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Rhythms of Mammalian Body Temperature Can Sustain Peripheral Circadian Clocks

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Summary

Background: Low-amplitude temperature oscillations can entrain the phase of circadian rhythms in several unicellular and multicellular organisms, including *Neurospora* and *Drosophila*. Because mammalian body temperature is subject to circadian variations of 1°C–4°C, we wished to determine whether these temperature cycles could serve as a *Zeitgeber* for circadian gene expression in peripheral cell types.

Results: In RAT1 fibroblasts cultured in vitro, circadian gene expression could be established by a square wave temperature rhythm with a ΔT of 4°C (12 hr 37°C/12 hr 33°C). To examine whether natural body temperature rhythms can also affect circadian gene expression, we first measured core body temperature cycles in the peritoneal cavities of mice by radiotelemetry. We then reproduced these rhythms with high precision in the liquid medium of cultured fibroblasts for several days by means of a homemade computer-driven incubator. While these "in vivo" temperature rhythms were incapable of establishing circadian gene expression de novo, they could maintain previously induced rhythms for multiple days; by contrast, the rhythms of control cells kept at constant temperature rapidly dampened. Moreover, circadian oscillations of environmental temperature could reentrain circadian clocks in the livers of mice, probably via the changes they imposed upon both body temperature and feeding behavior. Interestingly, these changes in ambient temperature did not affect the phase of the central circadian pacemaker in the suprachiasmatic nucleus (SCN) of the hypothalamus.

Conclusions: We postulate that both endogenous and environmental temperature cycles can participate in the synchronization of peripheral clocks in mammals.

Introduction

Most multicellular and several unicellular organisms possess an endogenous circadian clock that helps to adjust their physiology to daily cycles of light, temperature, and nutrient availability. Because this internal clock has a period that is only approximately 24 hr long, it must be reset daily by external cues. The most dominant of these so-called *Zeitgeber*s is light, which, in mammals, entrains rhythms in the suprachiasmatic nucleus (SCN) via ocular mechanisms, though other photoreceptive organs exist in other species (reviewed in [1, 2]). The central SCN clock then relays timing information to subsidiary clocks of similar molecular makeup in peripheral tissues, at least in part via diffusible signals [3] (reviewed in [4]). In the absence of a functional SCN, circadian gene expression does not persist in peripheral cell types such as liver [5, 6].

In organisms as diverse as cyanobacteria (*Synecococchus*), fungi (*Neurospora*), insects (*Drosophila*), and mammals, the molecular mechanisms generating circadian rhythms are thought to rely on feedback loops in gene expression. These involve interactions between positive oscillator components (e.g., *wc-1/wc-2* in *Neurospora*, *clk/cyc* in flies, *CLOCK/Bmal1* in mammals) and negative oscillator components (e.g., *frq* in *Neurospora*, *per/tim* in flies, and *Per1,2/Cry1,2* in mammals). The cycling clock gene products then directly or indirectly control the activity of clock-controlled genes, and thereby the physiological output pathways governed by the clock (reviewed in [7, 8]).

One prominent feature of circadian timing systems is that they are temperature compensated: the period length of their oscillations varies only slightly with temperature (Q_{10} between 0.8 and 1.2). Nevertheless, temperature cycles of low amplitudes have been shown to be capable of entraining the phase of circadian clocks in heterothermic organisms such as lizards, *Neurospora*, and *Drosophila* [9–11], and such cycles have been useful as tools to dissect clock mechanism [12–14]. In studies of cultured chick pineal neurons or mammalian SCN neurons, temperature changes could also phase shift intrinsic rhythmicity [15, 16]. However, in constantly homeothermic mammals such as marmosets and monkeys, environmental temperature cycles appear to have moderate effects at best upon circadian locomotor rhythms [17, 18]. The implicit conclusion of these analyses is that environmental temperature cannot easily perturb the central pacemaker of a homeothermic organism in vivo. Nevertheless, these studies did not address the question of whether temperature might serve as a *Zeitgeber* for oscillators in peripheral mammalian tissues. If so, core body temperature, which is itself subject to circadian oscillations, could participate in the synchronization of subsidiary oscillators in peripheral cell types, as has been suggested previously [14]. In this paper, we address this conjecture first in vitro by subjecting cultured fibroblasts to regimens of circadian temperature oscillation, and we show that both artificial and recorded natural body temperature rhythms can sustain circadian rhythmicity. We then extend these conclusions to a mouse in vivo by showing that abnormal environmental temperature cycles can result in the decoupling of peripheral oscillators from the central pacemaker in the SCN.

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Results

Square Waves of Temperature Can Entrain Circadian Rhythms of Cultured Fibroblasts

Depending upon the species, body temperature in mammals displays daily fluctuations of 1°C–4°C [19]. We wished to examine whether these temperature cycles might play a role in the entrainment of peripheral clocks. To begin to address this question, we first asked whether moderate 24 hr temperature oscillations (12 hr at 37°C, 12 hr at a lower constant temperature) could entrain circadian rhythms in cultured rat fibroblasts. Previous reports have shown that circadian gene expression can be elicited in these immortalized cell lines through various chemical signaling pathways [20–22]. After 6 days of entrainment with 12 hr temperature steps of 2°C–10°C, cells were harvested every 4 hr during 1 day, and the pattern of expression of several circadian genes was examined. RNase protection analysis showed that multiple clock and clock-controlled genes demonstrated circadian oscillations of transcript accumulation in cells subjected to temperature steps of 4°C. However, a 2° temperature fluctuation was insufficient to induce circadian expression of the clock output gene *Dbp*, and a 10° fluctuation induced apparently random fluctuations (Figure 1A). The oscillations in gene expression obtained with the Δ4°C temperature cycle are clock- rather than temperature-driven, because they persist in cells kept at constant temperature (37°C) after the temperature entrainment (Figure 1A). The data presented in Figure 1B attempt to dissect the resetting cue of such an entrainment. Cells were either kept at 37°C and shifted to 33°C (downshifted) or kept at 33°C and shifted to 37°C (upshifted). In each case, an abrupt 4° change in culture temperature was sufficient to induce very weak rhythmicity in gene expression. Although the circadian amplitude of this expression was very small, we find it significant that the induced gene expression was in opposite phases. We conclude that temperature shifts in both directions are potential circadian resetting cues, though multiple signals over several days are necessary to induce significant rhythmicity. This conclusion is underlined by the experiment presented in the bottom panel of Figure 1B, which compares circadian expression of the *Dbp* gene over 2 days after a single temperature drop or after 7 days of temperature entrainment. The repeated entrainment resulted in a 5-fold amplification of the amplitude of circadian *Dbp* expression relative to that induced by a single temperature change.

