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Sigrid Jacobshagen
Western Kentucky University, sigrid.jacobshagen@wku.edu

Bruce Kessler
Western Kentucky University, bruce.kessler@wku.edu

Claire Rinehart
Western Kentucky University, claire.rinehart@wkue.du

At Least Four Distinct Circadian Regulatory Mechanisms Required for All Phases of Rhythms in mRNA Amount

Sigrid Jacobshagen*[1], Claire Rinehart*, and Bruce Kessler[§]

*Department of Biology, [§]Department of Mathematics, Western Kentucky University, Bowling Green, KY, USA

Running title: Regulatory mechanisms for circadian rhythms in RNA amount

1. To whom correspondence should be addressed: Sigrid Jacobshagen, Department of Biology, Western Kentucky University, 1906 College Heights Blvd #11080, Bowling Green, KY 42101-1080, USA; phone: xx1-270-745-5994; fax: xx1-270-745-6856; e-mail: sigrid.jacobshagen@wku.edu

Abstract Since the advent of techniques to investigate gene expression on a large scale, numerous circadian rhythms in mRNA abundance have been reported. These rhythms generally differ in amplitude and phase. First studies on circadian rhythms of transcription on a large scale are also emerging. We investigated to what extent the same circadian regulatory mechanism of transcription can give rise to rhythms in RNA amount that differ in phase solely based on a parameter that is not regulated by the circadian clock. Using a discrete-time approach, we modeled a sinusoidal rhythm in transcription with various constant exponential RNA decay rates. We found that the slower the RNA is degraded the later the phase of the RNA amount rhythm compared to the phase of the transcriptional rhythm. However, we also found that the phase of the rhythm in RNA amount is limited to a time frame spanning the first quarter of the period following the phase of the transcriptional rhythm. This finding is independent of the amplitude and vertical shift of the transcriptional rhythm or even of the way RNA degradation is modeled. We confirmed our results with a continuous-time model, which also allowed us to derive a simple formula that relates the phase of a rhythm in mRNA amount solely to the phase and period of its sinusoidal transcriptional rhythm and its constant mRNA half-life. This simple formula even holds true for the best sinusoidal approximations of a non-sinusoidal rhythm of transcription and mRNA amount. When using our discrete-time approach to model constant rates of transcription with a sinusoidal RNA half-life, on the other hand, we found that varying the constant component of the system, i.e. the rate of transcription, does not change the phase of the rhythm in RNA amount. In summary, our data show that at least four distinct circadian regulatory mechanisms are required to allow for all phases in rhythms of RNA amount, one for each quarter of the period.

Key words Modeling, circadian rhythms, gene expression, phase, transcription, RNA stability

INTRODUCTION

Many behavioral, physiological, and biochemical activities in a variety of organisms have been reported to show a circadian rhythm. Circadian rhythms are those rhythms that continue under constant conditions with a period of about a day, that show temperature-compensation of their period, and that entrain to the daily environmental time cues such as light/dark or temperature changes (Johnson and Hastings, 1986). Circadian rhythms are based on the circadian clock, which is an endogenous, biochemically-based timer that regulates these rhythms through mechanisms summarily termed the output pathway.

Reports of rhythmic gene expression at the level of mRNA amount have been particularly numerous, because with the advent of techniques like microarray analysis it became possible to determine rhythms in mRNA amount on a large scale. In *Arabidopsis* for example, which was the first organism investigated in this manner (Harmer et al., 2000), 6% of the more than 8000 genes tested showed a statistically significant circadian rhythm in mRNA amount. These 453 rhythms differ greatly in amplitude and phase, with all six possible phases (due to the 4-hour-sampling intervals) well represented. Since the amount of an mRNA is determined by its rate of synthesis versus its rate of degradation, experiments to determine circadian transcription on a large scale have also been performed. Based on the enhancer-trap method with luciferase as reporter gene, even 36% of the 335 lines assayed in *Arabidopsis* showed a statistically significant circadian rhythm of transcription (Michael and McClung, 2003). Circadian rhythms of mRNA degradation have not been tested on a large scale yet. There is, however, some evidence

suggesting degradation rates in the form of a circadian rhythm for a few particular mRNAs (Lidder et al., 2005; Kim et al., 2005)

When investigating the model organism *Chlamydomonas reinhardtii* (Jacobshagen et al., 2001), we found that several of its genes show a circadian rhythm in mRNA amount albeit with various amplitudes and phases. For one of these genes, LHCB-1, we demonstrated that the circadian clock regulates the expression of this gene at the level of transcription (Jacobshagen et al., 1996). Our results prompted us to question whether the other genes with rhythms in mRNA amount show a circadian rhythm in transcription identical to LHCB-1, despite the differences in phase at the mRNA amount level. A few sample calculations revealed that even when two genes have the exact same circadian rhythm of transcription, their rhythms in mRNA amount can differ in phase solely because of a difference in their constant rate of mRNA degradation. These calculations showed that the gene with the slower mRNA degradation rate will have a later phase in its circadian rhythm of mRNA amount.

In order to more comprehensively investigate circadian transcription with various constant mRNA degradation rates, we turned to the method of discrete-time modeling. To our surprise we found that although the phase can vary depending on the constant RNA degradation rate chosen, there is a limit to how much it can vary. The phase of the rhythm in mRNA amount can occur no more than a quarter of the period later than the phase of the rhythm in transcription. If this is truly the case, then an organism needs at least four distinct mechanisms by which the circadian clock regulates gene expression in order to allow for all possible phases of rhythms in mRNA amount.

We confirmed our discrete-time modeling results by developing a solvable differential equation, yielding a continuous-time model with an explicit formula. The differential equation also allowed us to derive a simple formula for the phase of the rhythm in mRNA amount solely in terms of the phase and period of the sinusoidal transcriptional rhythm and the constant half-life of the mRNA. Since the sinusoidal mRNA amount rhythm has a period identical to the transcriptional rhythm, our formula may be used to calculate the phase of an underlying transcriptional rhythm from experimentally determined mRNA half-life and mRNA amount rhythm under the condition that the mRNA half-life is constant.

MATERIALS AND METHODS

DISCRETE-TIME MODELING

Discrete-time modeling (Fig. 1) was performed using the computer program STELLA version 8.1 at 0.25 h time-steps with Euler's Method chosen as numerical integration method. The amount of RNA was modeled as a "reservoir" that could not be negative. In all our models, the amount of RNA is expressed in "RNA units" which can represent equally well particular units of RNA amount or RNA concentration.

