Rhythms of differentiation and diacylglycerol in *Neurospora*

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Although the fungus *Neurospora crassa* is a relatively simple lower eukaryote, its circadian system may be more complex than previously thought. In this paper we review evidence suggesting that there may be several output pathways coupled in complex ways to a single oscillator, or that there may be more than one oscillator driving independent output pathways. We have described two new rhythms in *Neurospora* that are not tightly coupled to the rhythm of conidiation bands that is the standard assay for the state of the *Neurospora* circadian clock. The first is a rhythm in the timing of differentiation, i.e. the production of aerial hyphae and spores. Large regions of the mycelium differentiate synchronously, as if responding to a spatially widespread signal. This rhythm may be distinct from the timer that sets the determination switch controlling the spatial pattern of conidiation bands. The second new rhythm is an oscillation in the levels of the neutral lipid diacylglycerol (DAG). This rhythm is found in all regions of a colony and is not always in phase with the rhythm of conidiation bands. The DAG rhythm shares some characteristics with the differentiation rhythm and has the potential to act as the signal that induces rhythmic differentiation.

Keywords: *Neurospora*; circadian; clock; conidiation; diacylglycerol

1. INTRODUCTION

How complex is the *Neurospora* circadian clock? *Neurospora crassa* is a filamentous fungus—as such, it has the biochemical and subcellular complexity of a eukaryote, but has the macroscopic simplicity of a micro-organism, with fewer complications associated with multicellularity in higher eukaryotes such as differentiated tissue types and intercellular interactions. This might lead us to assume that rhythmicity in *Neurospora* is simple as well. From the beginning of research on *Neurospora* rhythms, one observable output has formed the basis for most assays of circadian clock function: the spatial pattern of bands of conidiation (asexual spore formation) formed by the growth front as it advances across the surface of solid agar medium (Pittendrigh *et al.* 1959). Even in liquid culture systems where conidiation is suppressed, the function of the circadian clock is assayed by transferring samples to solid agar medium and allowing the colonies to form conidiation bands (Perlman *et al.* 1981). A number of other circadian rhythms have been described, such as enzymatic activities and CO₂ output (for a review, see Lakin-Thomas *et al.* 1990) and rhythmic expression of clock-controlled genes (Bell-Pedersen *et al.* 2001). In the absence of evidence to the contrary, the simplest assumption is that all observable rhythms are controlled by a single output pathway driven by a single central oscillator.

A model for the mechanism of that oscillator has been developed based on the characteristics of the clock gene *frq* (including rhythms in the level of *frq* transcript and its

protein product(s)) and characterization of the white collar genes *wc-1* and *wc-2* and their effects on *frq* expression (for reviews, see Dunlap 1999 and Bell-Pedersen *et al.* 2001). This *frq*-based oscillator model is, however, insufficient to explain the properties of rhythmic conidiation in mutant strains lacking active *frq* or *wc* gene products (Aronson *et al.* 1994; Lakin-Thomas 2000; Lakin-Thomas & Brody 2000; Loros & Feldman 1986; Mellow *et al.* 1999). It is necessary therefore to postulate the existence of a *frq*-less oscillator (FLO) (Iwasaki & Dunlap 2000), which can drive rhythmic conidiation in the absence of *frq* function. The FLO is lacking some aspects characteristic of circadian rhythms, such as temperature and nutritional compensation, and light-entrainability. In strains with normal *frq* and *wc* function, the FLO may interact with *frq* and *wc* gene products to constitute a complete clock system with circadian properties and an output pathway controlling the conidiation rhythm. Thus the clock mechanism may be internally complicated but the output may still be simple. Based on our observations of two new rhythms that are not tightly coupled to the spatial pattern of bands, we discuss the possibility that the output of the *Neurospora* circadian system may be more complex than previously thought.

2. RHYTHMIC DIFFERENTIATION

As a *Neurospora* colony grows across the surface of solid agar medium under appropriate conditions, it produces the well-known pattern of bands and interbands that are formed by regions of dense growth (bands producing aerial hyphae and conidiospores) alternating with regions

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of thin, effuse growth (interbands with fewer aerial hyphae and spores). The production of conidiospores follows a defined developmental programme (Springer & Yanofsky 1989) and, like any developmental programme, the process of conidiation can be divided into two basic events: first, the decision is made as to which programme will be followed (determination); and second, the programme is executed (differentiation). The hyphae at the growth front of a colony make a choice as to whether they will be capable of conidiating to produce part of a band, or whether the conidiation programme will be turned off and they will produce part of an interband. Under some conditions, newly-formed hyphae begin differentiation almost immediately (as in figure 1c), suggesting that the determination decision must be made very soon after hyphal formation. This decision seems to be irreversible, as the interband regions of a colony never 'fill in' later by conidiating but remain thin indefinitely. The setting for this determination switch depends on the phase of the circadian clock in the hyphae at the growth front: during the late subjective night and early morning, the switch is set to 'band' and during the late day and early night it is set to 'interband'. Thus as the colony grows and newly-formed hyphae make their determination decisions, a spatial pattern of alternating bands and interbands is laid down and acts as a 'fossil record' of the state of that switch at the time a particular region was formed at the growth front.

