Cellular signalling and the complexity of biological timing: insights from the ultradian clock of *Schizosaccharomyces pombe*

Fred Kippert

Biological Timing Laboratory, Institute of Cell, Animal and Population Biology, University of Edinburgh, King's Buildings, Edinburgh EH9 3JN, UK (f.kippert@ed.ac.uk)

The molecular bases of circadian clocks are complex and cannot be sufficiently explained by the relatively simple feedback loops, based on transcription and translation, of current models. The existence of additional oscillators has been demonstrated experimentally, but their mechanism(s) have so far resisted elucidation and any universally conserved clock components have yet to be identified. The fission yeast, *Schizosaccharomyces pombe*, as a simple and well-characterized eukaryote, is a useful model organism in the investigation of many aspects of cell regulation. In fast-growing cells of the yeast an ultradian clock operates, which can serve as a model system to analyse clock complexity. This clock shares strict period homeostasis and efficient entrainment with circadian clocks but, because of its short period of 30 min, mechanisms other than a transcription/translation-based feedback loop must be working. An initial systematic screen involving over 200 deletion mutants has shown that major cellular signalling pathways (calcium/phosphoinositide, mitogen-activated protein kinase and cAMP/protein kinase A) are crucial for the normal functioning of this ultradian clock. A comparative examination of the role of cellular signalling pathways in the *S. pombe* ultradian clock and in the circadian timekeeping of different eukaryotes may indicate common principles in biological timing processes that are universally conserved amongst eukaryotes.

Keywords: *Schizosaccharomyces pombe*; ultradian clock; clock mutant; cellular signalling

1. INTRODUCTION

It is only a couple of years ago that, to many working in the field, the molecular basis of circadian clocks seemed largely solved. From cyanobacteria to mammals, a rather simple feedback loop, in which cycling proteins regulate the synthesis of their own messenger RNAs (mRNAs), appeared to be the molecular mechanism generating circadian rhythms (Dunlap 1999). However, it has since become obvious that there is much more to the orthodox models of 'interlocked positive and negative transcriptional/translational feedback loops' cannot explain (summarized by Lakin-Thomas 2000). As many presentations at the Complex Clocks Conference and in this issue of *Philosophical Transactions* show, the simplistic view is being superseded by an understanding that the mechanisms of circadian timekeeping are much more complex. Since only a small number of 'clock genes' have been identified to date, a higher complexity means that there must be (many) more components of the circadian system that are still waiting to be discovered.

Do we at least have some indications as to what other genes may be required for circadian timing? Substantial evidence points to a role for cellular signalling pathways in several aspects of circadian regulation, from simple

unicellular eukaryotes (Edmunds *et al.* 1992) to mammals (Gillette & Tischkau 1999). Their involvement upstream of the circadian oscillator in the transduction of environmental signals has been well demonstrated. There are also numerous reports of circadian rhythms in the concentrations of various second messengers, supporting a role for signalling pathways downstream of the oscillator, potentially bringing a plethora of cellular processes under clock control. The evidence for a direct involvement of cellular signalling pathways in the core oscillator(s) of the circadian system, however, is less persuasive. One reason may be the problems encountered in experimentally addressing this issue. Because these signalling pathways perform functions in other fundamental processes, such as growth and development, homozygous mutants may have severe defects or even be inviable. An example is the *Drosophila* 'clock gene' *double-time*, that encodes a subunit of casein kinase I. Besides its role in the circadian oscillator (Price *et al.* 1998), it is required for cell survival, proliferation and growth arrest in imaginal discs (Zilian *et al.* 1999). This makes it impossible to study the circadian clock in null mutants. Even if a signalling component is not strictly essential, its loss may result in reduced rates of growth or development.

In this and other respects, the ultradian clock of the fission yeast, *Schizosaccharomyces pombe*, is a more tractable system for addressing the role of cellular signalling pathways in a biological clock mechanism. The number of

Dedicated to Professor David Saunders on the occasion of his retirement.

genes that are essential is comparatively low and in many cases even deletion mutants for these genes can be made to grow using extragenic suppressor mutations, in the presence of which the essential gene becomes dispensable. Along with the ease of molecular genetic manipulations, this means that signalling pathways can be studied with high efficiency in this yeast. Below, I review the results obtained so far in a systematic investigation of the involvement of signalling pathways in the *S. pombe* ultradian clock, and put them into the context of our current knowledge of the relationship between signalling pathways and the circadian clock in different eukaryotes. This is not intended to be comprehensive but rather constitutes a selection of examples to illustrate the extent to which cellular signalling has been implicated in circadian clock function.

2. CIRCADIAN CLOCK COMPLEXITY: PROBLEMS AND QUESTIONS

The validity of the current transcriptional/translational feedback models for the circadian clock is being increasingly challenged (Lakin-Thomas 2000; Roenneberg & Merrow 2001). Numerous experimental findings are not compatible with the simplified view represented by these models. Most importantly, because of the residual rhythmicity seen in null mutants for the now classical 'clock genes' *frequency* in the fungus *Neurospora crassa* (Bell-Pedersen *et al.* 2001) and *period* in the fruitfly *Drosophila melanogaster* (Helfrich-Förster 2001), at least one additional oscillator has to be postulated for these organisms. Indeed, even for a unicellular organism, the dinoflagellate *Gonyaulax polyedra*, two circadian oscillators have been demonstrated that control different processes and respond differently to environmental signals (Roenneberg & Rehman 1998). The molecular basis of the postulated additional oscillator(s) has so far remained elusive, which is amazing in the light of the intense genetic and molecular biological investigation of circadian clocks.