Transcription rhythmic, RNA half-life constant

Transcription was modeled as a cosine wave with a period of 24 h, a phase where the minimum occurred at 0 min into the time domain, and an amplitude of 0.5 so that it was 1 RNA unit/h at its maximum and 0 RNA unit/h at its minimum (Enzyme_gene_TF = 0.5-0.5*COS((2*PI/24)*TIME) in Fig. 1). The inclusion of an Enz_Amplification_Factor that could be varied to a different value from one allowed for changes in amplitude of the transcriptional rhythm in order to analyze the effect of these amplitude changes on the resulting rhythm in RNA amount (Enz_Amplitude_Factor = 1 or variable in Fig. 1). The addition of a basic constant rate of transcription to be chosen by the experimenter allowed for the analysis of effects on the resulting rhythm in RNA amount from possible vertical shifts of the rhythm in transcription (Basal_Transcription = 0 or variable Fig. 1). The combination of all three parameters determined the rate of RNA synthesis in the model (INFLOWS: RNA_Transcription = Enzyme_gene_TF*Enz_Amplitude_Factor+Basal_Transcription in Fig. 1).

RNA degradation was modeled via exponential decay ([RNA] - [RNA] $2^{-\Delta t/h}$) for which the constant half-life (h) in hours could be chosen (OUTFLOWS: RNA_Degradation = (RNA-RNA*2^(-DT/halflife))/DT in Fig. 1). The phase of the rhythm in RNA amount (RNA(t) in Fig. 1) was determined by visually identifying the time of the highest or lowest value in the data table. Precision for the data table was set to "free float".

Transcription rhythmic, RNA degradation via Michaelis-Menten equation constant

In a different approach, RNA degradation was modeled via the Michaelis-Menten equation (V_{max} [RNA]/(K_M + [RNA]) in order to account for the action of an RNase. Various

values for both V_{max} and K_M could therefore be chosen to run the model with all other aspects identical to the model based on RNA exponential decay in Figure 1 (OUTFLOWS: RNA_Degradation = Vmax*RNA/(RNA+Km).

Transcription constant, RNA half-life or % RNA degradation rhythmic

When transcription was modeled at a constant rate, but the RNA half-life with a rhythm, the OUTFLOWS were modeled identical to Figure 1, except that the half-life was modeled as a cosine wave with a period of 24 h (halflife = (Amplitude_Factor-Amplitude_Factor*COS((2*PI/24)*TIME))+Vertical_Shift). This allowed us to vary the amplitude and vertical shift of the rhythm in RNA half-life. The constant rate of transcription was modeled to be chosen by the experimenter in RNA units/h.

To model a rhythm in % RNA degradation instead of RNA half-life, the % RNA degradation was modeled as a cosine wave with a 24 h period whose amplitude and vertical shift could be chosen (Percent_degradation = (Amplitude_Factor-Amplitude_Factor*COS((2*PI/24)*TIME))+Vertical_Shift). This rhythm was then integrated into the RNA degradation term (OUTFLOWS: RNA_Degradation = RNA*Percent_degradation).

CONTINUOUS-TIME MODELING

Some algebraic manipulations and the generation of graphs from our solutions were performed using the computer program *Mathematica*™.

RESULTS

DISCRETE-TIME MODELING

Rhythmic transcription, constant RNA half-life

We decided to model the rate of transcription as a sinusoidal wave, because many of the transcriptional rhythms measured with nuclear run-on assays or luciferase as a reporter gene generally resemble a sine wave (Liu et al, 1995; So and Rosbash, 1997; Michael and McClung, 2003). In addition, the seven circadian rhythms in mRNA amount that we detected in the model organism *Chlamydomonas reinhardtii* also resemble a sine wave (Jacobshagen et al., 2001). We decided to model RNA degradation via exponential decay, because when RNA degradation is measured experimentally, it usually follows this pattern and is therefore most commonly expressed in half-lives.

As demonstrated in Figure 2, a longer constant RNA half-life indeed leads to a later phase in the rhythm in RNA amount although the rhythm in the sinusoidal rate of transcription is the same as for the shorter constant RNA half-life. For the specific rhythm in transcription

modeled in this example (red in Fig. 2), the resulting rhythm in RNA amount has its peak only 0.25 h later than the transcriptional rhythm when the RNA half-life is 0.1 h (blue in upper panel of Fig. 2) but 3 h later when the constant half-life is lengthened to 2.5 h (blue in lower panel of Fig. 2). Identical results are achieved when comparing the troughs or any other phase reference points. This demonstrates that the phase of a rhythm in RNA amount can be shifted based on a parameter that is not controlled by the circadian clock. Figure 2 also demonstrates that the period of the RNA amount rhythm remains identical to the period of the transcriptional rhythm for both constant half-lives.

Limits to phase

When modeling longer and longer RNA half-lives, we discovered to our surprise that the phase of the resulting rhythm in RNA amount approached a final value. For our specific rhythm in transcription, the phase could not be more than 6 h later than the transcriptional rhythm (Fig. 3), which represents exactly a quarter of its 24 h period.

In order to understand what happens when very long RNA half-lives are chosen, it is instructive to look at the case of the extremely long half-life of 1000 h or 41.67 days (Fig. 4). In the beginning, the amount of RNA increases with a small amplitude rhythm on top of this general increase (upper panel in Fig. 4, note that the x-axis goes up to 1200 h instead of the 120 h in Fig. 2). However, when running the model long enough or starting out with the proper high amount of initial RNA, the RNA amount stabilizes to a high constant value on top of which it cycles with a stable, very low amplitude (lower panel in Fig. 4, note that the y-axis for the

amount of RNA goes from 719.4 to 723.4). Generally it can be said that the longer the RNA half-life, the more cycles it takes for the amount of RNA to stabilize when starting with an initial RNA amount of zero.

Table 1 summarizes the amount of RNA that can be found at the peak and trough for various RNA half-lives after the RNA amount has stabilized. Table 1 also shows that the amplitude when expressed as the amount of RNA that lies between the peak and the trough first increases with longer RNA half-lives but goes towards a final value for very long RNA half-lives (3.82 in this specific example). When the amplitude is expressed as the difference in RNA amount between peak and trough as a portion of the peak value, however, it continues to become smaller with longer RNA half-lives. For our example, the portion of RNA amount between the peak and the trough is only 0.5% of the peak amount when the extreme half-life of 1000 h is chosen (Table 1).