Natural Body Temperature Rhythms Can Sustain Circadian Rhythmicity In Vitro

Our results clearly show that abrupt temperature fluctuations can entrain daily oscillations in gene expression in mammalian cells, but they do not address the issue of whether complex natural body temperature rhythms, generally of lower amplitude, could contribute to the entrainment of peripheral oscillators. To examine this question, we designed and constructed a computer-driven incubator that is capable of reproducing complex temperature profiles with high precision in tissue culture dishes (see the scheme in Figure 2A). Core temperature profiles, recorded telemetrically in the peritoneal cavities of mice, were used to instruct the homemade computer-driven cell culture incubator, which in turn imposes these "natural" temperature fluctuations upon tissue culture cells. The data shown in Figure 2B indicate that this incubator mimics telemetrically recorded body temperature profiles in the medium of a culture dish with a temperature accuracy of about 0.1°C and a time resolution of 5 min.

Cells exposed to such natural temperature fluctuations during 7 consecutive days did not exhibit clear circadian gene expression (data not shown), suggesting that normal body temperature oscillations alone are not sufficient to reanimate molecular oscillators in peripheral cell types once they have dampened. However, it remained possible that natural body temperature profiles could participate in the maintenance of already-established circadian rhythms. To examine this conjecture, we first induced circadian cycles of gene expression in cultured fibroblasts by 3 days of 33°C/37°C square wave oscillations, as already described in Figure 1. This temperature regimen was then followed by 4 days of "natural body temperature" oscillations, which were telemetrically recorded in a mouse and reproduced in the culture dishes. The "natural" temperature oscillations were supplied in two different phases relative to the initial square wave, i.e., starting 72 hr or 84 hr after the beginning of entrainment. Thus, in this regimen, the two "natural" curves began at opposite clock phases. In parallel experiments, which served as a negative control, the temperature was left constant during the 4 days after entrainment with a square wave temperature profile. On the final day of the experiment, i.e., after 3 days of "natural temperature" entrainment, a dish of cells was harvested every 4 hr for 1 day, and gene expression was measured by real-time PCR or Northern blot hybridization. Temperature recordings from within a representative culture dish during each phase of this experiment are shown in all panels of Figure 3. Cells entrained by "natural temperature profiles" in one phase demonstrated circadian rhythmicity of multiple circadian genes at the end of the 6-day period (Figure 3A). Those entrained by "natural temperatures" in the other phase, or left at a constant temperature, displayed no conspicuous rhythmicity (Figures 3B and 3C). Although multiple clock genes showed circadian expression patterns after these endogenous temperature curves, the clock output gene *Dbp* did not (data not shown); this is a phenomenon that we do not fully understand. Nevertheless, the data presented in this section indicate that natural body temperature oscillations can delay the damping of cyclic gene expression in peripheral cell types and could thus contribute to the synchronization of peripheral oscillators. To achieve this effect, the presence of these low-amplitude temperature curves is continuously required. Unlike what is observed for the large-amplitude square waves in Figure 1, the rhythms sustained solely by natural temperature cycles are not spontaneous: they damp significantly 1 day after the elimination of the temperature cycles (data not shown). Since such temperature cycles are constantly present in animals, our data sug-