Once the determination decision has been made, the differentiation of aerial hyphae and conidiospores could occur at any time later without affecting the spatial pattern of bands. The differentiation programme might be activated immediately and then, depending on the constant time-lags built into the programme, spores might form immediately, or the hyphae could wait some constant length of time before spores appear. In either case, if we were to observe the growth of a colony by time-lapse photography, we would expect to see a wave of spore formation travelling smoothly along behind the growth front as it advances at a more-or-less steady rate. The results of such a time-lapse analysis (P. L. Lakin-Thomas, J. C. Thoen and V. D. Gooch, unpublished data) were unexpected: in colonies forming bands, there is no smooth travelling wave of differentiation. Instead, large regions of the colony differentiate simultaneously as if they are responding to a signal that is synchronous over a large region. The lack of correlation between the episodes of differentiation and the banding phase may indicate that the differentiation signal is independent of the circadian clock that controls the determination switch.

The analysis was carried out using a time-lapse video camera to observe colonies growing in long glass 'race tubes' in a constant-temperature incubator. Colonies were observed under a red safe-light, which is equivalent to constant darkness for this organism. The video image of each race tube was analysed by dividing it into a number of small segments or 'slices' (usually about 2.5 mm wide). The density of each slice was calculated at a number of time points (usually at 2 or 3 h intervals) and the change in density of each slice was determined. As a slice produced aerial hyphae, it increased in density rapidly, and the time interval during which the density increase was maximal was defined as the time of differentiation for that slice.

Figure 1. Time-lapse video analysis of rhythmic growth. Time is measured in hours from an arbitrary starting time. Position is measured in millimetres from an arbitrary starting position. The thin line indicates the position of the growth front at each time interval. Each dot represents the differentiation time of a particular 2.5 mm 'slice', identified by its position. Triangles mark the positions and times at which the growth front was laying down a region that would later become a band peak. Vertical bars near the *y*-axis indicate the positions of the band regions. (a) The *csp-1; bd; frq¹⁰* strain grown at 22 °C. (b) The *bd* strain grown at 25 °C. (c) The *bd* strain grown at 25 °C in a 12 L:12 D cycle, indicated by the white and black bars. White light was provided by cool-white fluorescent tubes at approximately $50 \mu\text{E m}^{-2} \text{s}^{-1}$.

The initial prediction was that each slice would differentiate in turn, according to its age, i.e. after the older slice behind it and before the younger slice ahead of it. When arrhythmic colonies were observed with this technique, that is precisely what was found. Strains carrying the *frq*¹⁰ mutation are usually arrhythmic under these particular growth conditions, and produce aerial hyphae and spores along the entire length of the race tube. As shown by figure 1a, slices differentiate about 7 or 8 h after they are formed at the growth front. As a result, a smooth travelling wave of differentiation follows along after the growth front at an interval of about 8 h.

Surprisingly, when colonies forming bands were observed, it was found that a number of neighbouring slices differentiated during the same time interval. In figures 1b and 1c, the *bd* strain (wild-type for *frq*) formed several bands during the experiment. The triangles mark the times and positions at which the growth front was forming a region that would later become the peak of a band. The vertical lines of dots indicate that a number of neighbouring slices differentiated at the same time, in contrast to the arrhythmic colony in figure 1a. The dotted lines extrapolate these times to the x-axis. In figure 1b, these episodes of simultaneous differentiation occurred about 4 or 5 h after the hyphae that would later become a band peak were laid down. These results indicate that the differentiation of a region into aerial hyphae and spores can be controlled somewhat independently of the determination decision at the growth front. A large region (a number of neighbouring slices) can delay differentiation and then develop simultaneously as if in response to a signal that is synchronous across a large region. This region usually corresponds to the first half of a band, as seen in figure 1b: the row of dots (the region of simultaneous differentiation) covers the positions just before the triangle marking the position of the band peak.

In the experiments reported in figure 1c, differentiation occurred at about the same time a band peak was being formed, rather than 4 or 5 h later as in figure 1b. The difference between figure 1b and figure 1c is that the colony in the latter was subjected to a light–dark cycle. Apparently the light–dark cycle can influence the timing of differentiation so that its relationship to the formation of a band peak can be altered. This may indicate the existence of two separate output pathways in the *Neurospora* circadian system, one that controls the determination event at the growth front and one that controls the differentiation into spores. The phase relationship between these two rhythms can be altered by environmental factors (in this case, the light–dark cycle).