Relevance to experiments

In experiments to determine rhythms in RNA amount through northern blot analysis, an amplitude of at least two fold is often considered significantly above background noise. A two-fold amplitude rhythm will show an RNA amount between peak and trough that is 50% of the peak amount. Consequently, such experiments would only be able to detect rhythms with a phase of up to about 4.9 h later than the phase of the transcriptional rhythm in our specific example (Table 1). They would not detect those rhythms whose phases are between 4.9 and 6 h later.

Effect of amplitude and vertical shift

We also investigated the effect of varying the amplitude of the transcriptional rhythm in our discrete-time model (Table 2). We did this because promoters that are regulated by the same circadian mechanism but differ in their strength will give rise to rhythms of transcription that differ in amplitude. A weak promoter will give rise to a lower amplitude than a strong promoter although the phase and period will remain the same. Not surprisingly, we found that the larger the amplitude of the transcriptional rhythm the larger the amplitude of the rhythm in RNA amount (compare amounts of RNA between peak and trough in Table 2). In fact, the amplitude of the rhythm in RNA amount changes by the same factor with which the amplitude of the transcriptional rhythm is changed. But more importantly, we also found that the phase of the rhythm in RNA amount is unaffected. The same constant RNA half-life will give rise to the same phase of the rhythm in RNA amount regardless of the amplitude of the transcriptional rhythm.

We also modeled our sinusoidal transcriptional rhythm with various vertical shifts by adding various constant rates of transcription (Table 2). Such a constant rate of transcription may account for a basic low-level promoter activity on top of which the activity of the promoter exhibits circadian regulation. In this case, the amplitude of the rhythm in RNA amount does not change, i.e. the amount of RNA between peak and trough is the same for a particular RNA half-life regardless of the vertical shift (Table 2). Only when this amount of RNA between peak and trough is expressed as a percent of the peak value will it become smaller as the vertical shift increases. However, the phase of the rhythm in RNA amount is again unaffected (Table 2). The same constant RNA half-life will give rise to the same phase of the rhythm in RNA amount

regardless of the vertical shift of the transcriptional rhythm.

In conclusion, neither changes in amplitude nor in vertical shift in our rhythmic transcription model will change the phase of the resulting rhythm in RNA amount. Instead, this phase, as compared to the transcriptional rhythm, depends solely on the particular constant RNA half-life chosen. Consequently, our finding that the phase of the rhythm in RNA amount is limited to between 0 and 6 h later than the phase of our 24-h-period transcriptional rhythm holds true regardless of the transcriptional rhythm's amplitude or vertical shift. Amplitude and vertical shift may, however, determine whether a rhythm in RNA amount can be detected experimentally or falls below the limit of detection.

RNA degradation via Michaelis-Menten equation

We also modeled RNA degradation using the Michaelis-Menten equation (V_{max} [RNA]/(K_M + [RNA]). This approach might be considered to better reflect the action of an RNase, the specific kind of enzyme that actually degrades RNA in organisms. The Michaelis-Menten equation contains two parameters that can be varied, V_{max} and K_M. When using various value combinations for these two parameters in our model, we principally obtained the same results as for the exponential decay model of RNA degradation. The phase of the rhythm in RNA amount may range between 0 and 6 h later than the phase of our transcriptional rhythm but not beyond (data not shown). However, not all V_{max} and K_M combinations fall under this pattern. Some combinations (extremely low V_{max} with extremely high K_M) will cause such a slow RNA degradation that the amount of RNA continues to increase without ever stabilizing to cycle

around final values. Some other combinations (extremely high V_{max} with extremely low K_M) will lead to such a fast RNA degradation that, due to the limitations of our discrete-time approach, the amount of RNA bounces between zero and a positive value. The amount of RNA under these conditions should remain zero at all times. It bounces, because the STELLA program executes the calculation of outflows from the RNA amount without inflows added but calculates the amount of RNA for the next time point from the outflows and the inflows. This problem can be circumvented by programming STELLA to calculate the outflows with the inflows added (replace RNA with RNA+(RNA_Transcription*DT)), although this has the disadvantage that the phase of the rhythm in outflows is now shifted to occur earlier by one time point interval. We also discovered that certain combinations of V_{max} and K_M lead to rhythms in RNA amount that do not strictly show a sine wave pattern. The consequence is that its phase cannot really be compared to the phase of the sinusoidal transcriptional rhythm since the shape of both rhythms differs. The lower panel in Figure 5 shows such an example.

Transcription constant but RNA half-life rhythmic

The amount of RNA might also show a rhythmic pattern due to a rhythm in RNA degradation instead of transcription. In order to investigate this kind of situation, we modeled a constant rate of transcription in combination with an RNA half-life that shows a sinusoidal pattern. Since an RNA half-life of 0 h leads to an instantaneous degradation of all RNA regardless of its amount, a situation not likely in an organism, we modeled the rhythm in RNA half-life with a trough of more than 0 h. For the two examples depicted in Figure 6, the cosine wave in RNA half-life has a period of 24 h, and either a peak of 1.25 h and a trough of 0.25 h

(pink in upper panel of Fig. 6) or a peak of 40.25 h and a trough of 0.25 h (pink in lower panel of Fig. 6) with a constant transcription rate of 1 RNA unit/h in both cases. Figure 6 demonstrates a general finding with this kind of modeling set-up. The resulting rhythm in RNA amount does not follow an exact sinusoidal pattern. The phase difference between the RNA amount rhythm and the RNA half-life rhythm has therefore a different value depending on whether the peak or the trough is used as reference point.

As demonstrated with the example in Table 3, we generally found that a change in the constant rate of transcription does not lead to a change in the phase of the rhythm in RNA amount. This phase, instead, can be changed by varying either the amplitude or the vertical shift of the rhythm in RNA half-life (Table 4). A larger amplitude or a larger vertical shift will lead to a later phase of the rhythm in RNA amount. Notice that due to the change in shape, the peak of the RNA amount rhythm may occur later than the quarter of the period limit we encountered with our rhythmic transcription model. However, when the average of the peak and trough is taken as an indication for the phase, it will not go beyond this quarter of the period limit.