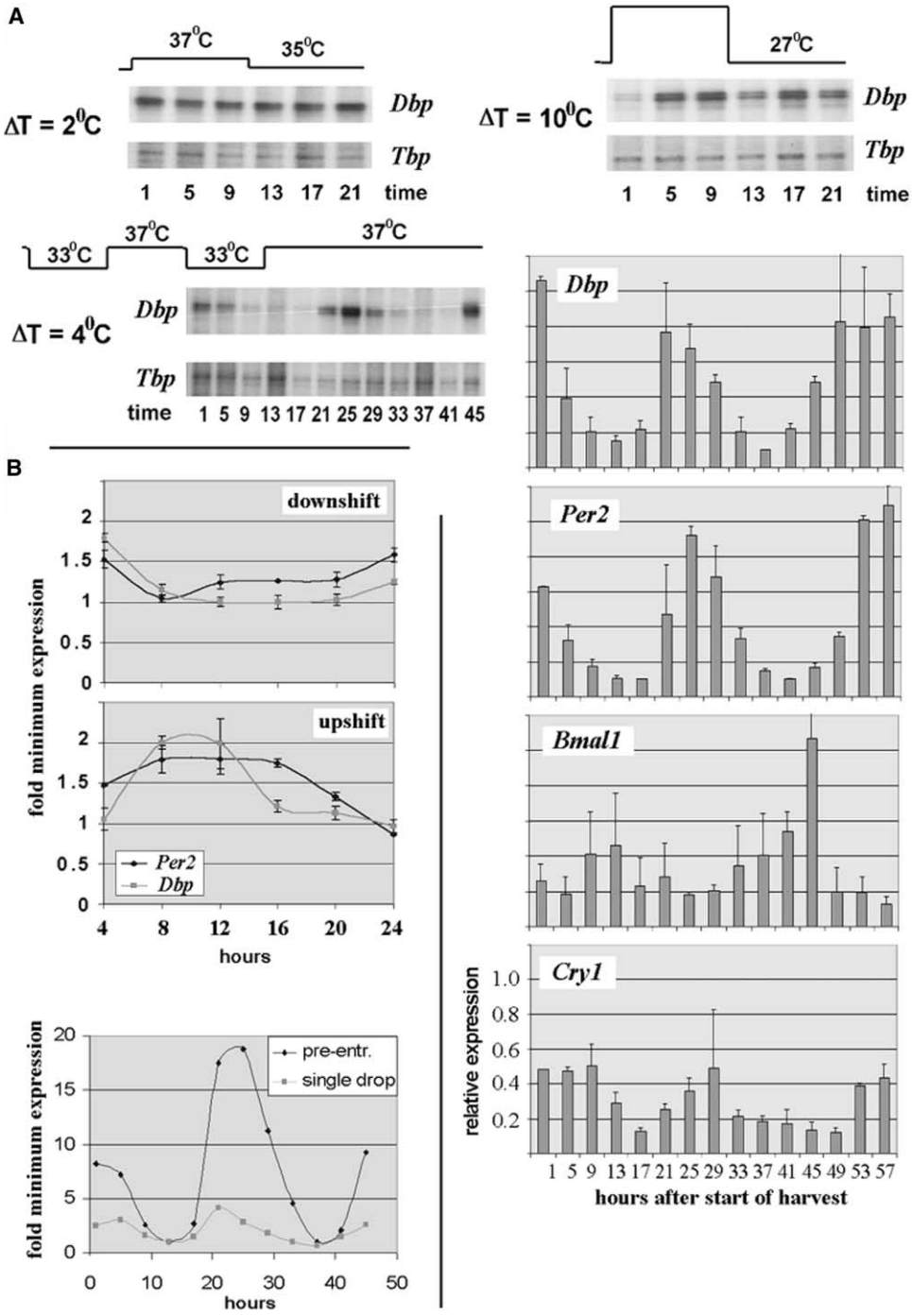

Figure 1. Effects of Temperature Square Waves upon Circadian Gene Expression in Cultured RAT1 Fibroblasts

(A) (Middle panel) For 6 days, confluent RAT1 fibroblast cells were incubated for 12 hr at 37°C, then for 12 hr at 33°C. Beginning on the seventh day, cells were kept constantly at 37°C. Beginning halfway through the sixth day, a plate of cells was harvested every 4 hr for the next 57 hr (t = 0 represents the beginning of the final 33°C incubation period). RNA extracted from these plates was examined by RNase protection, probing with fragments of the clock output gene *Dbp*. *Tbp*, a gene whose expression is constant throughout the day, was included as an internal control. Real-time PCR analysis was performed on the *Dbp*, *Per2*, *Bmal1*, and *Cry1* genes for two independent experiments, and the results are presented as histograms at the right. Mean values are shown, and their standard errors are quantified relative to maximal expression of the gene during the experiment. ANOVA analysis of the data sets against each other yielded the following values: for *Dbp*, $p = 3 \times 10^{-4}$; for *Per2*, $p = 1 \times 10^{-7}$; for *Bmal1*, $p = 0.03$; and for *Cry1*, $p = 0.002$. Regression analysis was performed to fit mean values to a theoretical sine function. The confidence values of this function relative to each data set were: for *Dbp*, $p = 5 \times 10^{-5}$; for *Per2*, $p = 1 \times 10^{-4}$; for *Bmal1*, $p = 2 \times 10^{-5}$; and for *Cry1*, $p = 8 \times 10^{-8}$. (Top panel) A similar experiment was performed, but oscillations between 37°C and 35°C ($\Delta T = 2$°C) and 37°C and 27°C ($\Delta T = 10$°C) were used. Cell harvests were performed on the sixth day only, without subsequent harvest of cells kept at constant temperature. Expression of the clock output gene *Dbp* was analyzed by RNase protection, with *Tbp* serving as a control.

(B) (Top panels) Cells were left at 37°C for 6 days, and were then shifted to 33°C (downshift) or the inverse (upshift). After the shift, cells were

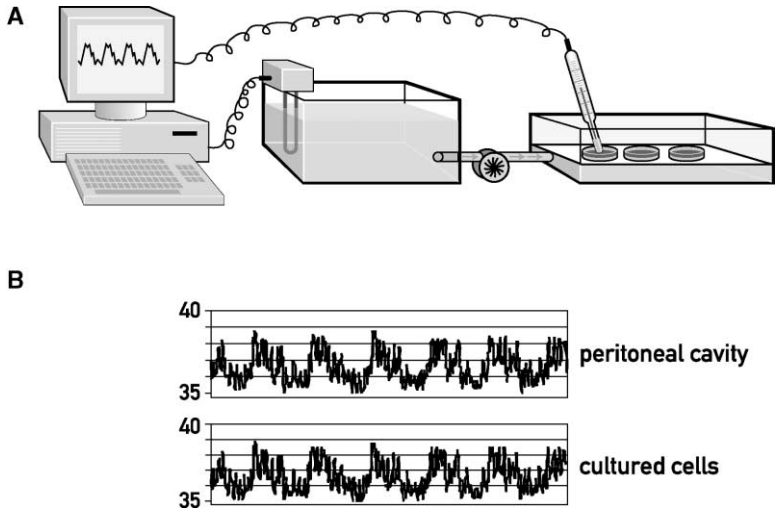

Figure 2. Mimicking of Natural Body Temperature Curves in Tissue Culture Dishes

(A) A scheme of the homemade incubator used in this study. Temperature was measured in the peritoneal cavity of a mouse every 5 min by radiotelemetry. These temperature readings were later transferred to a computer that controls a water bath. Water was pumped from the bath into a controlled-atmosphere incubator, where it circulated around flasks of cultured fibroblasts. Feedback from a temperature probe in the fibroblast culture milieu allowed the computer to recreate the mouse temperatures with a precision of 0.1°C in the milieu.

(B) A real-time body temperature curve in a culture dish. (Top panel) Recording of temperatures from the peritoneal cavity of a mouse for 6 days, as measured by Minimitter radiotelemetry devices. (Bottom panel) Recording of temperatures in flasks of RAT1 cells in our incubator programmed to mimic these mouse temperature profiles.

gest that temperature is likely to contribute to peripheral circadian gene expression.

