RUOFF, P. & RENSING, L. (1996). The temperature-compensated Goodwin model simulates many circadian clock properties. *J. theor. Biol.* **179,** 275–285.
RUOFF, P., MOHSENZADEH, S. & RENSING, L. (1996). Circadian rhythm and protein turnover: the influence of temperature on the period lengths of clock mutants simulated by the Goodwin Oscillator. *Naturwissenschaften* **83,** 514–517.
RUOFF, P., RENSING, L., KOMMEDAL, R. & MOHSENZADEH, S. (1997). Modelling temperature compensation in chemical and biological oscillators. *Chronobiol. Int.* **14,** 499–511.
RUOFF, P., VINSJEVIK, M., MOHSENZADEH, S. & RENSING, L. (1999a). The Goodwin model: simulating the effect of cycloheximide and heat shock on the sporulation rhythm of *Neurospora crassa*. *J. theor. Biol.* **196,** 483–494.
RUOFF, P., VINSJEVIK, M., MONNERJAHN, C. & RENSING, L. (1999b). The Goodwin oscillator: On the importance of degradation reactions in the circadian clock. *J. Biol. Rhythms* **14,** 469–479.
SARGENT, M. L. & BRIGGS, W. R. (1967). The effect of light on a circadian rhythm of conidiation in *Neurospora crassa*. *Plant Physiol* **42,** 1304–1510.
SCHROTT, E. L. (1981). The biphasic fluence response of carotenogenesis in *Neurospora crassa*: temporary insensitivity of the photoreceptor system. *Planta* **151,** 371–374.
SCHULZ, R., PILATUS, U. & RENSING, L. (1985). On the role of energy metabolism in *Neurospora* circadian clock function. *Chronobiol. Int.* **2,** 223–233.
TALORA, C., FRANCHI, L., LINDEN, H., BALLARIO, P. & MACINO, G. (1999). Role of a white collar-1-white collar-2 complex in blue-light signal transduction. *EMBO J.* **18,** 4961–4968.
WINFREE, A. T. (1980). *The Geometry of Biological Time*. New York: Springer-Verlag.
ZHULIN, I. B., TAYLOR, B. L. & DIXON, R. (1997). PAS domain S-boxes in Archea, Bacteria and sensors for oxygen and redox. *Trends Biochem.* **22,** 331–333.

APPENDIX A

A Simple Geometrical Model

It is illustrative to look at the implications of a simple geometrical model. The model describes the cycling of *frq*-mRNA where the phase of the oscillations is described by the phase angle Φ of a vector **I** sweeping on a circular ring [Fig. A1(a)]. For simplicity, we assume that the length of vector **I** is $|\mathbf{I}| = 1$ arbitrary unit (au). The cycling of *frq*-mRNA as a function of Φ is then a sine function [Fig. A1(b)]. In principle, Φ is a function of time and the angular velocity $d\Phi/dt$ may not be constant. A perturbation vector **P** can be