We also modeled rhythmic RNA degradation in the form of percent degradation with a cosine wave and obtained basically the same results. The phase of the rhythm in RNA amount does not change when varying the constant rate of transcription but does change when varying either the amplitude or the vertical shift of the rhythm in percent RNA degradation (data not shown). The shape of the rhythm in RNA amount is also not strictly sinusoidal (Figure 7).

In conclusion, although a rhythm in RNA amount may be due to a rhythm in transcription

or a rhythm in RNA half-life, these two possibilities differ greatly in the kind of parameters that determine the phase of the rhythm in RNA amount.

CONTINUOUS-TIME MODEL

The strict limits that we have discovered to the phase of a rhythm in RNA amount with respect to the phase of the sinusoidal transcriptional rhythm from which it is derived made us suspect a simple mathematical basis for these limits. We investigated this possibility by developing a differential equation for the amount of RNA resulting from rhythmic transcription and constant half-life-based RNA degradation.

Mathematics behind the continuous-time model

Let $R(t)$ be the amount of RNA present at a time t, in hours. Let $T(t)$ and $D(t)$ be the transcription and degradation rates, respectively, of the RNA in the system with respect to the time t in hours. By definition, the rate of change of the RNA present at time t is the difference of the transcription and degradation rates, given by

$$R'(t) = T(t) - D(t), \tag{1}$$

where $R'(t)$ represents the derivative of $R(t)$. This will be the basic underlying differential equation governing $R(t)$, the amount of RNA present at time t, regardless of how we model the degradation rate. We are initially assuming that the RNA decays exponentially, with a constant half-life h.

We will assume that the transcription rate $T(t)$ is a periodic function with period p, meaning that $T(t+p) = T(t)$ for all t. Assuming exponential decay, the average rate of RNA degradation over the interval $\left[t, t+\Delta t\right]$ is given by

$$\frac{1}{\Delta t}\left[R(t) - R(t)2^{-\frac{\Delta t}{h}}\right].$$

Then, by taking the limit using L'Hopital's Rule, we have

$$D(t) = \lim_{\Delta t \to 0} \frac{R(t)}{\Delta t}\left(1 - 2^{-\frac{\Delta t}{h}}\right) = R(t) \lim_{\Delta t \to 0}\left(-2^{-\frac{\Delta t}{h}} \ln 2\right)\left(-\frac{1}{h}\right) = \frac{\ln 2}{h} R(t).$$

Substituting into (1), we have that $R(t)$ will therefore be a solution to the differential equation

$$R'(t) + \frac{\ln 2}{h} R(t) = T(t) \tag{2}$$

with initial condition $R(0) = k$ for some constant k.

We will further assume that transcription is sinusoidal and that $T(t)$ has therefore the form

$$T(t) = a_0 + a_1 \cos\left(\frac{2\pi}{p} t\right) + b_1 \sin\left(\frac{2\pi}{p} t\right) \tag{3}$$

for some constants a_0, a_1, and b_1. Thus, $T(t)$ can be expressed in the form $a_0 + A\cos\left(\frac{2\pi}{p}t - \theta\right)$, with period p, mean value a_0, amplitude $A = \sqrt{a_1^{\,2} + b_1^{\,2}}$, and θ satisfying both $\cos\left(\frac{2\pi}{p}\theta\right) = \frac{a_1}{A}$ and $\sin\left(\frac{2\pi}{p}\theta\right) = \frac{b_1}{A}$.

Substituting equation (3) into the differential equation (2) yields a first-order differential equation that is solvable using Laplace transforms. Let $\Re(s)$ denote the Laplace transform of $R(t)$. Then, applying the Laplace transform term-by-term, we have

$$s\Re(s) - k + \frac{\ln 2}{h}\Re(s) = \frac{a_0}{s} + \frac{a_1 p^2 s + 2b_1 p\pi}{4\pi + p^2 s^2}.$$

Solving for $\Re(s)$, we have

$$\Re(s) = \frac{h}{hs + \ln 2}\left(k + \frac{a_0}{s} + \frac{a_1 p^2 s + 2b_1 p\pi}{4\pi + p^2 s^2}\right).$$

Applying the inverse Laplace transform term-by-term yields the general solution

$$\begin{aligned} R(t) = 2^{-\frac{t}{h}}\left[k - \frac{ha_0}{\ln 2} + \frac{hp\left(-pa_1 \ln 2 + 2h\pi b_1\right)}{4h^2\pi^2 + p^2(\ln 2)^2}\right] + \frac{ha_0}{\ln 2} \\ + \frac{hp}{\sqrt{4h^2\pi^2 + p^2(\ln 2)^2}}\left[a_1 \cos\left(\frac{2\pi}{p}t - \tan^{-1}\left(\frac{2\pi h}{p\ln 2}\right)\right) + b_1 \sin\left(\frac{2\pi}{p}t - \tan^{-1}\left(\frac{2\pi h}{p\ln 2}\right)\right)\right]. \end{aligned} \tag{4}$$

An example of how we can use this general solution to predict the RNA amounts for a particular periodic transcription rate, RNA half-life, and initial RNA value is shown in Figure 8. In this example, which is a replication of the discrete-time example in the lower panel of Figure 2, we show the RNA amount curve when $p = 24$ hours, $h = 2.5$ hours, and the transcription rate is given by

$$T(t) = 0.5 - 0.5\cos\left(\frac{\pi}{12}t\right).$$

Therefore, $k = T(0) = 0$, $a_0 = 0.5$, $a_1 = -0.5$, and $b_0 = 0$. Thus the continuous curve for the RNA amount at any time $t \geq 0$ is

$$R(t) = 2^{-\frac{t}{3}}\left[-\frac{5}{4\ln 2} + \frac{720\ln 2}{25\pi^2 + 576(\ln 2)^2}\right] + \frac{5}{4\ln 2} - \frac{30}{\sqrt{25\pi^2 + 576(\ln 2)^2}}\cos\left(\frac{\pi}{12}t - \tan^{-1}\left(\frac{5\pi}{24\ln 2}\right)\right).$$

The above solution, depicted graphically in Figure 8, shows that the initial discrete-time model depicted in Figure 2 is fairly accurate. Only close inspection reveals that the model in Figure 2 is slightly off in terms of amplitude and phase due to its resolution limits.

