A circadian clock in *Saccharomyces cerevisiae*

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Circadian timing is a fundamental biological process, underlying cellular physiology in animals, plants, fungi, and cyanobacteria. Circadian clocks organize gene expression, metabolism, and behavior such that they occur at specific times of day. The biological clocks that orchestrate these daily changes confer a survival advantage and dominate daily behavior, for example, waking us in the morning and helping us to sleep at night. The molecular mechanism of circadian clocks has been sketched out in genetic model systems from prokaryotes to humans, revealing a combination of transcriptional and posttranscriptional pathways, but the clock mechanism is far from solved. Although *Saccharomyces cerevisiae* is among the most powerful genetic experimental systems and, as such, could greatly contribute to our understanding of cellular timing, it still remains absent from the repertoire of circadian model organisms. Here, we use continuous cultures of yeast, establishing conditions that reveal characteristic clock properties similar to those described in other species. Our results show that metabolism in yeast shows systematic circadian entrainment, responding to cycle length and zeitgeber (stimulus) strength, and a (heavily damped) free running rhythm. Furthermore, the clock is obvious in a standard, haploid, auxotrophic strain, opening the door for rapid progress into cellular clock mechanisms.

entrainment | oscillation | yeast | temperature cycle

The circadian clock is a cell-based, regulatory network that controls processes from gene expression to behavior. These daily clocks, found in diverse organisms, share a set of signature properties (1). One of these is a free-running, circa 24-h (circadian) oscillation in constant conditions. The phenomenon of self-sustained rhythm, however, has never been the "aim" of evolution. It is per se not a prerequisite for the timing system but rather a consequence of how a daily timing system has developed in an environment that is utterly predictable in its alternation of light and darkness, warmer and colder temperatures, and numerous other qualities (2). Notably, many organisms do not show obvious free-running rhythms. For instance, the ascomycete, *Neurospora crassa*, suppresses daily, rhythmic circadian spore formation when CO_2 accumulates (3). The accidental discovery of a mutant strain that makes "bands" of spores once every 22 h in constant darkness—without exchanging the air to decrease CO_2 levels—permitted development of *Neurospora* as a clock model system (4). Even the banding strain of *Neurospora* appears arrhythmic in constant light, as do many animals. Yet, in the case of *Neurospora*, several transcript levels and the activity of the enzyme nitrate reductase are oscillating with a *circa* 24-h period despite no observable rhythms in spore formation (5, 6). When animals become arrhythmic in constant light, usually a decrease in irradiance will allow rhythmicity to emerge (7). These examples suggest that the expression of a free-running clock very much depends on conditions or that it is not a universal property of circadian clocks. They furthermore suggest that organisms for which circadian rhythms have not been described could still possess them.

In contrast to free-running rhythm, the major "task" of circadian clocks is to facilitate systematic synchronization of the organism with the cyclic environment (zeitgebers) (8). This active process, called entrainment, results in a stable phase relationship between the endogenous clock (the multitude of clock-controlled processes) and the exogenous cycle (environment; additional information on entrainment in *SI Materials*), the multitude of clock-controlled processes and the exogenous (environment) cycle (additional). This entrained phase varies systematically according to conditions such as strength (9) or period (T) (10) of the zeitgeber and the proportion of night and day (e.g., photoperiod or even light pulse), which also allows for seasonal adjustments (11). The process of entrainment remains poorly understood at the level of the cell although it organizes cellular biochemistry and metabolism to distinct temporal compartments. To this end, more genetic model systems that feature tools for cell biology research are needed and among the best candidates for this purpose is *Saccharomyces cerevisiae.*

There is a priori no reason to suspect that *S. cerevisiae* should be exempt from circadian regulation. Although yeast has been a denizen of laboratories for many decades, in nature, it is found in the soil and on many forms of biota and is thus subject to the same evolutionary pressures that have driven the development of circadian clocks in animals, plants, other fungi, and even in the rapidly dividing cyanobacteria (12, 13). The demonstration that circadian clocks confer an adaptive advantage in less than 10–20 generations is compelling (14, 15). It suggests that microbes will rapidly capture any spontaneous mutations that facilitate anticipation of environmental cycles.