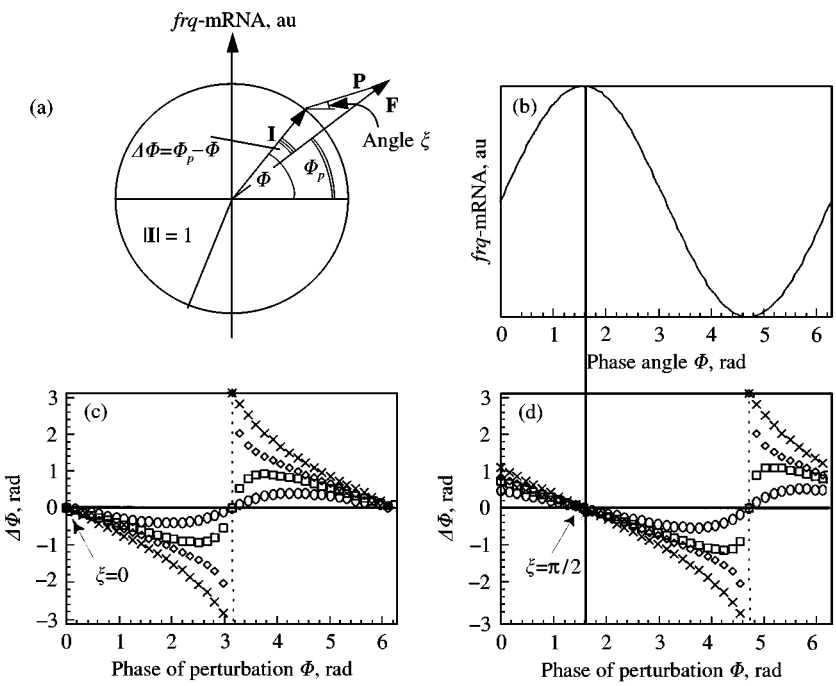

FIG. A1. (a) Describing *frq*-mRNA oscillations on a circular ring with radius $|\mathbf{I}|$. Vector **I** and phase angle Φ describe the phase of oscillation. **P** is a perturbing vector. The instantaneous perturbation leads to vector **F** and the final phase angle Φ_P. The phase shift is given by $\Delta\Phi = \Phi_P - \Phi$. Perturbing angle ξ defines zero phase shifts. (b) *frq*-mRNA as a (sinusoidal) function of phase angle. (c), (d) Calculated phase response curves as a function of $|\mathbf{P}|$ with $\xi = 0$ and $\pi/2$, respectively. (c) (○) $|\mathbf{P}| = 0.4$; (□) $|\mathbf{P}| = 0.8$; (◇) $|\mathbf{P}| = 1.1$; (×) $|\mathbf{P}| = 2.0$; (d) (○) $|\mathbf{P}| = 0.5$; (□) $|\mathbf{P}| = 0.9$; (◇) $|\mathbf{P}| = 1.1$; (×) $|\mathbf{P}| = 2.0$.

applied at a certain phase Φ, which results in a new vector $\mathbf{F}\cdot\mathbf{F}$ sweeps with the same angular velocity as $\mathbf{I}$ and with a constant phase difference $\Delta\Phi$ relative to $\mathbf{I}$. The phase difference is given by $\Delta\Phi = \Phi_p - \Phi$ [Fig. A1(a)]. In general, the orientation of the perturbing vector $\mathbf{P}$ is described by the angle ξ. It can easily be demonstrated that the phase angle after the perturbation [as shown in Fig. A1(a)], Φ_p, is given by the relationship

$$\Phi_p = \arctan\left\{\frac{|\mathbf{P}|\sin\xi + |\mathbf{I}|\sin\Phi}{|\mathbf{P}|\cos\xi + |\mathbf{I}|\cos\Phi}\right\}. \quad \text{(A.1)}$$

When $\xi = \Phi$, the geometry of Fig. A1(a) or inspection of eqn (4) shows that Φ_p becomes equal to Φ, which results in zero phase shifts. Figs A1(c) and A1(d) give calculated phase shifts (as a function of perturbation strength $|\mathbf{P}|$) when $\xi = 0$ and $\pi/2$, respectively. For $\xi = \pi/2$ zero phase shifts occurs at $\Phi = \pi/2$, which coincide with the *frq*-mRNA maximum [cf. Fig. 3(b) and (c)]. This simple model predicts in agreement with experiments that the effect of light on the *Neurospora* clock is apparently due to an (almost) instantaneous increase of *frq*-mRNA and describes, at least qualitatively, the light PRCs. As a consequence, a perturbation at the maximum *frq*-mRNA level leads to zero phase shift as found by Crosthwaite *et al.* (1995). For increased pulse lengths and for higher *frq*-mRNA production during the pulse, calculations with the relaxational model indicate that zero phase shifts no longer occur at the *frq*-mRNA maximum (Fig. 6).

APPENDIX B

Derivation of Equation (1)

The two perturbing instantaneous light pulses occur at phases Φ_1 and Φ_2, respectively (Fig. B1). $X(\Phi_1)$ and $X(\Phi_2)$ are the X concentrations at phases Φ_1 and Φ_2 *after* the perturbation. It is also assumed that after the light perturbation the transcription of the *frq* gene is inhibited. Times t_1 and t_2 are the phases when transcription starts again for the one-pulse perturbation at Φ_1 and for the two-pulse perturbation at Φ_1 and Φ_2, respectively. The additional delay $\Delta\Delta\Phi_{12}$ due to