Derived formula for difference in phase between transcriptional and RNA amount rhythm

The solution in (4) describes what experimental data should look like given the assumption of a sinusoidal transcription curve and constant exponential decay of the RNA. It is not purely periodic (see the first few hours in Figure 8 as an example) because of the first term,

but becomes mostly periodic over a period of time, since $\lim_{t\to\infty} 2^{-\frac{t}{h}} = 0$. (For example, in the curve shown in Figure 8, the magnitude of the exponential term is less than 0.1 after 11 hours.) After sufficient time has passed, the solution looks like

$$\frac{ha_0}{\ln 2} + \frac{hp}{\sqrt{4\pi^2h^2 + p^2(\ln 2)^2}}\left[a_1\cos\left(\frac{2\pi}{p}t - \tan^{-1}\left(\frac{2\pi h}{p\ln 2}\right)\right) + b_1\sin\left(\frac{2\pi}{p}t - \tan^{-1}\left(\frac{2\pi h}{p\ln 2}\right)\right)\right]. \qquad (5)$$

Therefore, we will draw conclusions from just the portion of the formula shown in (5).

The first term in (5) represents a vertical shift of $\frac{ha_0}{\ln 2}$. Thus, the vertical shift of the RNA amount is directly proportional to the half-life h; that is, multiplying the half-life by a factor of c will multiply the vertical shift by a factor of c. The second term gives the sinusoidal part of the steady-state solution, which has the same period p as the transcriptional rhythm. The amplitude of this rhythm equals the amplitude of the transcriptional rhythm multiplied by the factor $\frac{hp}{\sqrt{4\pi^2h^2 + p^2(\ln 2)^2}}$. Thus the amplitude is dependent upon but not directly proportional to h. This factor, and therefore also the amplitude, will become larger as h becomes larger, with the factor reaching a final value of $\frac{p}{2\pi}$.

But most importantly, the phase difference of the curve with respect to the transcription curve is

$$\frac{p}{2\pi}\tan^{-1}\left(\frac{2\pi h}{p\ln 2}\right).$$

Notice that the difference in phase is only dependent upon two parameters, the period p and the half-life h. In our example shown in Figure 8, the phase difference is $\frac{24}{2\pi}\tan^{-1}\left(\frac{2\pi(2.5)}{24\ln 2}\right) \approx 3.03$ hours. But notice in particular that, as $h \to 0$, the phase difference goes to $\frac{p}{2\pi}\tan^{-1} 0 = 0$, and as $h \to \infty$, the phase difference goes to $\frac{p}{2\pi} \cdot \frac{\pi}{2} = \frac{p}{4}$. It means that for our continuous-time modeling the phase of the RNA amount rhythm is limited to between 0 h and a quarter of the period later than the phase of the transcriptional rhythm and therefore that at least four circadian regulatory mechanisms are required to allow for all possible phases of rhythms in RNA amount.

Interestingly, the result for an infinite half-life h holds regardless of how we model RNA degradation. An infinite half-life corresponds to 0 degradation. In the Michaelis-Menten model for example, 0 degradation would correspond to $V_{\max} = 0$ or an extremely large value for K_M or both. If we assume 0 degradation, we can simply remove the degradation rate $D(t)$ from our differential equation in (1). If we then still assume a p-periodic transcription rate $T(t)$ as in (3), we have

$$R'(t) = a_0 + a_1 \cos\left(\frac{2\pi}{p}t\right) + b_1 \sin\left(\frac{2\pi}{p}t\right),$$

which is easily solvable, with solution

$$R(t) = a_0 t + \frac{p}{2\pi}\left(a_1 \sin\left(\frac{2\pi}{p}t\right) - b_1 \cos\left(\frac{2\pi}{p}t\right)\right) + k + \frac{pb_1}{2\pi},$$

or equivalently,

$$R(t) = a_0 t + \frac{p}{2\pi}\left(a_1 \cos\left(\frac{2\pi}{p}t - \frac{\pi}{2}\right) + b_1 \sin\left(\frac{2\pi}{p}t - \frac{\pi}{2}\right)\right) + k + \frac{pb_1}{2\pi}.$$

Notice that the periodic part of the solution also has the same period p as the transcription rate $T(t)$, with phase difference $\frac{p}{2\pi} \cdot \frac{\pi}{2} = \frac{p}{4}$ hours.

Rhythmic transcription rates that do not follow a sinusoidal pattern

Circadian rhythms of transcription might not necessarily resemble a sine wave. An extreme example would be a burst-like pattern where most of the time transcription is silent but about every 24 h it is high for a short period of time. We therefore also investigated the relationship between transcription and RNA amount for transcription with any periodic function in combination with constant exponential RNA decay using the continuous-time model.

Any periodic function of bounded variation (loosely, having finite arc-length over the period) can be expressed as infinite sum of trigonometric functions of the same period, known as a ***Fourier series***. We will therefore assume that

$$T(t) = a_0 + \sum_{n=1}^{\infty}\left(a_n \cos\left(\frac{2\pi n}{p}t\right) + b_n \sin\left(\frac{2\pi n}{p}t\right)\right) \quad (6)$$

for some constants a_0, a_n, and b_n, $n = 1, 2, \ldots$. Substituting equation (6) into the differential equation (2) again yields a differential equation that is solvable using Laplace transforms. Let

$\Re(s)$ denote the Laplace transform of $R(t)$. Then, applying the Laplace transform term-by-term, we have

$$s\Re(s) - k + \frac{\ln 2}{h}\Re(s) = \frac{a_0}{s} + \sum_{n=1}^{\infty}\left(\frac{a_n p^2 s + 2b_n np\pi}{4n^2\pi + p^2 s^2}\right).$$

Solving for $\Re(s)$, we have

$$\Re(s) = \frac{h}{hs + \ln 2}\left(k + \frac{a_0}{s} + \sum_{n=1}^{\infty}\frac{a_n p^2 s + 2b_n np\pi}{4n^2\pi + p^2 s^2}\right).$$