There are other observations that challenge the transcriptional/translational feedback loop models and it should suffice here to mention only one particularly striking example. In the giant alga, *Acetabularia mediterranea*, circadian rhythms persist for many days after experimental enucleation, demonstrating that nuclear transcription cannot be a core component of the circadian oscillator in this species (Woolum 1991).

Of concern is also the unresolved issue of the evolutionary origin of circadian clocks. Generally, universal properties of the eukaryotic cell have a monophyletic origin, having evolved once in an ancestral primitive eukaryote. It therefore seems puzzling that none of the components of the circadian machinery so far identified in various eukaryotes has homologues in all other eukaryotes. Some 'clock genes' such as *period*, *clock* and *cycle* have been identified in insects and vertebrates and seem to be conserved between them (Rosato & Kyriacou 2001), but there is no relationship to the known 'clock genes' in the fungus *Neurospora* (Bell-Pedersen *et al.* 2001) or the plant *Arabidopsis* (McWatters *et al.* 2001), which in turn bear no sequence similarity to each other or to those of the metazoa. There are three possible causes for the apparent lack of conservation in clock components: (i) the last

common ancestor did not have a circadian clock and these arose several times in independent branches by (convergent) evolution; (ii) the common ancestor did have a clock but this was lost when new clocks evolved along the same basic principles but using different components; and (iii) there are clock components conserved amongst all eukaryotes which have yet to be identified. Notably, in the genome of *S. pombe* there are no homologues of any of the *N. crassa* 'clock genes'. It appears most unlikely that these two ascomycetes should have evolved completely different circadian timekeeping mechanisms, and since an 'additional' oscillator operates in *N. crassa* (Bell-Pedersen *et al.* 2001), we may well find the components of this elusive oscillator to be conserved between the two ascomycetes and possibly beyond.

3. THE *S. POMBE* ULTRADIAN CLOCK: A UNIQUE MODEL SYSTEM

In fast-growing micro-organisms, ultradian clocks take control over many cellular functions, which are controlled by the circadian clock in slow-growing or non-growing cells (for review, see Kippert 1997a). Because these ultradian clocks share important properties with their circadian counterparts, similar molecular mechanisms may underlie both clocks. The ultradian clock of *S. pombe* is well characterized (for review, see Kippert & Hunt 2000). The major findings of this research are summarized in figure 1 and in this section I shall briefly review those features that make it ideally placed for the identification of oscillator mechanisms, in particular those that rely on components of cellular signalling pathways.

Temperature compensation is just one aspect of a general period homeostasis required for a functional circadian clock (Roenneberg & Merrow 1999). A general homeostasis has been demonstrated for *Neurospora*: different carbon and nitrogen sources (Sargent & Kaltenborn 1972) and a wide range of pH values (Ruoff *et al.* 2000), although having a great impact on growth rate, only slightly affected the free-running period. With the ultradian clock of *S. pombe* it has been possible to investigate period homeostasis over an even wider range of conditions. The period was found to be well temperature compensated, and was not affected by different carbon, nitrogen and sulphur sources, different pH values or different aeration conditions (Kippert 2001a). Particularly impressive is the 'metabolic compensation' when different carbon sources are metabolized by different pathways, i.e. via respiration or fermentation, which lead to distinct patterns of metabolism and gene expression. Figure 2a,b shows the ultradian rhythms of cultures growing on glucose (a) or glycerol (b) as the carbon source.

In contrast to the period homeostasis under steady-state conditions, the phase is easily affected by rapid changes in the environment. Synchronization to external signals is very effective, provided these are periodic (Kippert 2001a). Temperature cycles of only 2 °C difference result in rapid and efficient synchronization, demonstrating that the oscillator is very sensitive to changes in temperature. Other synchronizing treatments include cyclic transfer between media of high and low pH, of high and low osmolarity, and containing high and low concentrations of calcium. Synchronization through intercellular

Figure 1. Schematic representation of the ultradian clock of *S. pombe* (modified from Kippert & Hunt 2000). Individual cells can be synchronized by a variety of treatments provided they are periodic (e.g. temperature cycles with as little as 2 difference). Clock control has been observed for many basic cellular functions such as the cell cycle and metabolism. The period displays a strict homeostasis and is not affected by steady-state growth in different environments (temperature, medium type, carbon and nitrogen source). Mutants in many components of the major cellular signalling pathways have altered periods or are arrhythmic. For details see text (§ 3,4).

communication between the cells has been shown by mixing synchronous and asynchronous cultures (Kippert 2001a). This cellular crosstalk may be a physiologically relevant synchronizer within yeast colonies. The wide range of effective synchronization treatments indicates that the activation of several signalling pathways may be involved in transducing the respective signals.

Whereas transcriptional/translational feedback loops are undoubtedly important elements in circadian time-keeping, they are unlikely to have any role in the ultradian clock of *S. pombe*. Although such a mechanism is already most unlikely to account for the wild-type period of 30 min, it appears absolutely inconceivable for mutant periods as short as 14 min. Therefore, when looking for alternative mechanisms, the use of this ultradian model is particularly valuable. Moreover, *S. pombe* has, when grown at high rates under standard laboratory conditions, the remarkable ability to dispense with many of the components of cellular signalling pathways that are essential in higher organisms.