In figure 2, a number of experiments have been summarized by plotting only the times at which band peaks were formed, and the times at which a large region was differentiating simultaneously. Figures 2a and 2b reinforce the conclusion reached above, that a light–dark cycle can change the phase relationship between the two rhythms. Comparison of figure 2c with 2d indicates that a mutation at the *frq* locus can also change this phase relationship: the long-period *frq*⁷ mutation not only lengthens the period of both rhythms but also advances the phase of the differentiation rhythm relative to the band peaks. In figures 2e and 2f, the choline-requiring *chol-1* strain (Lakin-Thomas 1996, 1998) displays a very

Figure 2. Summary of time-lapse video experiments. Pairs of replicate race tubes are shown together. Triangles indicate the times at which the growth front was forming a region that would later become a band peak. Circles indicate times at which a large region was differentiating simultaneously. Times have been normalized so that the first band peak in each race tube occurs at 24 h after an arbitrary start time. (a) The *bd* strain grown at 25 °C. (b) The *bd* strain grown at 25 °C in a 12 L:12 D cycle. (c) The *csp-1; bd* strain grown at 22 °C. (d) The *csp-1; bd; frq*⁷ strain grown at 22 °C. (e) The *csp-1; chol-1 bd* strain grown at 22 °C. (f) The *csp-1; chol-1 bd; frq*¹⁰ strain grown at 22 °C.

long period for both rhythms, but the phase relationship between them is no longer regular and two episodes of differentiation sometimes occur within one band cycle (top tube in figure 2e). A second episode of differentiation can also be seen in the second tube of figure 2a. Figure 2f is a strain carrying both the *chol-1* mutation and the *frq*¹⁰ mutation. This strain produces rhythmic banding under conditions of choline depletion (Lakin-Thomas & Brody 2000) and figure 2f demonstrates that the rhythm in differentiation can also be observed in this strain under long-period conditions. This indicates that the product of the *frq* gene is not required for either the determination or differentiation rhythms.

3. RHYTHMIC DIACYLGLYCEROL

Chol-1 mutants have a defect in their ability to synthesize the important membrane phospholipid phosphatidylcholine and require choline supplementation for normal growth and rhythmicity. Choline depletion also affects total lipid composition (Hubbard & Brody 1975; Juretic 1977). When cultures are depleted of choline, the growth rate is slow, the period of the conidiation rhythm is very long (about 60 h) and temperature compensation of that period is poor (Lakin-Thomas 1996, 1998).

In order to investigate the biochemical basis for altered rhythmicity in *chol-1* strains, we have assayed the levels of various lipids and have looked for correlations with period. We found that the level of the neutral lipid diacylglycerol (DAG) correlates with period under conditions of choline depletion, unlike the membrane phospholipids

such as phosphatidylcholine and phosphatidylethanolamine (Ramsdale & Lakin-Thomas 2000). DAG is an intermediate in lipid synthesis pathways in fungi (Chopra & Khuller 1984) but is better known as an intracellular signalling molecule in animal cells (Hodgkin *et al.* 1998). This led us to look more closely at DAG levels in *Neurospora*. We have found that DAG levels are rhythmic in all parts of the colony, that the rhythm is driven by a light-sensitive oscillator and that rhythmicity does not require the product of the *frq* gene (Ramsdale & Lakin-Thomas 2000).

For the experiments reported in figure 3, samples of mycelia were collected from cultures grown on agar plates overlaid with cellophane. Lipids were extracted and DAG was assayed as described (Ramsdale & Lakin-Thomas 2000). For figure 3*a*, samples were collected from the most recently formed mycelium at the growth front and the front was followed as the colony expanded. For figures 3*b* and 3*c*, the same band or interband region was repeatedly sampled. In all three cases, a rhythm in DAG was found, indicating that DAG is rhythmic in all areas of the colony. The implication is that the DAG rhythm is not simply a product of rhythmic differentiation since it persisted in regions that had completed development, as well as at the growth front where differentiation had not yet begun. The phase of the DAG rhythm was set by the light-to-dark transition in these experiments (Ramsdale & Lakin-Thomas 2000), as expected of a rhythm that is driven by a light-sensitive oscillator. In figure 3*d*, the long-period *frq*⁷ strain displays a bimodal DAG rhythm in the first band cycle followed by a long-period DAG cycle, suggesting that the *frq* gene can influence the period of the DAG rhythm. However, in figure 3*e*, the DAG rhythm is seen to persist in a *frq*¹⁰ mutant that has no functional *frq* gene product. We find that under these growth conditions (on cellophane in large culture dishes) colonies of *frq*¹⁰ produce a short-period banding rhythm early during growth (figure 3*e*). The DAG rhythm also has a short period (about 12 h). This is similar to the short period seen in the banding rhythm (Loros & Feldman 1986) and deduced from temperature entrainment experiments (Merrow *et al.* 1999) with *frq*⁹, another mutant without a functional *frq* gene product.