Applying the inverse Laplace transform term-by-term yields the general solution

$$R(t) = 2^{-\frac{t}{h}}\left[k - \frac{ha_0}{\ln 2} + \sum_{n=1}^{\infty}\frac{hp}{4h^2n^2\pi^2 + p^2(\ln 2)^2}\left(-pa_n \ln 2 + 2hn\pi b_n\right)\right] + \frac{ha_0}{\ln 2}$$
$$+ \sum_{n=1}^{\infty}\frac{hp}{\sqrt{4h^2n^2\pi^2 + p^2(\ln 2)^2}}\left[a_n \cos\left(\frac{2\pi n}{p}t - \tan^{-1}\left(\frac{2\pi hn}{p\ln 2}\right)\right) + b_n \sin\left(\frac{2\pi n}{p}t - \tan^{-1}\left(\frac{2\pi hn}{p\ln 2}\right)\right)\right]. \quad (7)$$

An example of how we can use this general solution to predict the RNA amounts for a particular periodic transcription rate, half-life, and initial value is shown in Figure 9. In this example, we show the RNA amount curve when $p = 24$ hours, $h = 3$ hours, $k = 1$, and the transcription rate is given by

$$T(t) = 1.5 + 0.5\cos\left(\frac{\pi}{12}t\right) + 0.5\sin\left(\frac{\pi}{12}t\right) + 0.2\cos\left(\frac{\pi}{6}t\right) - 0.5\sin\left(\frac{\pi}{6}t\right).$$

Note that the two curves no longer have the same shape, thus the concept of a "phase difference" between the two curves is no longer valid. However, since the trigonometric

functions that form the basis for the Fourier series are orthogonal under the $L^2[0,p]$ norm, we may form a best-fit sinusoidal for both the transcription curve $T(t)$ and the RNA amount curve $R(t)$, after the effects of the exponential part of the solution fade, by truncating both trigonometric formulas to just the constant term and the trigonometric functions of period p. The approximations of the curves appear in Figure 9 as dashed lines of the same color. We may then talk about the phase difference of the sinusoidal best approximations. The phase difference of these best approximations will still be limited to $\frac{p}{4}$. In the example shown in Figure 9, the phase difference between the best sinusoidal fits is $\frac{24}{2\pi}\tan^{-1}\left(\frac{2\pi(3)}{24\ln 2}\right) \approx 3.24$ hours.

DISCUSSION

We believe we have shown that under the special case of the circadian clock regulating transcription but not RNA degradation, rhythms in RNA amount of all possible phases can only be achieved through a minimum of four distinct mechanisms the circadian clock employs to regulate transcription. This follows necessarily from our finding that the phase of the rhythm in RNA amount under such conditions is limited to a time frame spanning the first quarter of the period following the phase of the transcriptional rhythm. The limit holds true regardless whether discrete- or continuous-time modeling is used. It is independent of the amplitude and vertical shift of the transcriptional rhythm or the way RNA degradation is modeled, and also applies to the best sinusoidal approximation of a transcriptional rhythm that has a non-sinusoidal pattern.

Our findings make it particularly interesting to inspect the phases found for rhythms in transcription determined on a large scale in order to evaluate whether they show only four precise phases, each a quarter of the period apart. For the 128 different circadian rhythms of transcription in *Arabidopsis* discovered through the enhancer-trap method with luciferase as reporter (Michael and McClung, 2003), the phases as determined by their time of peak vary considerably. However, it is noteworthy that only six out of these 128 rhythms do not show a phase of exactly either subjective dawn, midday, dusk or midnight (CT 0, 6, 12 or 18) within the range of their standard deviation (see Table III in the supplement to Michael and McClung, 2003). Similar experiments in *Drosophila* (Stempfl et al., 2002) were unfortunately mainly carried out in light/dark cycles so that additional direct light effects cannot be excluded and for the experiments carried out in constant darkness the phase of the rhythms are not given. Our findings also raise the question of whether RNA degradation is generally rather constant or shows a circadian rhythm instead. Methods to determine RNA decay rates on a large scale were recently developed (Gutierrez et al., 2002; Raghavan and Bohjanen, 2004) but have not been applied to this question yet. For a few particular mRNAs, there is some evidence for a circadian rhythm in RNA processing/degradation (Frisch et al, 1994; So and Rosbash, 1997) or more specifically RNA degradation (Kim et al, 2005; Lidder et al., 2005; Garbarino-Pico et al., 2007).

An entirely different picture arises when modeling RNA degradation under control of the circadian clock with constant transcription. In this case, varying the non-rhythmic component of the system, i.e. the transcription rate, will not alter the phase of the RNA amount rhythm. Although this is an interesting finding, it is also the only conclusion we can draw with real confidence from this set-up. Since changing the amplitude of the exponential RNA decay rhythm

also changes the shape of the RNA amount rhythm, the concept of "phase difference" between RNA decay and RNA amount rhythm does not really apply. We therefore did not pursue this set-up further. However, we can at least say that the peak as well as the trough of the RNA amount rhythm changes its relationship to the respective parameter of the RNA decay rhythm when the amplitude or vertical shift of the RNA decay rhythm is changed. And when the average of the peak and the trough is taken as reference point, the phase will not be later than a quarter of the period compared to the phase of the RNA decay rhythm.

Modeling circadian transcription with various forms of RNA degradation has been reported before, although not with the same intention as we have done here. Modeling was mainly performed in order to fit actual experimental data for a particular gene and therefore be able to conclude on aspects of the expression of this gene not experimentally determined. So and Rosbash (1997), for example, have already demonstrated that a longer constant RNA half-life will lead to a later phase in the RNA amount rhythm. However, only when modeling rhythmic RNA degradation in addition to circadian transcription were they able to fit their nuclear run-on data with their mRNA amount data for the *per* gene. Therefore, they argued that the circadian clock must regulate the expression of the *per* gene not only at the transcriptional level but also at the level of RNA stability or processing. Similarly, Kim and coworkers (2005) modeled rhythmic transcription and rhythmic RNA degradation for the *AANAT* gene. Rhythmic RNA degradation was modeled in this case based on the amount rhythms of three heterogeneous nuclear ribonucleoproteins, which they showed to mediate degradation by specifically binding to the 3' UTR of the *AANAT* mRNA. Gachon and coworkers (2006) finally modeled circadian transcription based on their promoter binding activity data for three rhythmic transcription

factors. They then modeled RNA degradation via constant RNA half-lives in an effort to identify possible target genes of the transcription factors based on the phase of the rhythm in target RNA amount. Interestingly, their simulations produced only phases of up to 4.5 h later than the transcriptional rhythm despite their 24 h periods. However, they did not model extremely long RNA half-lives, possibly because they were only interested in rhythms of RNA amount that are experimentally detectable.