With the genome sequenced and deletion mutants readily available from the community or easily constructed, the haploid *S. pombe* is well suited to a systematic, functional-genomics-type screen for clock mutants. So far, more than 200 mutant strains of *S. pombe* have been examined. In the majority of cases, in which the gene was not essential, these were deletion mutants. Essential genes were included in this screen, as reduced function or gain-of-function mutants, only in cases of special interest, e.g. when the analysis of deletion mutants had already

indicated that a particular pathway was required for normal clock function. The screen has already identified a remarkable number of mutants that show aberrant period length (outside the wild-type range of 28–35 min) or arrhythmicity (Kippert 2001c). Fifty-four strains had shorter or longer periods, ranging from 14 to 84 min, and a further 29 strains were arrhythmic. This high proportion of ‘clock mutants’ is of course related to the fact that mutants in ‘candidate genes’ were preferentially selected for this screen, but it still contrasts remarkably with the small number of genes identified in previous screens for circadian clock mutants. The majority of the *S. pombe* ‘clock mutants’ involved genes that encode components of major signalling pathways, and these are discussed in the remainder of this review.

The screen also included a selection of other house-keeping genes and it is important to note that none of the mutants in biosynthetic or metabolic pathways showed an aberrant clock phenotype. For example, even a mutant that was completely devoid of mitochondrial DNA displayed normal period length and temperature compensation (figure 2c,d). Notably, this mutant also has normal circadian rhythms (F. Kippert, unpublished data) when entrained by temperature cycles (mitochondrial cytochrome *b* is required for photosynchronization (Kippert *et al.* 1991)). This lack of effect contrasts with the suggested role for metabolism in the circadian system of *N. crassa* (Roenneberg & Merrow 1999; Morgan *et al.* 2001). In this fungus, several mutants with defects in metabolic or biosynthetic pathways showed changes in the

Figure 2. Ultradian rhythmicity in *S. pombe*. The standard parameter is the septation index that indicates the percentage of dividing cells. (a, b) Cultures of the wild-type were grown in 2% glucose (a) or 5% glycerol (b), synchronized by temperature cycles (15 min at 27 °C and 15 min at 33 °C) and then released to constant 30 °C. The lower mean septation index for the glycerol-grown culture reflects the lower growth rate on this carbon source (generation time 6.4 h versus 2.7 h for the glucose-grown culture in this experiment). (c, d) Following the standard synchronization treatment, a mutant strain devoid of mitochondrial DNA was shifted to constant 25 °C (c) or 35 °C (d).

circadian period. However, since these changes were only minor, it may simply be that the ultradian and circadian clocks of *S. pombe* are shielded from metabolic variations to an even higher degree. Whatever the cause for the differences, the results obtained with *S. pombe* do not support any universal role for metabolism in biological clocks.

4. CELLULAR SIGNALLING AND CLOCK FUNCTION

In this section, I shall take major signalling pathways and briefly point to what is known about their involvement in the circadian system of various eukaryotes, and relate this to recent findings on the ultradian clock of *S. pombe*. I shall distinguish between input pathways (in which environmental signals are transmitted to a central oscillator), the oscillator itself and output pathways (by which cellular functions are brought under control of this oscillator). However, it should be noted that one major realization of recent years is that this conceptual dissection of the circadian system, although very useful, has been an oversimplification. It is now clear that components of an input pathway may be regulated by the oscillator and that an output can feed back onto the oscillator (Roenneberg & Merrow 2001). With the signalling pathways to be discussed here, the story may be even more complicated because it is quite possible that, as well as acting on the input, on the output and in the oscillator itself, they may also be involved in the regulation of input pathways by the oscillator and in feedback from the output back to the oscillator. It is therefore questionable whether it will be possible to assign a component to a unique category. For the present discussion, I shall remain with the simplified input–oscillator–output

scheme merely as an organizing principle, identifying input components as acting *upstream* of the oscillator and output components as those acting *downstream*.

(a) *cAMP/protein kinase A (PKA) and mitogen-activated protein (MAP) kinase signalling*

cAMP is the classic second messenger, involved in a plethora of signalling and regulatory processes. Not surprisingly, there is a substantial number of reports implicating cAMP and PKA signalling in various aspects of the circadian system. For example, this pathway has been shown to be involved in transmitting environmental signals to the suprachiasmatic nuclei (SCN)—the mammalian master clock (Tischkau *et al.* 2000)—and also transduces non-neuronal signals in peripheral tissues (Balsalobre *et al.* 2000). Circadian rhythms in cAMP have been observed in different experimental systems including the flagellate *Euglena gracilis* (Carré *et al.* 1989), heads of *Drosophila* (Levine *et al.* 1994) and the mammalian SCN (Prosser & Gillette 1991), and it is difficult to envisage how these cAMP oscillations could not take part in regulating some output from the clock. In *Drosophila*, mutations in the regulatory (Park *et al.* 2000) and catalytic (Majercak *et al.* 1997) subunits of PKA lead to a loss of locomotor rhythmicity under constant conditions. Because the mRNA for the ‘clock gene’ period cycles normally in these flies, the defect appears to be downstream of the oscillator. A role for cAMP/PKA signalling in the oscillator itself may be indicated by the observation that mutations in the *dunce* gene, coding for a cAMP-specific phosphodiesterase, resulted in period shortening (Levine *et al.* 1994). These flies also exhibited changes in their phase-resetting response to light pulses, indicating an involvement in the light-input pathway too. Thus, in

Figure 3. Ultradian rhythm in cytoplasmic free calcium in *S. pombe*. Light emission (in relative light units, RLU) was measured in a Berthold microplate luminometer from a strain expressing the photoprotein aequorin under control of the strong constitutive *nmt1* promoter. The culture was synchronized and maintained as in figure 2.