Environmental Temperature Cycles Can Alter Circadian Gene Expression in Liver

To assess the possible importance of temperature cycles to circadian rhythmicity in vivo, we analyzed the effects of altered environmental temperature on cyclic gene expression in intact animals. Mice were exposed to 24 hr temperature cycles (12 hr 37°C, 12 hr 24°C) either in phase with light (warm days, cold nights) or out of phase with it (warm nights, cold days). After 6 days of such treatment, we analyzed the phase of circadian gene expression in these animals and in control animals kept at constant temperature. In the SCN, the expression of the clock output gene *Dbp* measured by in situ hybridization maintained the same phase, irrespective of the different temperature regimens, with maximum expression at ZT5 (Figure 4A). By contrast, in the liver, the phase of *Dbp* expression was almost completely inverted and showed a maximum of ZT23 rather than the usual ZT9 when temperature cycles were reversed from what would be expected in natural habitats, where days are warmer and nights are colder. Similar temperature-induced changes were observed for the expression of *Per2* and *Cry1* (Figure 4B). The in situ hybridization experiments presented in Figure 4A also indicate that the phase of circadian *Dbp* mRNA accumulation is inverted in brain areas other than the SCN, although the amplitude of this cycle is relatively low when compared to that observed in liver (and other peripheral tissues). "Normal" temperature cycles (i.e., warm during the day, cold during the night) did not alter the phase of circadian rhythms, and these cycles even moderately reinforced their amplitude in the liver (Figure 4B and data not shown).

Telemetric core temperature recordings of mice subjected to "normal" and "inverted" temperature cycles are shown in Figure 4C. Whereas these Balb-C mice normally display a temperature (and activity) peak in the late afternoon and early evening, mice subjected to warmth at night display a new peak of temperature in the late evening and early morning. Such an inversion of natural body temperature cycles could help to explain the inversion of peripheral circadian gene expression that we observe.

The presented data indicate that inverted environmental temperature cycles are able to reverse circadian rhythms in peripheral oscillators in the liver without altering those of the central clock in the SCN. Such decoupling has also been observed in studies of restricted feeding, in which food availability at aberrant times (during the day) could also resynchronize peripheral oscillators without affecting the SCN [23, 24]. We therefore considered the possibility that the changes in peripheral gene expression observed in mice subjected to warm nights arose simply because of changes in their feeding patterns. We thus measured the feeding frequency of these mice with an infrared beam placed above their food source, and we obtained a feeding profile for normal mice, warmed mice, and food-entrained mice (Figure 5A). The phase of the feeding cycles of mice warmed at night was indeed changed by an average of 6.5 hr, as mice demonstrated a marked preference for late-afternoon rather than early-evening meals when heated. Nevertheless, circadian gene expression changed

harvested and analyzed by Northern blot hybridization or real-time PCR as in (A). Mean values from two experiments were quantified. The mean values along with their standard errors are given in the graphs. The values shown are normalized relative to minimum gene expression, and times are shown relative to the temperature shift. (Bottom panel) The data for *Dbp* expression monitored in an independent "temperature-downshift" experiment lasting 48 hr are compared to those recorded after a 6-day entrainment, similar to that shown in (A). Values have been normalized relative to minimal expression, arbitrarily given a value of 1. Note that this extended recording suggests that the temperature downshift elicited a low-amplitude cycle of *Dbp* expression.

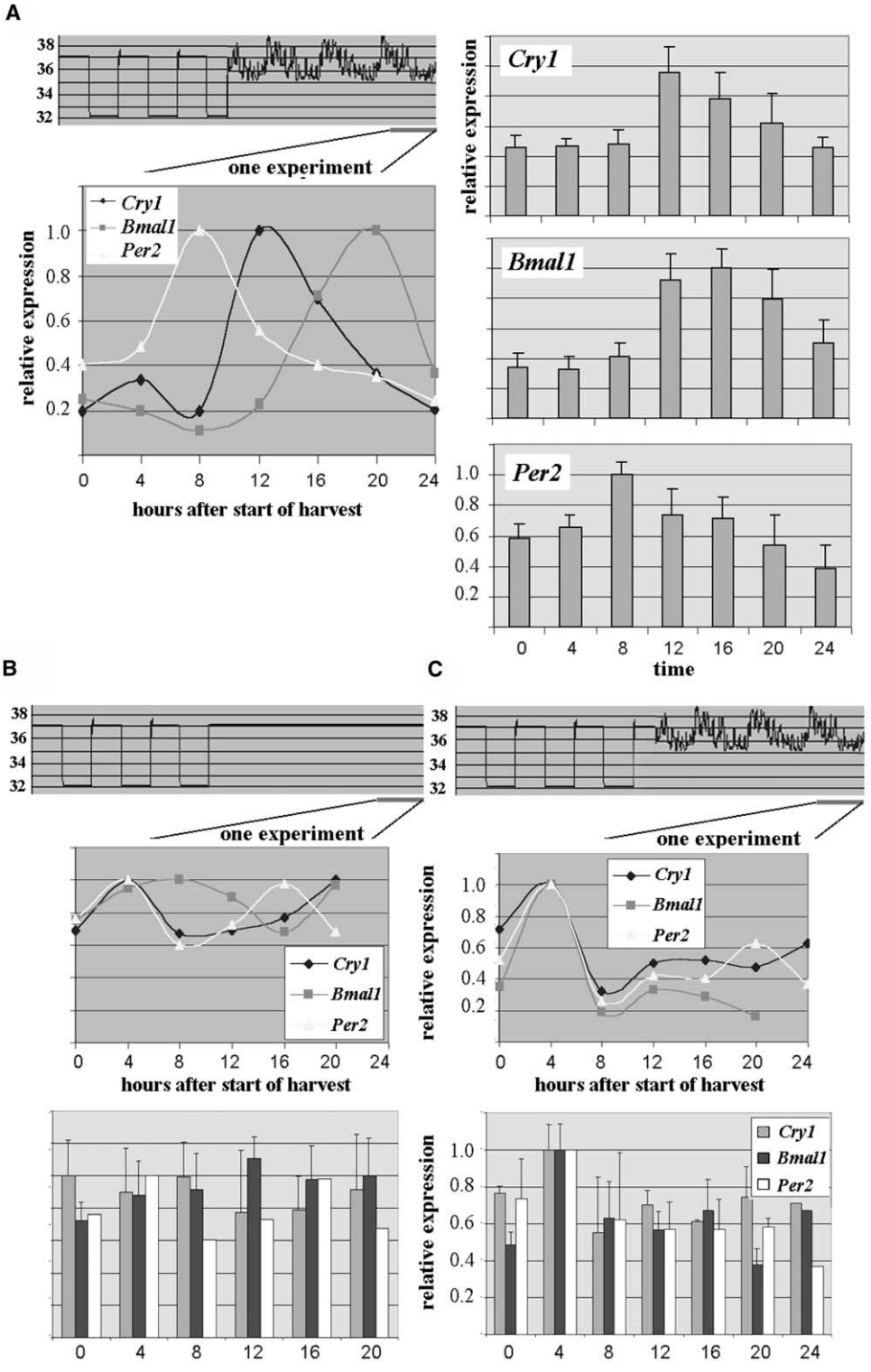

Figure 3. Effects of Natural Temperature Curves upon Circadian Gene Expression in Cultured RAT1 Fibroblasts