In this report, we show circadian regulation in the budding yeast. We approach the problem first via entrainment, showing systematic synchronization to environmental cycles according to established principles that have been demonstrated in fungi, plants, and animals (16, 17). Using conditions and methods derived from entrainment experiments, we investigate free running rhythms, both at the physiological and molecular levels. For these experiments, we developed a fermentor culture system that maintains cells in a nutritionally stable environment for weeks to months. Short (so-called ultradian) rhythms in metabolism and gene expression have been reported in similar cultures (18–21) when minimal medium is used and when pH levels are strictly controlled. However, when searching for a circadian regulation, it seems disadvantageous to clamp pH because it can serve as a read-out of daily metabolic fluctuations (22). We, therefore, let the culture freely establish its own pH levels. Furthermore, we used a rich, complex culture medium that would support a higher level of metabolism than minimal medium.

Results and Discussion

Fermentor cultures were subjected to temperature cycles with a period of 24 h, to generate a rhythmic environment (11 h at 21 °C and 11 h at 28 °C—unless otherwise specified—with 1-h transitions between temperatures; Fig. 1*A*). Dissolved oxygen (dO_2) in

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the media fluctuated with a period of 24 h, presumably reflecting daily alterations in metabolic rate. Under these conditions, ultradian oscillations were absent. Similar to dO_2, daily rhythms in pH were also observed. The incoming media (pH 6.3) was "conditioned" by the cells to a mean level of ≈pH 5. The pH oscillated around this set point, in synchrony with the temperature cycle and corresponding to fluctuations of roughly 10^7 H^+/day/cell.

The oscillations in dO_2 and protons could simply represent *passive*, temperature-dependent changes in metabolic rates (called masking) (23). Alternatively, the temperature cycle could entrain a circadian system that *actively* regulates the timing of the observed oscillations. Both mechanisms have been reported for biological clocks in response to environmental cycles, and established protocols exist that can distinguish between these two (16). Furthermore, zeitgebers can induce a mixture of masking and entrainment, evident in the responses of many organisms to daily light:dark cycles. In *Drosophila*, a shock response is observed at light transitions, yet these are preceded by a gradual increase in activity that is controlled by the circadian clock (24). The activity in mice is acutely suppressed at light onset, whereas their activity would have continued in dim light or sustained darkness (25). Masking has even been noted at the molecular level, with RNA from the clock gene *frequency* being rapidly induced at all times when lights come on although protein is produced selectively depending on the elapsed time from midnight (11). By simply changing the structure of the zeitgeber cycle, synchronization by circadian (active) versus masking (passive) processes can be discerned (16).

If the oscillations in the fermentors were passive responses, the phase relationship between the external (temperature) and the internal (metabolic) rhythms should be independent of conditions (e.g., of the zeitgeber's period). If the oscillations were actively produced by an entrained timing system, then these phase relationships should change systematically. And finally, if they were a product of both mechanisms, the waveform of the oscillations should change in addition to changing its phase angle. We, therefore, subjected cultures to shorter temperature cycles (T = 23.7 and 23.5 h), which should delay their phase in relationship to this slightly shorter temperature cycle (10, 17). Consistent with the predictions for a circadian timing mechanism that actively entrains—and inconsistent with a passive response—we observed that the pH oscillation in the yeast cultures showed delayed phases relative to the 24-h temperature cycle (Fig. 1*B*). The delays were as much as 4–5 h, with a more or less preserved wave form: the delay was similar for the peaks, the troughs, as well as the halfway transitions between peaks and troughs. This is similar to observations in circadian systems where essentially opposite entrained phases can be achieved by changing the cycle length (17, 26) and contrasts synchronization in noncircadian cycles (Fig. S1).

There were, however, subtle changes in the curves' shapes between the different cycles, indicating that *passive* responses to the temperature changes also occur. We therefore used additional protocols to distinguish active versus passive mechanisms of synchronization. Circadian entrainment should also result in changed phase relationships when the zeitgeber strength is altered (9, 16). We changed zeitgeber strength by simply shifting the temperature so that it cycled between 18 °C and 25 °C. The structure of the cycle remained the same, with 11 h at 25 °C, a 1-hour transition to the lower temperature, 11 h at 18 °C, and then a 1-hour transition back to 25 °C. The phase angle of the pH rhythm was delayed by 6 h relative to the 21/28 °C cycles, moving the pH peak from the cold to the warm phase (Fig. 2*A*). Again, these changes are more consistent with *active* entrainment than with *passive* responses. A high-amplitude cycle from 15 to 30 °C (Fig. S2), approximating what is experienced in nature, yielded a completely different wave form relative to Figs. 1 and 2. The stronger zeitgeber drove a steep increase in protons in the media with the onset of the warm phase of the incubation, followed by a relaxation back to lower levels. This appears to be more passive in its characteristics than the other entraining protocols.