Drosophila cAMP/PKA signalling appears to have multiple roles: upstream and downstream of the oscillator and potentially within it.

In *S. pombe*, the four enzymes making up the core of this signalling pathway—adenylate cyclase, cAMP-specific phosphodiesterase, regulatory and catalytic subunits of PKA—are each encoded by only a single gene. Remarkably, deletion mutants for any of the four genes are viable (although somewhat impaired in several cellular functions). Regarding adenylyl cyclase, this means that *S. pombe* is viable without a trace of cAMP, which may be unique amongst eukaryotes. However, the deletion mutant does not remain viable in stationary phase for any extended period of time and frequently stops growing in less favourable conditions, which means that circadian clock experiments cannot be performed with it. With regard to the ultradian clock, the deletion mutants of all four key enzymes are arrhythmic (Kippert 2001c). Arrhythmicity could arise anywhere, from defects in input, output or oscillator (and, of course, at multiple points). Notably, experiments with a range of agonists and antagonists of cAMP/PKA signalling have all produced effects on the period, indicating that this pathway may be important (also) in the oscillator itself (Kippert 2001c; F. Kippert, unpublished data).

Interest in MAP kinase cascades has arisen in the circadian field only recently but there is already evidence for a role in both input and output pathways of the vertebrate circadian system. Circadian rhythms in the phosphorylation status, and thus activity, of a MAP kinase, and a time-of-day-dependent effect of light on phosphorylation was found for the mammalian SCN (Obrietan *et al.* 1998) and the chick pineal gland (Sanada *et al.* 2000). Like cAMP/PKA signalling, a MAP kinase pathway is also involved in transducing signals to the circadian oscillators in peripheral tissues (Akashi & Nishida 2000). Of particular interest are the interactions between these two pathways, as discussed below.

Regarding MAP kinase signalling, *S. pombe* is again a very simple organism as it has only three MAP kinase cascades. The most intensively studied of these, the *sty1*⁺ MAP kinase cascade, is important in the cell cycle and in stress responses (Wilkinson & Millar 2000) but is not

essential. It also has a crucial role in the ultradian clock; mutants in most of its components showed aberrant clock phenotypes (Kippert 2001c), with deletions in any of seven components resulting in complete arrhythmicity. A particularly interesting case is given by the two histidine kinases, which act in a histidine kinase–phosphorelay system at the top of the *sty1*⁺ cascade (Buck *et al.* 2001). The deletion of one resulted in period shortening, whereas the deletion of the other resulted in period lengthening; the simultaneous deletion of both caused arrhythmicity.

These two enzymes belong to the small number of *S. pombe* proteins that have PAS domains (a protein interaction module named after the three proteins in which it was first identified, i.e. *period*, *aryl hydrocarbon receptor* and *single-minded* (for a review, see Gu *et al.* (2000)). Much has been made of the occurrence of PAS domains in diverse ‘clock proteins’. So far, clock components that share PAS domains, but not any further sequence similarity, have been found in animals (Rosato & Kyriacou 2001), plants (McWatters *et al.* 2001) and fungi (Bell-Pedersen *et al.* 2001), indicating that PAS domains may be a central feature of the oscillator mechanism. However, it should be considered that all proteins with PAS domains appear to be involved in the reception and/or transduction of signals: whereas in unicellular organisms, fungi and plants, the signals appear to come from the environment, in animals many of the signals arise from within the organism (Gu *et al.* 2000). Because biological clocks receive continuous input from their environment, it is not at all surprising that so many PAS domain proteins are found to affect circadian clocks. Instead, regarding their general signalling role, one might be more surprised to come across a PAS protein that is *not* involved with the clock in some way. Indeed, as far as the *S. pombe* ultradian clock is concerned, all of the PAS-domain-containing proteins investigated so far are required for its normal functioning (Kippert 2001c).

Individual signalling pathways do not normally act on their own but are part of a complex network of interactions. For example, in the SCN (Gillette & Tischkau 1999; Golombek *et al.* 2000), as well as in peripheral tissues (Akashi & Nishida 2000; Balsalobre *et al.* 2000), multiple

signal-transduction pathways are interacting in the entrainment to external signals. A particularly tight interaction is seen between the cAMP/PKA and MAP kinase pathways. In many instances, they converge onto the cAMP-response-element binding protein (CREB), input signalling to the circadian oscillator in vertebrates being just one example (De Cesare & Sassone-Corsi 2000; Tischkau *et al.* 2000). Downstream of the oscillator, CREB appears to have a major role in the circadian regulation of gene expression: many of the genes expressed with a circadian rhythm have CREs in their promoters to which the CREB transcription factor binds (Obrietan *et al.* 1999), and CREs can confer circadian transcription of reporter genes (Belvin *et al.* 1999; Obrietan *et al.* 1999). From *Drosophila*, there is an indication for an involvement of CREB in the oscillator itself: a loss-of-function mutant of dCREB2, a *Drosophila* homologue, exhibited a shortened period of the locomotor rhythm (Belvin *et al.* 1999).