(A) Plates of confluent RAT1 cells were incubated alternately for 12 hr at 37°C and 12 hr at 32°C during 3 consecutive days. Cell temperatures were then maintained identical to a mouse peritoneal temperature profile (see Figure 2) during the 4 following days. The temperature profiles to which the cells were exposed during the 7-day period are shown in the top panel. On the seventh day, a plate of cells was harvested every 4 hr. Its RNA was analyzed by real-time PCR for the transcripts of the clock genes *Cry1*, *Bmal1*, and *Per2*. The values shown are first normalized relative to the noncircadian *Gapdh* mRNA, and then to the maximal corrected values determined for each transcript. On the left, the results from a single experiment are presented; on the right, the mean values and their standard errors from three to five independent experiments are presented as histograms (see the Experimental Procedures). The following significance values were obtained: for data sets compared to

phase even more (9.5 hr). Because inverted food availability changes feeding patterns even more, and also dramatically changes body temperature in mice (see below), we conclude that both factors might play a role in the reversal of peripheral circadian gene expression.

Temperature Profiles from Food-Inversed Mice Can Inverse Circadian Gene Expression in Cultured Cells

Inversed feeding regimens not only alter peripheral circadian gene expression in mice, but they also radically alter mouse body temperature rhythms. When mice are fed only during the day, the inverse of their usual habits, their core temperature drops sharply at night (by up to 4°) (Figure 5B, [24]). To see whether these temperature curves could also maintain circadian rhythmicity in cultured cells, we performed the same experiment that was used for normal temperature curves in Figure 3. Plates of RAT1 cells were entrained for 3 days by using square wave temperature profiles, and were then subjected to 4 days of temperature profiles recorded intraperitoneally in a mouse fed only during the day. On the fourth day, a plate of cells was harvested every 4 hr (at the same time relative to the start of the experiment as for the "normal" curves in Figure 3), and the expression patterns of multiple circadian genes were measured by real-time PCR (Figure 5C). These food-inverted temperature curves are also capable of maintaining circadian rhythmicity in tissue culture fibroblasts. The engendered rhythms are less robust, but the phase of gene expression in these cells is opposite to that observed for cells entrained by "normal" temperature curves. Hence, it is possible that body temperature profiles could contribute to inversion of peripheral circadian gene expression in mice.

In spite of the altered core temperature profiles observed in daytime-fed mice and their ability to entrain cultured cells, body temperature changes are not required for food-driven phase adjustments in peripheral gene expression — at least not in all mammals. Interestingly, in mice, superposition of the circadian environmental temperature regimens of Figure 4 upon the inverted feeding regimen of Figure 5 resulted in body temperatures and rhythms of circadian gene expression that closely mimicked those obtained with inverted feeding alone. The same holds true when the two are calculated to produce conflicting phases (data not shown). Thus, feeding is dominant over body temperature, but it also controls it. Daytime feeding also efficiently inverts the phase of peripheral circadian gene expression in the rat (F. Damiola, N. Le, and U.S., unpublished data) but does not affect circadian core temperature rhythms significantly in this much larger rodent (data not shown). Thus, the phenomena of temperature-cycle entrainment and feeding entrainment can be induced by independent mechanisms.

Discussion

The observation that circadian fluctuations of temperature can influence the phase of circadian rhythmicity in mammalian cells in vivo and in vitro adds new complexity to the picture of the entrainment of peripheral oscillators in mammals. Since the discovery that most or all cells of metazoan organisms contain semi-independent "slave" circadian oscillators that are dependent upon a central clock, investigators have searched for a signal by which the central clock might communicate timing information to them [4, 25–28]. In mammalian systems, numerous hormonal resetting cues have been shown to act upon peripheral oscillators both in vivo and in vitro (e.g., glucocorticoids, growth factors, cAMP, and calcium signaling), but so far none has been shown to be essential to their circadian rhythmicity [20, 29]. Rather, current models suggest that peripheral rhythms may be sustained by a complex amalgam of signaling pathways.

On a cellular level, the experiments presented above show that circadian rhythms of temperature fluctuation can sustain or entrain circadian rhythmicity in a cell-autonomous fashion; thus, we propose that body temperature could be among the entrainment cues for peripheral oscillators in the intact animal. Mammals possess an endogenous circadian rhythm of core body temperature of 1°C–4°C [19]. At least in part, these rhythms are thought to be governed indirectly, via the control of sleep-wake cycles and activity patterns by the SCN [28, 30]. As expected in such a regulatory scheme, circadian body temperature fluctuations are indeed dependent upon an intact SCN [31]. Hence, cell-autonomous circadian entrainment by SCN-driven body temperature cycles could help sustain the rhythmicity of peripheral clocks [14]. While mouse temperature cycles do not engender spontaneous rhythmicity in vitro, they slow the damping of existing rhythms. Since these cycles are normally constantly present in animals, it is plausible that they could fulfill a similar function in vivo. Feeding patterns probably perform a role analogous to that of temperature. Also governed by sleep-wake cycles, and thus by the SCN, they too could serve as an indirect route by which the SCN entrains peripheral clocks under normal circumstances. Together, experiments with feeding and temperature rhythms, as well as studies with known signaling components [20–22, 28, 29], emphasize that none of the time cues controlled by the SCN is exclusive in entraining peripheral oscillators. Rather, this task may be accomplished via multiple mechanisms, some of which are indirect (see Figure 6).