Although the dO_2 and pH rhythms shift their peaks in the same direction when the zeitgeber strength changes (Fig. 2*B*), their respective responses to the altered zeitgeber conditions are clearly different. Whereas the dO_2 rhythm shows a predominantly passive response with a strong increase at the transition to the warmer temperature and a drastic change in waveform, the pH rhythm shows the typical properties of an output of an entrained

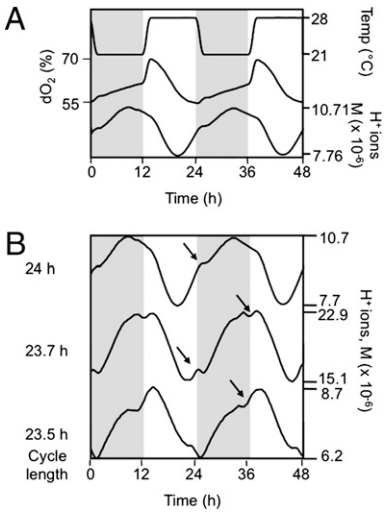

Fig. 1. Temperature cycles induce oscillations in dO_2 and protons in media. Gray panels represent cool temperature; open panels represent warm temperature. (*A*) The experimental protocols used temperature cycles, shown here from 21 °C to 28 °C (*Top*), which support oscillations in dissolved O_2 (*Middle*) and pH (*Bottom*). Note that here and in the subsequent figures, the pH is converted to proton concentration. (*B*) In sub-24-h T cycles, the oscillations in H^+ concentrations occur later within the temperature cycle. The top line shows the H^+ levels in a 24-h temperature cycle and the bottom line shows the oscillation in a 23.5-h cycle. The *Middle* tracing is a 23.7-h cycle. Arrows indicate where the shape of the curve changes, indicating *passive* changes in the oscillation because of the zeitgeber transition (see text).

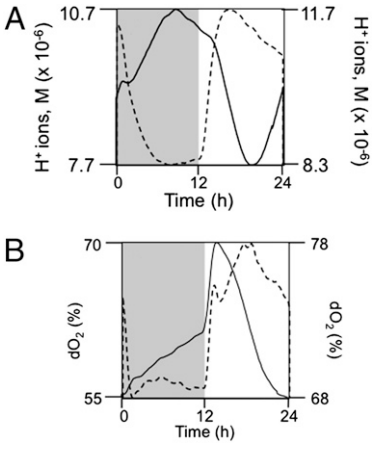

Fig. 2. Phase relationships change with zeitgeber strength. (*A*) In lower temperature cycles (18 °C to 25 °C; dashed line), the peak of the H^+ oscillation moves into the warm phase, later than the peak in warmer cycles (21 °C to 28 °C; solid line). (*B*) In the same cultures, the relationship between the peaks of the dO_2 oscillations and the temperature cycle is largely preserved (solid and dashed lines as for *A*).

clock, shifting its entire waveform in response to zeitgeber strength with little change in shape.

Systematic circadian entrainment (different phase relationships in different *T* cycles and zeitgeber strengths with a preservation of waveform) are qualities of both robust, self-sustained, free-running rhythms and of weak oscillators that rapidly damp in constant conditions (17). To investigate whether the yeast timing system is a weak or a robust circadian oscillator, we released cultures after temperature entrainment to constant temperature. The oscillation in proton concentrations damped in under two cycles (Fig. 3; see also Fig. S3). The phenomenon of damping has been noted previously in, among others, plants (27, 28) and in cell culture using mouse and rat fibroblasts and organ explants (29–32). Some organisms even dispense of circadian rhythms if cycling environmental conditions recede, such as in an arctic summer or winter (33). The yeast timing system shows canonical properties of a circadian clock controlled by a weak, damped oscillator (at least under the culture conditions applied here).