When comparing promoter sequences of the many genes exhibiting a circadian rhythm in mRNA amount in *Arabidopsis* (Harmer et al., 2000), an "evening element" could be identified in the promoter of genes with a peak towards the end of the subjective day. This same evening element was also found in the study on *Arabidopsis* large-scale circadian transcription (Michael and McClung, 2003). When the evening element is exchanged with the CCA1-binding site previously shown to be important for morning-specific transcription, the phase of the transcriptional rhythm changes with its peak occurring towards subjective dawn (Michael and McClung, 2002). It is interesting that similar promoter elements specific for subjective midday or midnight could not be discovered. This finding together with the likelihood of circadian rhythms in mRNA decay raises the possibility that a distinct circadian regulatory mechanism might consist of a "classical" circadian transcriptional mechanism in combination with a circadian RNA decay mechanism. When attempting to model this situation using again a sinusoidal RNA half-life, we encountered the same problem as modeling a sinusoidal RNA half-life with constant transcription, i.e. the shape of the RNA amount rhythm is not strictly sinusoidal and varies when amplitude or vertical shift of the half-life rhythm are varied. Proper modeling of rhythmic RNA degradation needs perhaps to be reevaluated after experimental data

on such a rhythm have been determined with high time resolution.

Our model did not take into account possible effects of RNA processing or RNA nuclear export. These processes can be regulated (Terry et al., 2007; Keene, 2007) although there is currently no evidence that they might be the target of regulation by the circadian clock. If we assume that these processes occur with a combined constant exponential rate like we assumed for RNA degradation, we can simply expand our model by connecting two such models in series. The outflows of the first model unit, representing the processed/exported RNA, serve as the inflows to the next model unit. The amount of processed/exported RNA, which would be equal to the amount of degraded RNA in the single model, is proportional to the amount of RNA not processed/exported and therefore also shows a sinusoidal rhythm with a phase identical to the rhythm in not processed/exported RNA. The overall phase difference between the transcriptional rhythm and the rhythm of mature mRNA in the cytosol is now the sum of the phase differences caused by the half-life of each individual model unit. Consequently, the amount of mature mRNA in the cytosol can have a circadian rhythm up to half a period later, one quarter due to RNA processing/export and one quarter due to RNA degradation. This would, however, require that RNA processing/export rates are very slow. In addition, the amplitude of this rhythm in mature mRNA in the cytosol would become extremely small when expressed as % of the peak value for phases of between one and two quarters of the period later than the transcriptional rhythm. Therefore, by adding RNA processing/export with a constant half-life to our model, the minimally required distinct circadian regulatory mechanisms to allow for all phases of RNA amount rhythms reduces from four to two, but at the price of very low amplitudes for phases between one and two quarters of the period later than the transcriptional rhythm.

Our model may also be modified to include competition of two processes that occur with constant half-lives, like for example RNA processing/export and RNA degradation within the nucleus. The half-lives of processing and degradation, h_1 and h_2, respectively, will combine to give an overall half-life of $h = \frac{h_1 h_2}{h_1 + h_2}$. The half-lives of both competing processes therefore determine the phase of the resulting rhythm in processed RNA that arrives in the cytosol, whereas its amplitude is determined by the factor $\frac{\ln 2}{h_1} \frac{hp}{\sqrt{4\pi^2 h^2 + p^2 (\ln 2)^2}}$.

We succeeded in developing a simple formula that relates the phase of a rhythm in mRNA amount solely to the phase and period of its sinusoidal transcriptional rhythm (or its best sinusoidal approximation) and its constant mRNA half-life. This formula is

$$\phi^{RNA} - \phi^{Tr} = \frac{p}{2\pi} \tan^{-1}\left(\frac{2\pi h}{p \ln 2}\right)$$

where ϕ^{RNA} symbolizes the phase of the rhythm in RNA amount, ϕ^{Tr} the phase of the rhythm in transcription, *p* the period of the transcriptional as well as the RNA amount rhythm and *h* the constant half-life of the RNA. This formula might prove useful. If any three of the four parameters have been experimentally determined with reasonably high accuracy and time resolution, it will be possible to calculate the fourth parameter using this formula. It might also be used to evaluate how well techniques like nuclear run-on or luciferase reporting truly reflect transcription. However, all these uses depend on the RNA decay rate being constant and therefore on a parameter that has not been sufficiently investigated yet.

Our findings also raise the possibility that many of the genes with "constant" amounts of mRNA are actually transcribed with a circadian rhythm but have an RNA decay rate that is very slow. Would there be an advantage to regulating the expression of a gene in this manner? It probably would not make much difference for the organism from the viewpoint of this particular gene and its function. However, it might be advantageous to an organism that lives in a predictably changing environment to commit its machinery to transcribing a particular gene during a certain time of day when resources are more plentiful or interference with other functions is minimized.

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Figure/Table legends:

Figure 1: Discrete-time modeling design. The program STELLA was used to model circadian transcription with constant RNA half-lives to determine the resulting rhythms in RNA amount. **Upper panel:** The components and their connections. **Lower panel:** The mathematical basis for each component. The square symbolizes the amount of RNA. The two circles with arrows symbolize the RNA transcribed (inflows) and degraded (outflows). The four simple circles symbolize the specific parameters for either the inflows or the outflows. The amount of RNA is expressed in RNA units, the rate of transcription in RNA units/h and the RNA half-life in h. Note that the rate of transcription is modeled as a cosine wave with a period of 24 h, a peak of 1 RNA units/h and a trough of 0 RNA units/h if not further modified in amplitude or vertical shift.

Figure 2: The phase of the rhythm in RNA amount varies with the constant RNA half-life chosen for identical rhythms in transcription. Graphs were obtained from running the model in Fig. 1. The rate of transcription in RNA units/h is shown in red, the amount of RNA in RNA units is shown in blue. **Upper graph:** Constant RNA half-life of 0.1 h yielding a phase for the rhythm of RNA amount 0.25 h later than the transcriptional rhythm. **Lower graph:** Constant RNA half-life of 2.5 h yielding a phase for the rhythm of RNA amount 3.0 h later than the transcriptional rhythm.