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one another, for *Cry1*, $p = 0.02$; for *Bmal1*, $p = 0.003$; and for *Per2*, $p = 0.02$; for mean values compared to a fitted sine function, for *Cry1*, $p = 0.007$; for *Bmal1*, $p = 0.06$; and for *Per2*, $p = 0.04$.

(B) An experiment similar to that presented in (A) was performed, but cells were left constantly at 32°C after 3 days of square wave preentrainment. On the seventh day, cells were harvested as before, and the gene expression patterns of *Cry1*, *Bmal1*, and *Per2* were determined by real-time PCR as described above. The top panel shows results from a single experiment; the bottom panel shows averaged results from two to three experiments and their standard errors.

(C) An experiment similar to the one shown in (A) was performed, but this time the natural temperature curve was shifted by 12 hr relative to (A). Results are presented as for (B).

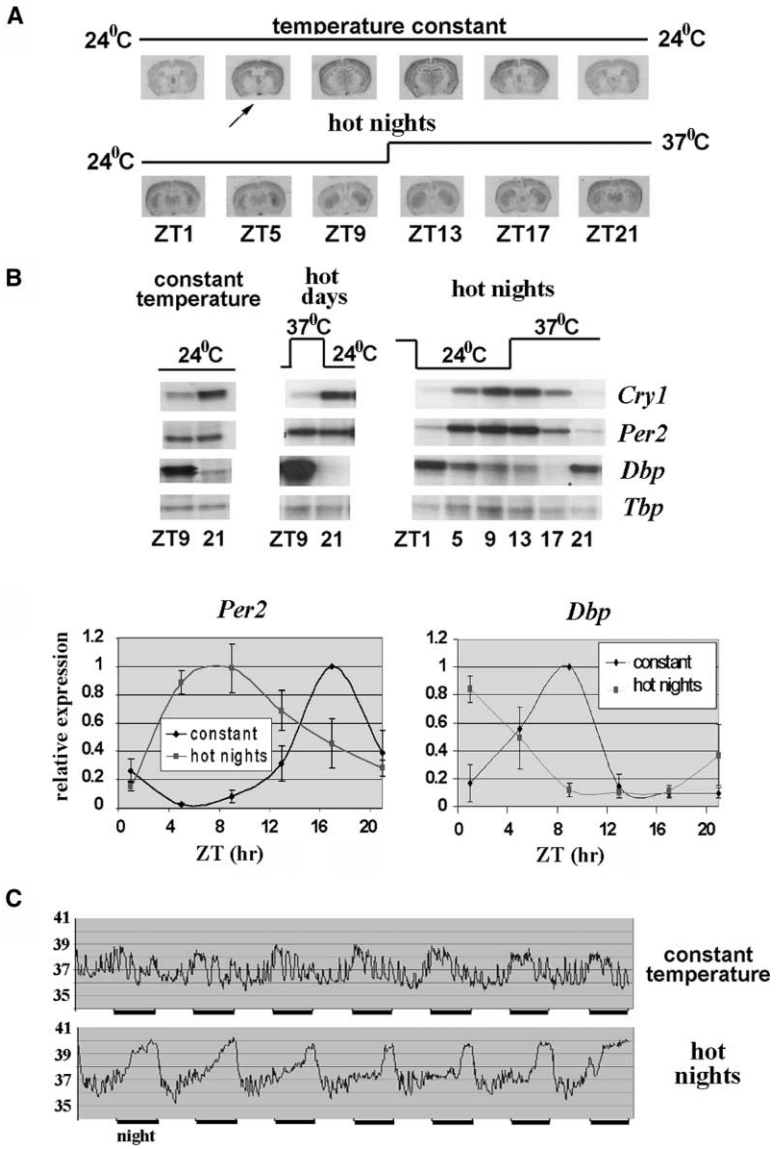

Figure 4. Circadian Cycles of Environmental Temperature Can Phase Shift Circadian Gene Expression in the Liver without Affecting the SCN

For 6 days, mice were kept for 12 hr in light at 24°C and for 12 hr in darkness at 37°C, or vice-versa (light/37°C, dark/24°C). Alternatively, mice were kept at a constant temperature of 24°C for 12 hr in light and for 12 hr in darkness. On the sixth day, a mouse was sacrificed every 4 hr, and its brain and liver were prepared for in situ hybridization and RNase protection analysis, respectively.

(A) In situ hybridizations of mouse coronal brain sections taken above the optic chiasma, using an antisense *Dbp* cRNA probe. The SCN is shown by the arrows. For this experiment, mice were kept in LD conditions at a constant temperature (top panel), or in light at 24°C and darkness at 37°C (bottom panel), as described above. Note that in the bottom panel, the phase of circadian *Dbp* mRNA accumulation in the cortex and in other non-SCN brain regions is nearly the opposite of that observed in the top panel. This finding indicates that the circadian phase in these brain regions imitates that of the periphery.

(B) (Top panel) An RNase protection assay showing the expression of *Dbp*, *Per2*, *Cry2*, and *Tbp* in livers from mice harvested as described above. (Bottom panel) A graph of means (±SE) of *Per2* and *Dbp* mRNA levels from two different tissues (liver, kidney) in two sets of mice kept at constant temperature and two sets of mice warmed at night.

(C) Peritoneal temperature profiles from mice kept under the conditions described above.

On an organismic level, we have also shown that circadian temperature differences comparable to day-night temperature changes in the environment can phase shift or reinforce peripheral clocks in mice without affecting the light-controlled central clock in the SCN. Such a mechanism could fine-tune peripheral clocks to their environment. In small, semitransparent organisms like *Drosophila* and zebrafish, light is able to phase shift peripheral oscillators directly [25, 32]. In opaque vertebrates, another solution must be employed. As discussed above, feeding habits provide one solution; nevertheless, irregular food availability in the wild could cloud this signal in some species. Environmental temperature usually follows day-night patterns, so circadian temperature oscillations might also provide an ideal *Zeitgeber* to peripheral tissues, even in mammals.