Interactions between cAMP/PKA and MAP kinase signalling are also widely observed in lower eukaryotes. *S. pombe* is no exception and fundamental processes, such as the cell cycle (Wilkinson & Millar 2000) and gene expression in response to environmental signals (Neely & Hoffman 2000; Bonnet *et al.* 2000), are under the integrated, and mostly antagonistic, control of both pathways. The ultradian clock exerts control over the cell cycle (Kippert 2001b) and energy metabolism (Kippert & Read 2001), and it seems likely that much of this control involves the cAMP/PKA and *styI*⁺ MAP kinase pathways. In this context, it should be noted that several genes that are under the integrated control of the two pathways are transcribed with a circadian rhythm in *S. pombe* (F. Kippert, unpublished data). Deletion of *atfI*⁺, a member of the CREB transcription factor family acting downstream of the *styI*⁺ MAP kinase, results in arrhythmicity with respect to both the ultradian (Kippert 2001c) and the circadian clock (F. Kippert, unpublished data). This indicates that a role in the output exerted by the cAMP/PKA and MAP kinase pathways may be conserved at least between fungi and animals.

(b) *Calcium and phosphoinositide signalling*

Calcium is such a versatile and universal regulator of cellular functions (for review, see Berridge *et al.* 2000) that it is hardly imaginable that it should not be involved, at some level, in the circadian system. The cytoplasmic concentration of calcium rises in synaptic transmission, by which the circadian pacemaker neurons receive signals (Gillette & Tischkau 1999), as well as in response to a wide variety of environmental stimuli, including both blue and red light, in plants (Trewavas & Malho 1998). It therefore seems reasonable to assume a role for calcium signalling upstream of the circadian oscillator. Circadian rhythms in cytoplasmic free calcium have been observed in plants (Johnson *et al.* 1995) and in SCN neurons (Colwell 2000). As with the circadian oscillations in cAMP, it seems unlikely that these are not part of some output pathway.

Periodically shifting *S. pombe* cultures between media with low and high concentrations of calcium synchronizes the ultradian clock, indicating that calcium can have an input on the oscillator (Kippert 2001a). Using a strain expressing the photoprotein aequorin as an indicator of

cytoplasmic free calcium, it has been demonstrated that the intracellular calcium concentration follows an ultradian rhythm (figure 3). There is also a circadian rhythm in cytoplasmic free calcium in stationary-phase cells (F. Kippert, unpublished data), indicating that in both fast-growing and non-growing cells these calcium oscillations may contribute to the rhythmic regulation of a wide variety of different functions. Because such rhythms in cytoplasmic calcium have been found in fungal, plant and animal species, they may represent a general pattern and possibly a conserved component of the circadian system.

Regarding the ultradian clock of *S. pombe*, arrhythmicity has been found for several deletion mutants of proteins involved in calcium signalling and homeostasis (Kippert 2001c) but, more significantly, altered periods indicative of an affected oscillator have been observed. The most striking case is that of calmodulin mutants. *S. pombe* has only a single calmodulin gene and, although a deletion mutant is not viable, it is possible to eliminate calcium binding to one or more of the four calcium-binding domains (Moser *et al.* 1995). Mutants defective in one of the two N-terminal calcium-binding domains showed period lengthening, whereas those defective in one of the two C-terminal domains showed period shortening (Kippert 2001c). Their respective effects on the period were augmented when both calcium-binding domains of the N- or C-terminal halves of the protein were eliminated. A mutant with a defect in subcellular calcium distribution showed highly erratic rhythms, a phenotype observed only in this particular mutant (F. Kippert, unpublished data). Taken together, these results strongly suggest that calcium signalling is crucially involved in the ultradian oscillator of *S. pombe*.

Although phosphoinositide (PI) signalling is in many circumstances intricately linked with calcium signalling, it has not attracted much specific attention in circadian research. However, an important role may be inferred from the effect of lithium on the period of biological clocks, which is now thought to be brought about by interference with PI signalling. Of the many pharmacological agents that have been tested for their effects upon circadian clocks, lithium is the only one that has produced a consistent period-lengthening effect in essentially all cases studied (Klemfuss 1992). The lengthening is by up to 20%, as found for the isolated eye of the mollusc *Bulla gouldiana* (Khalsa *et al.* 1993).

Lithium, at subtoxic concentrations, also lengthens the period of the ultradian clock of *S. pombe* (figure 4a,b). The effect is concentration dependent with a lengthening of up to 50%, the largest effect found for any biological clock so far (Kippert & Diebold 2001). The ultradian clock of *S. pombe* is therefore a good experimental system with which to investigate the underlying molecular basis. Molecular genetic and physiological analysis of lithium effects, mainly on viability and development, in a number of organisms has indicated three different potential targets of lithium (Williams & Harwood 2000), including the enzyme inositol monophosphatase, which is required for the maintenance of PI signalling. This enzyme has been identified as the target in lithium's effect on the period of the *S. pombe* ultradian clock: the regulated over-expression of a human inositol monophosphatase gene, in

Figure 4. Effect of interference with PI signalling on the ultradian clock of *S. pombe*. (a) Wild-type strain; (b) wild-type strain in the presence of 20 mM lithium chloride; (c) wild-type strain in the presence of 1 mM neomycin; (d) strain expressing profilin under control of the strong constitutive *nmt1* promoter. The cultures were synchronized and maintained as in figure 2.

a dose-dependent manner, reversed period lengthening (Kippert 1997b; F. Kippert, unpublished data).

