How Temperature Changes Reset a Circadian Oscillator

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Circadian rhythms control many physiological activities. The environmental entrainment of rhythms involves the immediate responses of clock components. Levels of the clock protein FRQ were measured in *Neurospora* at various temperatures; at higher temperatures, the amount of FRQ oscillated around higher levels. Absolute FRQ amounts thus identified different times at different temperatures, so temperature shifts corresponded to shifts in clock time without immediate synthesis or turnover of components. Moderate temperature changes could dominate light-to-dark shifts in the influence of circadian timing. Temperature regulation of clock components could explain temperature resetting of rhythms and how single transitions can initiate rhythmicity from characteristic circadian phases.

Common elements are emerging in the molecular mechanisms of circadian rhythms and in the ways that these mechanisms respond to environmental cues, including light and temperature (*1–6*). Phase resetting by light is understood in terms of rapid light-induced responses of central clock components. However, a description of how small, naturally occurring temperature cycles can reset a clock is lacking.

The universality and the sensitivity of temperature resetting indicate that temperature is a major factor in the entrainment of clocks, one which is commonly regarded as being secondary to light in importance as a zeitgeber for entrainment (*7*). Temperature shifts mimic the effects of light or darkness in most organisms (*5, 8–14*), with high temperature eliciting responses that are similar to responses to light and low-temperature darkness. Thus, light and temperature cycles reinforce each other. Circadian clocks can be extremely sensitive to temperature changes; in insects, lizards, and fungi, clocks can be entrained by temperature cycles that oscillate only 1° to 2°C (*14–16*). Temperature effects on the *Neurospora* circadian system, in particular, are well described (*9, 10, 16*), as is the general pattern in which molecular components are assembled, forming a feedback loop that is central to the system (*1, 3, 17, 18*). We have sought to understand how a day-phase oscillator that is characteristic of many eukaryotes could be reset by temperature steps. Here we describe a mechanism for temperature resetting of the *Neurospora* clock that could be generalizable to other systems and show that, contrary to expectation, temperature can be a stronger zeitgeber than light in *Neurospora*.

Rapid light-induced increases of *frq* mRNA and FRQ protein are associated with light-induced clock resetting in *Neurospora* (*3, 17*). Because temperature treatments mimic light entrainment, we speculated that they could yield similar molecular responses as well. Additionally, because the peak level of the FRQ oscillation increases dramatically with increasing temperature (*17, 18*), we surmised that the set point about which FRQ levels oscillate could be higher at higher temperatures and that this could explain how shifting the organism from one temperature to another would phase-shift the clock. To examine this possibility, we performed protein immunoblot and Northern (RNA) blot analyses to determine the rhythmic expression of FRQ protein and *frq* mRNA at temperatures within the physiological temperature range for rhythmicity (16° to 32°C). The levels of FRQ and *frq* mRNA were rhythmic at 21° and 28°C (Fig. 1A), but the average level about which FRQ oscillated at 28°C was much higher than the level at 21°C; the peak level of FRQ at 28°C was about three times as high as the peak level at 21°C, and the FRQ trough level at 28°C was slightly greater than the FRQ peak at 21°C. Additionally, as expected, temperature determined the ratio of the two FRQ forms; there was rela-