Finally, it has been previously shown that the phase of peripheral oscillators can be uncoupled from that of the central clock by restricting food availability to rodents. If nocturnal rodents, such as rats or mice, receive meals during the day, peripheral oscillators will phase shift to be in resonance with feeding time, but the light-driven central clock in the SCN remains unaltered [23, 24]. In such a case, it would be inconvenient to have a conflicting signal to peripheral oscillators furnished by temperature. Fortunately, this dilemma is already neatly resolved. Damiola et al. have shown previously that body temperature rhythms are altered by restricted feeding regimens [24]. We show here that these rhythms can also sustain peripheral circadian rhythmicity, but in an inversed phase from that sustained by normal temperature rhythms. Thus, although cycles of body temperature are not necessary for peripheral reentrainment by food availability, they do not conflict with it either. Our data suggest rather that the two are related cues that act independently, with feeding dominant over temperature in rare cases where the two conflict. Interestingly, the SCN may be somehow involved in the relationship between food intake and body temperature: in SCN-lesioned rats in which body temperature rhythms are abolished, prolonged food deprivation no longer changes body temperatures [33].

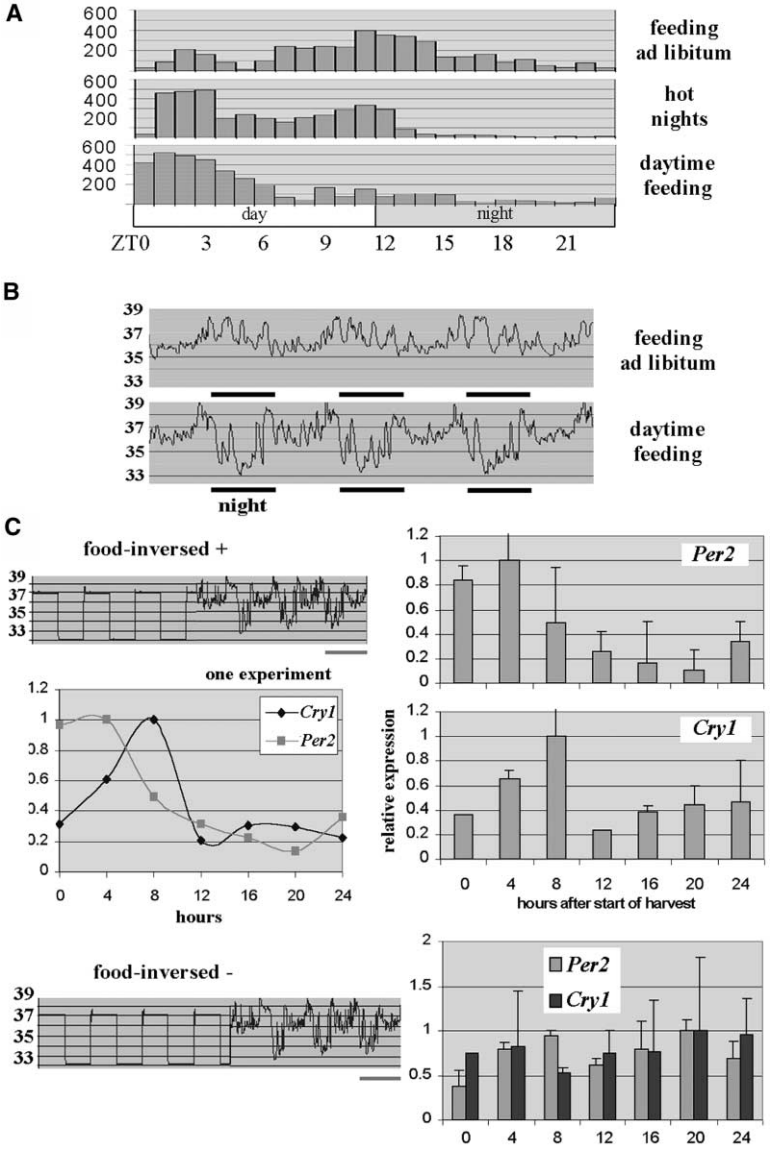

Figure 5. Core Body Temperatures of Daytime-Fed Mice Can Phase Shift the Circadian Rhythms of Cultured Fibroblasts

(A) Feeding patterns of mice kept at constant temperature with food ad libitum (top panel), of mice warmed at night as in Figure 4 (middle panel), and of mice receiving food exclusively during the day (bottom panel). Feeding was measured with an infrared beam positioned directly above the food tray, using a device designed by Minimitter. The charts presented show the average number of seconds that a mouse spent breaking the beam per hour (*y* axis), as a function of the time of day (*x* axis). For the top two panels, n = 4 mice; for the bottom panel, n = 2 mice.

(B) Comparison of intraperitoneal temperature profiles of mice fed ad libitum and mice fed exclusively during the day.

(C) For 3 consecutive days, plates of confluent RAT1 cells were incubated for 12 hr at 37°C and for 12 hr at 32°C. Cell temperatures were then maintained identical to mouse intraperitoneal temperatures for 4 additional days in two different phases relative to the initial entrainment, using temperature readings taken from mice fed only during the day. The temperatures recorded in the culture dishes during the described 7-day period are shown in the left panel. On the seventh day, a plate of cells was harvested every 4 hr, and the RNA was examined by real-time PCR for the transcripts of the clock genes *Cry1*, *Bmal1*, and *Per2*. Mean values from two experiments, and their standard errors, are shown in the right panel; these values are normalized relative to the noncircadian gene *Gapdh* and are plotted relative to the maximal expression of the gene during the experiment. For the panel labeled "food-inversed +" (top panel), a single experiment is also shown. ANOVA statistical analyses were conducted on the values obtained for *Per2* and *Cry1* transcripts to verify the significance of the circadian rhythms observed. The fit of the two data sets relative to each other for *Per2* mRNA is p = 0.01, and for *Cry2* mRNA, the fit is p = 0.06. The fit of the mean values relative to a theoretical sine function is p = 0.001 for *Per2* mRNA, and p = 0.005 for *Cry1* mRNA.