The inhibition of inositol monophosphatase can lead to a reduction in the cellular concentration of inositol, and the 'inositol-depletion hypothesis' (Berridge *et al.* 1989) is now a widely accepted explanation for many of lithium's effects. One consequence is a reduction also of inositol-containing phospholipids, in particular phosphatidylinositol 4,5-bisphosphate (PIP_2), which is the main substrate of phospholipase C (PLC). Interference with PLC might therefore result in an effect similar to that of lithium. Neomycin, although not specific, has been widely used in the study of PI signalling because it binds to PIP_2 and

thereby interferes with the access of PLC to its substrate. Indeed, neomycin also lengthens the period of the ultradian clock (figure 4c). A similar way to interfere with PLC is to overexpress the structural protein profilin, which also binds to PIP_2 and, predictably, it too lengthens the period of the ultradian clock (figure 4d). And not surprisingly, a deletion mutant for the single PLC gene of *S. pombe* is rendered completely arrhythmic (Kippert 2001c). Considering the universality of the lithium effect, it seems most likely that PI signalling has a conserved role in the circadian system of all eukaryotes.

5. CONCLUSIONS AND PROSPECTS

The systematic screen for mutants with effects on the ultradian clock of *S. pombe* has clearly demonstrated that the major cellular signalling pathways have a crucial role in this timekeeping mechanism. Considering the involvement of these pathways in the transduction of environmental signals and in the control of many cellular functions, it seems most likely that they act both upstream and downstream of the ultradian oscillator. The apparent arrhythmicity of many deletion mutants could be due to the interruption of a signalling chain on either side of the oscillator. However, there are also a considerable number of mutants that show aberrant periods. Along with the effects on period produced by various agonists and antagonists of these signalling pathways, this suggests a participation of signalling pathways in rhythm generation *per se*. Because (i) a transcription/translation-based feedback mechanism cannot account for the ultradian clock, and (ii) mutants in metabolic pathways have normal rhythms, we may expect to find a complex network of interactions between cellular signalling pathways to be at the heart of the ultradian oscillator.

How is this relevant to the investigation of circadian clock mechanisms? As the examples cited here have shown, there is considerable evidence for a similar involvement of signalling pathways in circadian timekeeping. It is therefore important to move on now from the preconception of 'dedicated clock genes', which has biased most previous screens, and instead look for oscillator components that are involved in other cellular functions at the same time. It is well known that eukaryotes make use of the same repertoire of signalling molecules and pathways for very different regulatory purposes. For example, for all signalling pathways discussed here we find an involvement in long-term potentiation and memory formation (Alberini 1999). It would therefore not be at all surprising if they had a crucial, and potentially conserved, role also in the circadian clock.

To describe in exact detail the role of signalling pathways at different positions within the ultradian timekeeping system of *S. pombe* will be a challenging task. However, it has to be considered that until now, only the role of core components of the signalling pathways has been explored. There are many proteins on the periphery that interact with the core components at various points, and an extension of the systematic screen may therefore result in the identification of components of the signalling network that act exclusively at one position, upstream or downstream of the oscillator or in the oscillator itself. As the results already obtained show, the reverse genetic

approach is most appropriate here and *S. pombe* is an ideal organism for its exploitation. This will ultimately tell us how the signalling pathways fit into the complex working of the ultradian clock of this simple yeast, and provide insights into where and how to look for similar mechanisms in more complex eukaryotes.

I am indebted to Paul Hunt (Institute of Cell, Animal and Population Biology, University of Edinburgh) for critical reading and discussion of the manuscript.