The general explanation for damping under constant conditions is either loss of sustained rhythms at the level of the individual cell or desynchronization of a population of individual sustained cellular oscillators via small, stochastic changes in period. In the latter case, a change in the average free-running period is not anticipated. Here, the period lengthens as it is damping, suggesting that the former scenario is in play, namely that the timing system itself is impacted. A hallmark of the fermentor culture system—indeed, our goal in using it—is achieving a stable state for weeks or even months at a time with respect to the cell number, nutrition, etc. The same state is revisited from 1 day to the next. However, on release to the free run, the yeast culture is no longer stable as evidenced by increasing cell number, decreasing pH of the media, and increasing amplitude of the oscillation before it damps to nonrhythmicity. There may also be trivial reasons for lack of self-sustained rhythms in yeast, namely that we are following the wrong clock outputs. In *Neurospora*, several mRNA transcripts oscillate in the absence of any obvious circadian rhythm (5). Furthermore, these transcripts can fail to entrain when the frequency of the circadian rhythm becomes long, as in the case of the mutant *frq*^{7}, with a circa 29-h free-running period. This example is akin to a biological *T* cycle, with one oscillatory system running at 29 h and another at circa 24 h. Each is outside the other's range of entrainment.

Circadian clocks are controlled by a transcriptional–translational feedback loop, posttranscriptional processes, or a mixture of the two (34–36). *S. cerevisiae* has no clear orthologs of the transcription factors (clock genes) that mediate circadian regulation in fungi or animals, so we targeted likely circadianly regulated output pathways. Trafficking of ions in and out of cells is well understood in yeast, thus we have used this physiology to identify clock-controlled gene expression, a first step to understanding clock mechanisms in yeast. We focused on those genetic components involved in pH regulation, with consideration to the media used in our experiments, YPD. It supplies a rich source of amino acids but—in this form—nitrogen is expensive to metabolize. In comparison, ammonium is efficiently shuttled into the glutamine synthesis pathway, a gateway for production of multiple amino acids. Furthermore, yeasts have been shown to secrete ammonium during colony development on a time scale of day(s) as a means of intercellular communication (37). MEP2p and GAP1p are ammonium and amino acid permeases, respectively, and both are similarly regulated by nitrogen catabolite repression (38). We therefore measured their RNA concentrations over 48 h after release from 24-h temperature entrainment to constant conditions. Their RNAs show a high amplitude oscillation in constant conditions with expression mirroring that of the pH oscillation (Fig. 4), with a peak in RNA concentration about 3 to 6 h before the media reaches the lowest pH. In entrained conditions, MEP2 and GAP1 RNAs precede the pH oscillation much as in the free-running condition (Fig. S4). The periodic transport of amino acids and ammonium to the cytoplasm would increase cytoplasmic pH, as they carry protons into the cell. The plasma membrane H^+-ATPase, PMA1p, maintains intracellular pH by controlled extrusion of protons in response to their increase in the cytoplasm (39, 40) leading to secretion of excess protons and creating the observed oscillations. This may be a manifestation of clock regulation of metabolism in yeast, as cellular pathways are coordinated for optimal function. The general strategy of metabolic regulation is a fundamental property of clocks as demonstrated in higher organisms (41–44).

Although circadian clocks are found widely in nature, they have not yet been scrutinized in *S. cerevisiae*. An extensive literature describes ultradian rhythms in yeasts (e.g., 18–21), and it was recently suggested that these short rhythms could be used as building blocks for longer circadian rhythms (45). Although this is formally possible, we see no evidence for ultradian oscillations under the conditions used for these experiments. Several decades ago, experiments purported to show circadian rhythms of cell division in bulk cultures of yeast (46), but these findings were never independently repeated. In these fermentor cultures, the cell division rate is approximately once every 9 h and there is no apparent rhythm in cell division, which indicates gating of this process to a specific time of day. The temperature cycle protocols applied here reveal a circadian timing mechanism in *S. cerevisiae* that can systematically entrain and that rapidly damps in constant conditions. Furthermore, we have shown clock-controlled molecular rhythms in gene expression of a key metabolic pathway that can be further used to investigate circadian behavior as well as to search for clock genes in yeast. These observations open the door

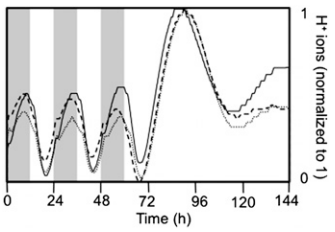

Fig. 3. The yeast oscillator rapidly damps in constant temperature. Cultures were entrained to a 24-h temperature cycle (21 °C to 28 °C) and released to constant conditions (28 °C). The relative H^+ concentration of the cultures are shown.