Conclusions

Most mammals demonstrate circadian fluctuations of core body temperature. We have shown that these cycles can help in sustaining the rhythmicity of peripheral circadian oscillators in vitro. We further show that fluctuations of environmental temperature can phase shift

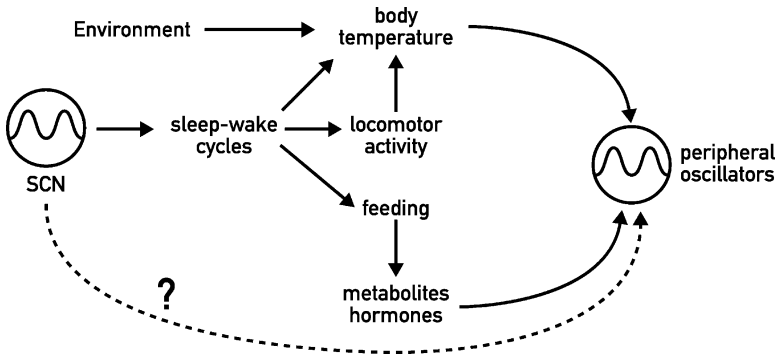

Figure 6. A Model Depicting the Phase Entrainment of Peripheral Oscillators by the SCN and Environmental *Zeitgebers*

The master pacemaker in the SCN, whose phase is reset daily by the photoperiod, may entrain peripheral oscillators mainly by indirect timing cues. By determining activity and rest phases, the SCN sets feeding time and rhythmic locomotor activity. In turn, these could influence the amplitude of body temperature cycles. Both feeding time and temperature rhythms then serve as *Zeitgebers* for peripheral circadian oscillators. The detailed mechanisms by which feeding and temperature synchronize peripheral clocks remain to be identified.

peripheral circadian rhythms in vivo. Taken together, our results suggest that temperature has been coopted as one of a growing family of *Zeitgeber*s for peripheral circadian clocks that help maintain the harmony of an organism with its environment.

Experimental Procedures

Cell Culture, Animal Husbandry, and RNA Preparation

RAT1 fibroblasts were cultured in Dulbecco's modified Eagle's medium with 5% fetal bovine serum in an incubator containing 5% CO_2. Prior to harvest, cells were rinsed with PBS. RNA from cells was prepared as described previously [34]. Mice used in this study were Balb-C. After sacrifice, brains were dissected and frozen for 5 min in isopentane at $-20°C$, then stored at $-80°C$. Livers were perfused with PBS and homogenized in a buffer containing guanidium thiocyanate, and RNA was prepared exactly as described elsewhere [35].

In Situ Hybridization

Coronal brain sections of 12 μm thickness were prepared according to standard procedures, and hybridizations were performed using previously published protocols [36]. Probes for the *Dbp* gene in sense and antisense configurations were constructed by using full-length cDNA constructs as described [37].

RNase Protection, Real-Time PCR, and Northern Analyses

Antisense RNA probes for the *Per1*, *Per2*, *Per3*, *Cry1*, *Dbp*, and *Tbp* messages were produced from linearized DNA templates by transcription with T7 polymerase. RNase protection reactions were then performed with 10 μg RNA per reaction. Details of probe sequences and reaction conditions are available in [24]. Quantitation was performed with a Biorad Personal FX phosphorimager and was normalized relative to the noncircadian *Tbp* transcript. For real-time PCR, 0.5 μg RNA was reverse transcribed with Superscript enzyme according to the protocols of the manufacturer (Invitrogen), in a total reaction volume of 10 μl. Reactions were then diluted 5-fold, and 4 μl was used for Taqman PCR using standard Applied Biosystems reaction conditions and reagents, in a total reaction volume of 20 μl. Real-time PCR was performed by using Applied Biosystems instrumentation and Taqman probes as described elsewhere [38]. Expression levels were normalized relative to the expression of the noncircadian *GAPDH* gene. For Northern blot analysis, 10 μg denatured RNA per sample was electrophoresed in 1% agarose/TBE gels and was transferred to Nytran membrane (Millipore). Ribosomal RNA was visualized with methylene blue staining of the membrane. Northern hybridizations against the *Bmal1* and *Cry1* transcripts were performed by using probes and methods described previously [39]. The transcript levels were quantified by densitometry phosphorimaging or real-time PCR. Mean values and standard errors of the mean were calculated for each data set where appropriate, and ANOVA analysis was used to calculate the significance of the data sets relative to one another and the significance of the mean values relative to a regression-fitted sine function. Between two and five data sets were used in each case, as specified in the figure legends. In the case of Figure 3A, a bootstrapping analysis was also used. Of three circadian genes analyzed for five data sets, in one data set the average circadian phase was 4 hr earlier across each gene, and in another it was 4 hr later. Hence, the mean phase of all genes in these two sets was adjusted by $+4$ hr and -4 hr, respectively. As described in the legend of Figure 3, p values after this bootstrapping were: for *Cry1*, $p = 0.02$; for *Bmal1*, $p = 0.003$; and for *Per2*, $p = 0.02$. Without phase adjustment, $p = 0.12$ for *Cry1*, $p = 0.01$ for *Bmal1*, and $p = 0.04$ for *Per2*. Because of these two adjustments, time in this experiment represents a "circadian time" in which $t = 0$ is defined as the time at which the harvest was begun for the three principal data sets, and this circadian time corresponds to the time of minimum expression of the *Cry* gene. *y* axis values are expressed as a fraction of the maximal measured expression of a gene during the experiment.

Telemetric Recordings

All telemetric recordings were performed with software and PDT-4000 transmitter hardware purchased from Minimitter Corporation. Transmitters were surgically introduced into the abdominal cavity behind the gut as previously detailed [24], and the peritoneal temperature was sampled every 5 min. Measurements of feeding frequency and duration were performed with an infrared beam placed just above the food tray, in a device designed by Minimitter (DP-24 data port with feeding frequency monitor). Beam breaks were tabulated with the Vitalview software system, also designed by Minimitter.

Computer-Controlled Temperature of Cultured Cells

Temperature measurements recorded by Minimitter telemetric devices were downloaded to a computer, and homemade software was used to instruct a Haake K20/DC50 computer-controlled circulating water bath. Water from this bath was pumped into channels in an aluminum block upon which cell culture flasks rested in a CO_2-controlled atmosphere. A separate temperature probe from the computer was placed directly into cell flasks to monitor the actual temperature of the culture medium. These measurements were recorded for publication and were also used as a source of dynamic feedback to permit more accurate instruction of the water bath by the computer on a constant basis. Minimitter instructions were met with 0.1°C tolerance within the measured 5-min intervals.

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