REFERENCES

- Akashi, M. & Nishide, E. 2000 Involvement of the MAP kinase cascade in resetting of the mammalian circadian clock. *Genes Dev.* **14**, 645–649.
- Alberini, C. M. 1999 Genes to remember. *J. Exp. Biol.* **202**, 2887–2891.
- Balsalobre, A., Marcacci, L. & Schibler, U. 2000 Multiple signaling pathways elicit circadian gene expression in cultured Rat-1 fibroblasts. *Curr. Biol.* **19**, 1291–1294.
- Bell-Pedersen, D., Crosthwaite, S. K., Lakin-Thomas, P. L., Merrow, M. & Vinsjevsk, M. 2001 The *Neurospora* circadian clock: simple or complex? *Phil. Trans. R. Soc. Lond. B* **356**, 1697–1709. (DOI 10.1098/rstb.2001.0968.)
- Belvin, M. P., Zhou, H. & Yin, J. C. 1999 The *Drosophila* dCREB2 gene affects the circadian clock. *Neuron* **22**, 777–787.
- Berridge, M. J., Downes, C. P. & Hanley, M. R. 1989 Neural and developmental actions of lithium: a unifying hypothesis. *Cell* **59**, 411–419.
- Berridge, M. J., Lipp, P. & Bootman, M. D. 2000 The versatility and universality of calcium signalling. *Nature Rev. Mol. Cell Biol.* **1**, 11–21.
- Bonnet, C., Perret, E., Dumont, X., Picard, A., Caput, D. & Lenaers, G. 2000 Identification and transcription control of fission yeast genes repressed by an ammonium starvation growth arrest. *Yeast* **16**, 23–33.
- Buck, V., Quinn, J., Soto Pino, T., Martin, H., Saldanha, J., Makino, K., Morgan, B. A. & Millar, J. B. A. 2001 Peroxide sensors for the fission yeast stress-activated mitogen-activated protein kinase pathway. *Mol. Biol. Cell* **12**, 407–419.
- Carré, I. A., Laval-Martin, D. L. & Edmunds Jr, L. N. 1989 Circadian changes in cyclic AMP levels in synchronously dividing and stationary-phase cultures of the achlorophyllous ZC mutant of *Euglena gracilis*. *J. Cell Sci.* **94**, 267–272.
- Colwell, C. S. 2000 Circadian modulation of calcium levels in cells in the suprachiasmatic nucleus. *Eur. J. Neurosci.* **12**, 571–576.
- De Cesare, D. & Sassone-Corsi, P. 2000 Transcriptional regulation by cyclic AMP-responsive factors. *Prog. Nucleic Acid Res. Mol. Biol.* **64**, 343–369.
- Dunlap, J. C. 1999 Molecular bases for circadian clocks. *Cell* **96**, 271–290.
- Edmunds Jr, L. N., Carré, I. A., Tamponnet, C. & Tong, J. 1992 The role of ions and second messengers in circadian clock function. *Chronobiol. Int.* **9**, 180–200.
- Gillette, M. U. & Tischkau, S. A. 1999 Suprachiasmatic nucleus: the brain's circadian clock. *Recent Prog. Horm. Res.* **54**, 33–58.
- Golombek, D. A., Ferreyra, G. A., Katz, M. E., Marpegan, L., Pinto, F. D., Alfonso, T. F. & Yannielli, P. C. 2000 Neurochemistry of mammalian entrainment: signal transduction pathways in the suprachiasmatic nuclei. *Biol. Rhythm Res.* **31**, 56–70.
- Gu, Y. Z., Hogenesch, J. B. & Bradfield, C. A. 2000 The PAS superfamily: sensors of environmental and developmental signals. *A. Rev. Pharmacol.* **40**, 519–561.
- Helfrich-Förster, C. 2001 The activity of *Drosophila melanogaster* is controlled by a dual oscillator system. *J. Insect Physiol.* **47**, 877–887.
- Johnson, C. H., Knight, M. R., Kondo, T., Masson, P., Sedbrook, J., Haley, A. & Trewavas, A. 1995 Circadian oscillations of cytosolic and chloroplastic free calcium in plants. *Science* **269**, 1863–1865.
- Khalsa, S. B., Michel, S. & Block, G. D. 1993 The effects of lithium on a neuronal *in vitro* circadian pacemaker. *Chronobiol. Int.* **10**, 321–330.
- Kippert, F. 1997a Temperature-compensation of ultradian rhythms: a general homeostasis of period length identifies ultradian clocks as timekeeping devices. *Chronobiol. Int.* **14**, 469–479.
- Kippert, F. 1997b Lithium affects the ultradian clock of *Schizosaccharomyces pombe* by inhibition of inositol monophosphatase. *Biochem. Soc. Trans.* **15**, S602.
- Kippert, F. 2001a This is a real clock: superb period homeostasis of ultradian oscillations in *Schizosaccharomyces pombe*. (Submitted.)
- Kippert, F. 2001b Stages of the cell division cycle are under ultradian clock control in fast growing *Schizosaccharomyces pombe*. (Submitted.)
- Kippert, F. 2001c Multiple signalling pathways are central to the operation of the *Schizosaccharomyces pombe* ultradian clock. (Submitted.)
- Kippert, F. & Diebold, S. S. 2001 Lithium effects on viability and the ultradian clock in *Schizosaccharomyces pombe*. (Submitted.)
- Kippert, F. & Hunt, P. 2000 Microbial ultradian clocks: from behavioural observation to functional genomics. *BioEssays* **22**, 16–22.
- Kippert, F. & Read, N. R. 2001 Metabolism is under ultradian clock control in fast growing *Schizosaccharomyces pombe*. (Submitted.)
- Kippert, F., Ninnemann, H. & Engelmann, W. 1991 Photosynchronization of the circadian clock of *Schizosaccharomyces pombe*: mitochondrial cytochrome *b* is an essential component. *Curr. Genet.* **19**, 103–107.
- Klemfuss, H. 1992 Rhythms and the pharmacology of lithium. *Pharmacol. Ther.* **56**, 53–78.
- Lakin-Thomas, P. L. 2000 Circadian rhythms: new functions for old clock genes. *Trends Genet.* **16**, 135–142.
- Lakin-Thomas, P. L., Gooch, V. D. & Ramsdale, M. 2001 Complex rhythms in *Neurospora*. *Phil. Trans. R. Soc. Lond. B* **356**, 1711–1715. (DOI 10.1098/rstb.2001.0966.)
- Levine, J. D., Casey, C. I., Kalderon, D. D. & Jackson, F. R. 1994 Altered circadian pacemaker functions and cyclic AMP rhythms in the *Drosophila* learning mutant *dunce*. *Neuron* **13**, 967–974.
- McWatters, H. G., Roden, L. C. & Staiger, D. 2001 Picking out parallels: plant circadian clocks in context. *Phil. Trans. R. Soc. Lond. B* **356**, 1735–1743. (DOI 10.1098/rstb.2001.0936.)
- Majercak, J., Kalderon, D. & Ederly, I. 1997 *Drosophila melanogaster* deficient in protein kinase A manifests behavior-specific arrhythmia but normal clock function. *Mol. Cell. Biol.* **17**, 5915–5922.
- Morgan, L. W., Feldman, J. F. & Bell-Pedersen, D. 2001 Genetic interactions between clock mutations in *Neurospora crassa*: can they help us to understand complexity? *Phil. Trans. R. Soc. Lond. B* **356**, 1717–1724. (DOI 10.1098/rstb.2001.0967.)
- Moser, M. J., Lee, S. Y., Klevit, R. E. & Davis, T. N. 1995 Ca^{2+} binding to calmodulin and its role in *Schizosaccharomyces pombe* as revealed by mutagenesis and NMR spectroscopy. *J. Biol. Chem.* **270**, 20 643–20 652.
- Neely, L. A. & Hoffman, C. S. 2000 Protein kinase A and mitogen-activated protein kinase pathways antagonistically regulate fission yeast *fbp1* transcription by employing different