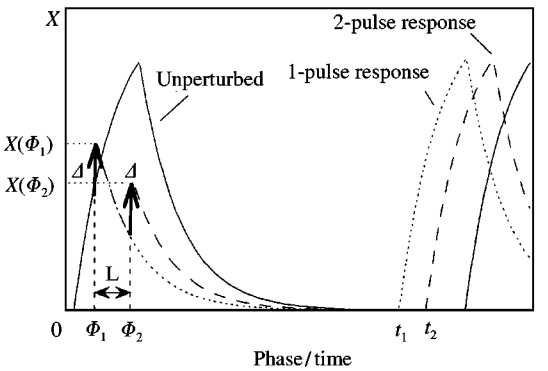

FIG B1. Representation of two-pulse light perturbation. After each perturbation in X (*frq*-mRNA), production of X (transcription in *frq*-mRNA) is inhibited, and each perturbation leads to an increase Δ in X. Φ_1 and Φ_2 are the phases of the first and second pulse, respectively, where $L = \Phi_2 - \Phi_1$. t_1 and t_2 are times when X production starts again. The additional delay due to the second pulse is given by $t_1 - t_2$.

the second pulse can therefore be written as

$$\Delta\Delta\Phi_{12} = t_1 - t_2. \quad \text{(B.1)}$$

Due to the first-order relaxation in X, t_1 and t_2 are expressed as

$$t_1 = \Phi_1 - \frac{1}{k_4}\ln\left(\frac{X_{min}}{X(\Phi_1)}\right), \quad \text{(B.2)}$$

$$t_2 = \Phi_1 - \frac{1}{k_4}\ln\left(\frac{X_{min}}{X(\Phi_2)}\right), \quad \text{(B.3)}$$

where X_{min} is the (minimum) X concentration at which production (transcription) of X starts again. Concentration $X(\Phi_2)$ is written as (Fig. B1)

$$X(\Phi_2) = \Delta + X(\Phi_1)\exp\{-k_4(\Phi_2 - \Phi_1)\}$$
$$= \Delta + X(\Phi_1)\exp(-k_4 L). \quad \text{(B.4)}$$

Inserting eqn (B.4) into eqn (B.3), and then calculating $\Delta\Delta\Phi_{12}$ [eqn (B.1)], we get

$$\Delta\Delta\Phi_{12} = (\Phi_1 - \Phi_2) - \frac{1}{k_4}\ln\left(\frac{X_{min}}{X(\Phi_1)}\right) + \frac{1}{k_4}\ln\left(\frac{X_{min}}{\Delta + X(\Phi_1)\exp(-k_4 L)}\right), \quad \text{(B.5)}$$

which is identical with eqn (1).

APPENDIX C

Abbreviations and Explanations

bd	*band* mutant strain of *N. crassa*
breakpoint	abrupt transition between phase delays and advances in PRCs when phase shift is plotted against phase of perturbation. The breakpoint is an artefact, because it does not occur in Winfree's "new phase vs. old phase" plot (Winfree, 1980) (Fig. 6)
CT*n*	circadian time *n*. CT*n* is the time/phase equal to *n*/24 of the circadian oscillators period length
Δ	amount of *frq*-mRNA produced by a light pulse transduced by a partially recovered P-state [eqns (1) and (3)]
Δ_0	amount of *frq*-mRNA produced by a light pulse transduced by a fully recovered P-state [eqn (3)]
$\Delta\Delta\Phi_{12}$	additional phase shift due to a second light pulse [eqn (1)]
F-P-U states	proposed states of the *Neurospora* photoreceptor or the light signal transduction pathway [reaction sequence LD]
Φ_i	phase of rhythm at position "*i*" [eqn (1), Fig. B1]
frq^+	*frequency* gene in *N. crassa* wild type
frq^7	mutated allele "7" of *frequency* (*frq*) gene in *N. crassa*
FRQ	protein produced by *frequency* (*frq*) gene
k_i	the rate constant of reaction *Ri* (Fig. 1)
L	time interval between two successive light pulses, $L = \Phi_2 - \Phi_1$ [Fig. B1]
PAS	protein domains leading to homo- or heterodimerization
PRC	phase response curve
t	time [eqns (2) and (3)]
t_i	time at position "*i*" [Fig. B1]
τ_{rp}	relaxation time for the recovery of P-state in darkness [eqn (3)]
wc-1, *wc-2*	*white-collar* mutations "1", "2" of *N. crassa*
WCC	white-collar complex