Figure 3: The phase of the rhythm in RNA amount has limits. Phase values were obtained from running the model in Fig. 1 with various RNA half-lives. The phase of the rhythm in RNA amount is expressed as delayed with respect to the phase of the rhythm in transcription.

Figure 4: For very long constant RNA half-lives, the amount of RNA increases for many cycles before it stabilizes to oscillate around final values. Graphs were obtained from running the model in Fig. 1 with a constant RNA half-life of 1000 h. The rate of transcription in RNA units/h is shown in red, the amount of RNA in RNA units is shown in blue. **Upper graph:** The amount of RNA for the first 50 cycles when the initial amount of RNA is zero. **Lower graph:** The amount of RNA after it has stabilized to cycle around final values because the model was run for an extended period of time. Note that the y-axis for the amount of RNA goes from 719.4 to 723.4

Figure 5: Modeling RNA degradation based on the Michaelis-Menten equation instead through exponential decay. The rate of transcription in RNA units/h is shown in red, the amount of RNA in RNA units is shown in blue. The rhythm in transcription is identical to that in Fig. 2 and 4. **Upper graph:** V_{max} is 40 RNA units/h and K_M is 200 RNA units. The rhythm in RNA amount shows a sine wave pattern. **Lower graph:** V_{max} is 1.5 RNA units/h and K_M is 1 RNA unit. The rhythm in RNA amount does not show a strict sine wave pattern.

Figure 6: Modeling a rhythmic RNA half-life with a constant rate of transcription. The RNA half-life in hours is shown in pink, the amount of RNA in RNA units is shown in blue. In both graphs, transcription was modeled at a constant rate of 1 RNA unit/h and the RNA half-life as a cosine wave with a period of 24 h. **Upper graph:** The RNA half-life was modeled with an amplitude of 0.5 h and a vertical shift of 0.25 h. **Lower graph:** The RNA half-life was modeled with an amplitude of 20 h and a vertical shift of 0.25 h.

Figure 7: Modeling rhythmic % RNA degradation with a constant rate of transcription. The % RNA degradation is shown in pink, the amount of RNA in RNA units is shown in blue. For both graphs, the transcription rate was modeled at a constant rate of 1 RNA units/h and the % RNA degradation as a cosine wave with a period of 24 h. **Upper graph:** RNA degradation was modeled with an amplitude of 0.25% and a vertical shift of 0%. **Lower graph:** RNA degradation was modeled with an amplitude of 2.5% and a vertical shift of 0%.

Figure 8: Example graph derived from the differential equation developed for a sinusoidal rhythm of transcription. The rate of transcription in RNA units/h is shown in red, the amount of RNA in RNA units given by the solution (4) to the differential equation (2) is shown in blue. In this example, the period p is 24 h, the RNA half-life h is 2.5 h, the initial amount of RNA is 0 RNA units, and the periodic transcription rate is given by $T(t) = 0.5 - 0.5\cos\left(\frac{\pi}{12}t\right)$; that is, $k = 0$, $a_0 = 0.5$, $b_0 = 0$, and $a_1 = -0.5$. The blue curve is the result of substituting these values into (4).

Figure 9: Example graph derived from the differential equation developed for any periodic transcription. The rate of transcription in RNA units/h is shown in red, the amount of RNA in RNA units given by the solution (7) to the differential equation (2) is shown in blue. In this example, the period p is 24 h, the RNA half-life h is 3 h, the initial amount of RNA is 1 RNA unit, and the periodic transcription rate is given by $T(t) = 1.5 + 0.5\cos\left(\frac{\pi}{12}t\right) + 0.5\sin\left(\frac{\pi}{12}t\right) + 0.2\cos\left(\frac{\pi}{6}t\right) - 0.5\sin\left(\frac{\pi}{6}t\right)$; that is $k = 1$, $a_0 = 1.5$, $b_0 = 0$,

$a_1 = 0.5$, $b_1 = 0.5$, $a_2 = 0.2$, and $b_2 = -0.5$. The blue curve is the result of substituting these values into (7). The dashed lines are best sinusoidal approximations.

Table 1: Characteristics of the rhythm in RNA amount due to a specific rhythm in transcription and various constant RNA half-lives. The rate of transcription was modeled as a sine wave with a period of 24 h, an amplitude of 0.5 RNA units/h, and a vertical shift of 0 RNA units/h (see Fig. 1). "Δ Phase" refers to the time the phase of the rhythm in RNA amount is delayed with respect to the phase of the rhythm in transcription. "Δ RNA peak to trough" refers to the amount of RNA at the peak minus that of the trough. "*" denotes that these parameters are expressed in RNA units.

Table 2: Changes in amplitude or vertical shift of the rhythm in transcription do not change the phase of the rhythm in RNA amount. Amplitude and vertical shift parameters given in the table are that of the modeled cosine wave-rhythm in transcription whose period was always 24 h. As in Table 1, "Δ Phase" refers to the time the phase of the rhythm in RNA amount is delayed with respect to the phase of the rhythm in transcription, "Δ RNA peak to trough" refers to the amount of RNA at the peak minus that of the trough, and "*" denotes that these parameters are expressed in RNA units.

Table 3: Changes in the constant rate of transcription do not change the phase of the rhythm in RNA amount when it is driven by a rhythm in RNA half-life. The rhythm in RNA half-life was modeled as a cosine wave with a period of 24 h, an amplitude of 0.5 h and a vertical shift of 0.25 h. "Δ Phase peak" refers to the time the phase of the rhythm in RNA amount is

delayed with respect to the phase of the rhythm in RNA half-life when the peak was used as reference point, "Δ Phase trough" to the time this phase is delayed when the trough was used as reference point, "Δ RNA peak to trough" refers to the amount of RNA at the peak minus that of the trough, and "*" denotes that these parameters are expressed in RNA units.

Table 4: Change in amplitude or vertical shift in the rhythm of RNA half-life leads to a change in phase of the rhythm in RNA amount. Data were obtained by running each specified cosine wave-rhythm in RNA half-life with at least the four constant rates of transcription of 0.3, 1, 3, and 10 RNA units/h. "Δ Phase peak" refers to the time the phase of the rhythm in RNA amount is delayed with respect to the phase of the rhythm in RNA half-life when the peak was used as reference point, "Δ Phase trough" to the time this phase is delayed when the trough was used as reference point.