- modes of action at two upstream activation sites. *Mol. Cell. Biol.* **20**, 6426–6434.
- Obrietan, K., Impey, S. & Storm, D. R. 1998 Light and circadian rhythmicity regulate MAP kinase activation in the suprachiasmatic nuclei. *Nature Neurosci.* **1**, 693–700.
- Obrietan, K., Impey, S., Smith, D., Athos, J. & Storm, D. R. 1999 Circadian regulation of cAMP response element-mediated gene expression in the suprachiasmatic nuclei. *J. Biol. Chem.* **274**, 17 748–17 756.
- Park, S. K., Sedore, S. A., Cronmiller, C. & Hirsh, J. 2000 Type II cAMP-dependent protein kinase-deficient *Drosophila* are viable but show developmental, circadian, and drug response phenotypes. *J. Biol. Chem.* **275**, 20 588–20 596.
- Price, J. L., Blau, J., Rothenfluh, A., Abodeely, M., Kloss, B. & Young, M. W. 1998 *double-time* is a novel *Drosophila* clock gene that regulates PERIOD protein accumulation. *Cell* **94**, 83–95.
- Prosser, R. A. & Gillette, M. U. 1991 Cyclic changes in cAMP concentration and phosphodiesterase activity in a mammalian circadian clock studied *in vitro*. *Brain Res.* **568**, 185–192.
- Roenneberg, T. & Merrow, M. 1999 Circadian systems and metabolism. *J. Biol. Rhythms* **14**, 449–459.
- Roenneberg, T. & Rehman, J. 1998 Survival in a temporal world—the circadian programme of the marine unicell *Gonyaulax*. In *Microbial responses to light and time* (ed. M. X. Cadick, S. Baumberg, D. A. Hodgson & M. K. Phillips-Jones), pp. 237–250. Cambridge University Press.
- Roenneberg, T. & Merrow, M. 2001 Circadian systems: different levels of complexity. *Phil. Trans. R. Soc. Lond. B* **356**, 1687–1696. (DOI 10.1098/rstb.2001.0969.)
- Rosato, E. & Kyriacou, C. P. 2001 Flies, clocks and evolution. *Phil. Trans. R. Soc. Lond. B* **356**, 1769–1778. (DOI 10.1098/rstb.2001.0961.)
- Ruoff, P., Behzadi, A., Hauglid, M., Vinsjevik, M. & Havas, H. 2000 pH homeostasis of the circadian sporulation rhythm in clock mutants of *Neurospora crassa*. *Chronobiol. Int.* **17**, 733–750.
- Sanada, K., Hayashi, Y., Harada, Y., Okano, T. & Fukada, Y. 2000 Role of circadian activation of mitogen-activated protein kinase in chick pineal clock oscillation. *J. Neurosci.* **20**, 986–991.
- Sargent, M. L. & Kaltenborn, S. H. 1972 Effects of medium composition on circadian conidiation in *Neurospora*. *Plant Physiol.* **50**, 171–175.
- Tischkau, S. A., Gallman, E. A., Buchanan, G. F. & Gillette, M. U. 2000 Differential cAMP gating of glutamatergic signaling regulates long-term state changes in the suprachiasmatic circadian clock. *J. Neurosci.* **20**, 7830–7837.
- Trewavas, A. J. & Malho, R. 1998 Ca²⁺ signalling in plant cells: the big network! *Curr. Opin. Plant Biol.* **1**, 428–433.
- Wilkinson, M. G. & Millar, J. B. A. 2000 Control of the eukaryotic cell cycle by MAP kinase signaling pathways. *FASEB J.* **14**, 2147–2157.
- Williams, R. S. B. & Harwood, A. J. 2000 Lithium therapy and signal transduction. *Trends Pharmacol. Sci.* **21**, 61–64.
- Woolum, J. C. 1991 A re-examination of the role of the nucleus in generating the circadian rhythm in *Acetabularia*. *J. Biol. Rhythms* **6**, 129–136.
- Zilian, O., Frei, E., Burke, R., Brentrup, D., Gutjahr, T., Bryant, P. J. & Noll, M. 1999 *double-time* is identical to *discs overgrown*, which is required for cell survival, proliferation and growth arrest in *Drosophila* imaginal discs. *Development* **126**, 5409–5420.