When the phase of the DAG rhythm is compared with the phase of the banding rhythm (indicated by the triangles marking band peaks in figure 3), it can be seen that band peaks often, but not always, coincide with DAG troughs (see in particular figures 3*a* and 3*c*). The DAG rhythm is also sometimes bimodal, producing two peaks of DAG for one banding cycle, as in figure 3*d*. These observations indicate that the DAG rhythm is not strongly coupled to the banding rhythm.

4. CONCLUSIONS

What do the data on rhythms in differentiation and rhythms in DAG tell us about the complexity of rhythmicity in *Neurospora*? Can all of the observed rhythmic phenomena be accounted for by assuming a single oscillator mechanism driving a single output pathway, or will it be necessary to look for multiple oscillators and/or

Figure 3. Diacylglycerol (DAG) rhythms. DAG was assayed relative to the residual dry weight (RDW) after extraction. Except where indicated, the curve is a three-point running average of data collected every 2 h. (Data points and standard errors have been omitted for clarity but are shown in Ramsdale & Lakin-Thomas (2000).) Triangles indicate the times at which the growth front was forming a band peak. Strains carrying the *chol-1* mutation were grown in constant darkness on 200M choline, which restores wild-type growth rates and rhythms. (a-c) The *csp-1; chol-1 bd* strain. (a) Samples were collected from the growth front. (b) Samples were collected from a band region. (c) Samples were collected from an interband region. (d) The *csp-1; chol-1 bd; frq*⁷ strain. Samples were collected from the growth front. (e) The *csp-1; chol-1 bd; frq*¹⁰ strain. Samples were collected from the growth front. Data were collected every 3 h, and the curve was drawn point-to-point and is not a running average. DD, constant darkness.

complex output pathways, either in the same spatial unit or in different regions and/or tissues?

The banding pattern is probably determined at the growing front in the newly made hyphae. The actual differentiation into aerial hyphae and conidiospores

occurs mainly behind the growing front, in older hyphae that might be biochemically different from the growth front. We know from Dharmananda & Feldman (1979) that plugs of mycelium taken from old areas will produce bands when transferred to fresh agar medium, and the phase of the new bands indicates that an oscillator is running in old areas. However, the period of this oscillator appears to lengthen as the hyphae age, as shown by the later phase of the banding pattern produced from old hyphae as compared with young hyphae (Dharmananda & Feldman 1979). The differentiation rhythm we have seen in old hyphae may therefore be driven by an oscillator with somewhat different kinetics (and possibly different components) from the oscillator at the growth front that determines the banding pattern. This could account for the lack of strict correlation between the phase of the band-determining events at the growth front and the episodes of synchronous differentiation in older areas.

The same argument may not apply to the DAG rhythm. In common with the differentiation rhythm, there is not a strict correlation between the peaks and troughs of the DAG rhythm and the occurrence of conidiation bands. However, this lack of correlation is seen even when the same region, i.e. the growth front, is assayed for both DAG and band phase (figures 3*a* and 3*d*). This indicates that the two rhythms are not in phase with each other, although the oscillator(s) that drive them must be in the same tissue. Either there are two independent sources of timing information (two oscillators) at the growth front, or there is one oscillator driving two outputs that are coupled to it in complicated ways.

Could the DAG rhythm and the differentiation rhythm be related? DAG is well-known as a signalling molecule in animal cells (Hodgkin *et al.* 1998), although such a role has not yet been demonstrated in *Neurospora*. DAG is rhythmic not only at the growth front but also in older areas of the colony where differentiation occurs. Both rhythms sometimes produce two peaks per band cycle and their phases are not always correlated with the phase of the banding rhythm (figures 2 and 3). Rhythmic DAG levels therefore have the potential to act as the differentiation signal across a large region of the mycelium. We have not yet assayed both DAG and differentiation simultaneously in the same colony to see if there is a strict correlation between the two.

Other rhythms in *Neurospora* have not been analysed in sufficient detail to reveal whether or not their phases are strongly coupled to the banding rhythm. In particular, it would be very interesting to assay the rhythms in gene expression of *frq* and clock-controlled genes (Bell-Pedersen *et al.* 2000) in colonies expressing the banding pattern on solid media. Until now, all assays of gene expression have been carried out in liquid medium in which the banding rhythm is not expressed. Will the phase of gene expression rhythms correlate with bands, or DAG rhythms, or differentiation rhythms? An answer to this could provide some insight into the relationships between these various rhythms and a better indication of the complexity of the *Neurospora* system.

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