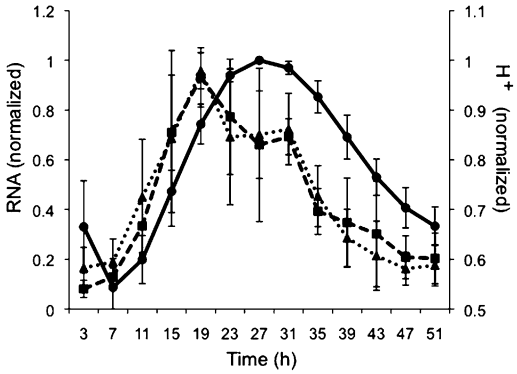

Fig. 4. Oscillations in gene expression and in the pH of the media are synchronized. MEP2 and GAP1 RNA were measured (three independent, experimental replicates) in cell extracts from free-running cells in constant conditions. The solid line shows the H^+ oscillation, the dashed line is MEP2 RNA, and the dotted line is GAP1 RNA. The RNA values are normalized using tubulin RNA levels. Averages ± SD are shown.

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for new approaches to elaborating circadian clock mechanisms and behaviors in eukaryotes. Budding yeast is especially attractive as it invites utilization of the multiple genomewide toolkits that facilitate high-throughput protocols.

Materials and Methods

Yeast Strain and Culture Conditions. The strain used throughout this study was *S. cerevisiae* FY1679-2B (*MATα ura3-52 leu2Δ1 TRP1 his3Δ200 GAL2*; EUROSCARF, Frankfurt am Main, Germany). All experiments were performed in fermentors (APPLIKON) to facilitate control and monitoring of the cultures. The 1-L culture vessels were inoculated with overnight cultures grown from single colonies in YPD (1% yeast extract, 2% peptone, 2% dextrose). The remainder of the experiment was then performed using YPD plus 10 mL l^{-1} Sigma Antifoam A. A batch culture at 30 °C lasted ≈36 h. When a rapid decrease in dO_2 was observed, the culture was starved for an additional 4 h. Cultures were then operated in continuous mode (a constant rate of media inflow and outflow) with agitation at 750 rpm, aeration at 150 mL min^{-1}, and dilution at 0.09–0.1 h^{-1} (unless otherwise specified). The dO_2 and the pH were monitored online; the dilution rate was monitored offline. The cell number was stable at around $2–3 \times 10^9$ cells/mL. It usually took about 2 weeks for the culture to become stable, such that it showed the same phase angle each day for weeks or months. Transitions from one cycle condition to another (i.e., from 24 to 23.5 h in length) would take up to a week to stabilize at a new entrained phase.

Zeitgeber Cycles. Half of each cycle was spent in high temperature (25 °C, 28 °C, or 30 °C), the other half in low temperature (15 °C, 18 °C, or 21 °C), with temperature transitions occurring over 60 min to decrease masking. The temperature of the room was maintained at 18 °C; the temperature of the cultures was maintained using a programmable water bath (Lauda).

RNA Preparation. A total of 1×10^9 yeast cells were collected and frozen in liquid nitrogen. Under free-running conditions, cells were harvested every 4 h over 2 days of a free run, starting 2 h after the temperature transition from cold to warm. Under entrained conditions, cells were harvested every 3 h over 24 h. Yeast total RNA was prepared using a modified version of the hot phenol RNA extraction protocol (47). The frozen yeast pellet was suspended in 400 μL AE buffer (50 mM NaOAc pH 5.3 and 10 mM EDTA); 40 μL 10% SDS and 400 μL acidic phenol were added. The cells were disrupted by vortexing and then heated at 65 °C for 30 min. The samples were cooled, centrifuged, and the aqueous phase was reextracted with 400 μL acidic phenol followed by chloroform. RNA samples were purified and concentrated using a NucleoSpin RNA II kit (Macherey-Nagel).

RT-PCR Analysis. cDNA was prepared according to standard methods (ABI reagents). One microliter of template cDNA was analyzed in triplicate for each primer set. Primers were designed with Primer Express software (ABI). The sequences were:

GAP1-Fw, 5′-TTGTTCTGTCTTCGTCACCGC-3′,
GAP1-Rv, 5′-TACGGATTCACTGGCAGCAAG-3′,
MEP2-Fw, 5′-CAGATGCGGAAGAAAGTGGAC-3′,
MEP2-Rv, 5′-GGGTGATACCCACTAGGCCAG-3′;
TUB1-Fw, 5′-TCCATTGCTGAGGCTTGGAA-3′,
TUB1-Rv, 5′-ACCAGTGGACGAAAGCACGTT-3′.

PCR reactions were performed according to standard methods.

Data Analysis. The output files from the fermentors were analyzed with ChronosX software (48).

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