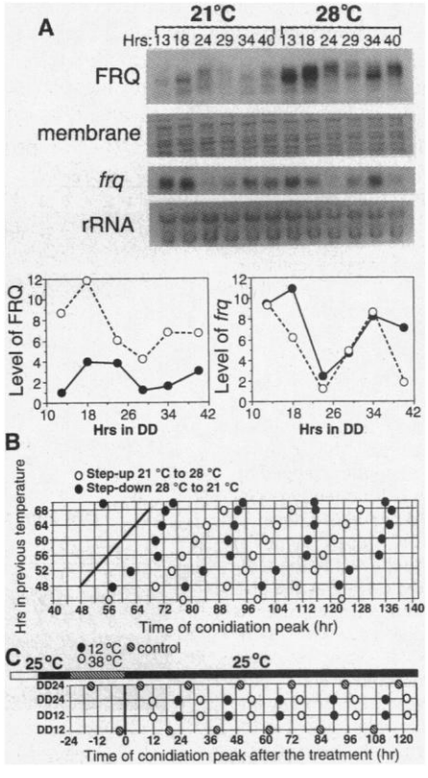

Fig. 1. The effects of temperature on the oscillation in *frq* RNA and FRQ and the effects of temperature steps on the phase of the rhythm. **(A)** The ambient temperature determines the average level about which FRQ amounts oscillate. Protein immunoblot and Northern blot analyses show molecular rhythms of *frq* mRNA and FRQ protein in cultures held in constant darkness at 21° or 28°C (top). In the densitometry data (bottom), the results from the hybridizations have been normalized either to ribosomal RNA or to total protein (as represented by amido black staining) as appropriate (21°C, solid circles; 28°C, open circles). Schematic representations of race tube assays show that the overt rhythm is affected by steps between temperatures within the temperature range permissive for rhythmicity (B) or by temperature steps from outside to within the physiological temperature range (C). Six separate sets of cultures were grown on race tubes (*30*), and these sets were stepped from one temperature to another at the times indicated by the diagonal black bar; one set of control cultures for each temperature were not stepped. The position of each circle indicates the average time when the center of the band of conidiation occurred in a set of equivalent tubes (*30*). **(B)** Reciprocal temperature steps within the physiological range do not produce equivalent effects on the clock. Cultures were adjusted by either a temperature step-up (from 21° to 28°C) or a temperature step-down (from 28° to 21°C). Step-ups result in strong resetting; the time of the conidiation peaks parallels the time of transfer, with the first peak always occurring at ~21 hours after the transfer. In contrast, step-downs result in weaker resetting; the time of the conidiation peaks occurs between 5 and 14 hours after the step and does not completely parallel the transfer line. **(C)** Large temperature steps from outside to within the physiological range reset the clock to characteristic times. Beginning from two time points about half a circadian cycle apart, DD12 (grown for 12 hours in constant darkness at 25°C) and DD24 (grown for 24 hours in constant darkness), cultures were transferred in darkness and incubated at either 12° or 38°C for 24 hours before being released back into darkness at 25°C. The clock was reset to the same phase regardless of the phase at the time of the temperature shift; the 12°C incubation reset the clock to about CT0 (subjective dawn), and the 38°C incubation reset the clock to about CT12 (subjective dusk).

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tively more full-length FRQ [FRQ(1–989)] than small FRQ [FRQ(100–989)] at higher temperatures (*18*). However, the level of *frq* mRNA was about the same at both temperatures. Similar experiments performed at other temperatures (from 18° to 30°C) produced results that were consistent with this pattern: the higher the temperature, the higher the average level about which FRQ oscillated. The Q_{10} value (the increase in the amount of FRQ for each 10°C increase in temperature) of the FRQ peak level was about 4, perhaps reflecting the temperature dependence of the threshold level of FRQ required for rhythmicity (*18*). Because the level of *frq* mRNA showed little change with temperature, a temperature-responsive posttranscriptional regulatory mechanism contributes to determining the average level of the FRQ expression at all times of day.

Because the function of FRQ is important to the *Neurospora* clock, the finding that the FRQ oscillation range is different at different temperatures potentially provides a molecular explanation for clock resetting by temperature steps. The same absolute amount of FRQ must correspond to different circadian times at different temperatures; therefore, when a cell is transferred from one temperature to another, the clock will be reset to the subjective time that corresponds to the actual amount of FRQ in the cell as viewed in the context of the new temperature. The size of temperature step–induced phase shifts is a function of the circadian time and the magnitude of the temperature treatments (*9, 10, 16*), and large temperature steps elicit strong resetting (*16*), moving the phase of the clock to a restricted part of the circadian day; temperature step-ups reset the clock to about circadian time (CT) 0 (subjective dawn), and temperature step-downs reset the phase to between CT9 and CT12 (subjective dusk) (Fig. 1, B and C) (*9, 10, 16*).

CT0 corresponds to the low point of the FRQ oscillation, and CT12 corresponds to a point when FRQ is highly expressed and phosphorylated (*17, 18*). Because even the peak level of FRQ at 21°C was slightly below the trough level at 28°C, we expected that temperature step-ups from 21° to 28°C would always reset the clock to a time that corresponds to the low point of the FRQ cycle, about CT0 (Fig. 1B). Steps from 21° to 28°C at any time did indeed completely reset the clock, and the new phase (about CT0, the low FRQ point in the cycle) was totally independent of the old phase, which was consistent with our prediction that every time point at 21°C is treated as the trough point of the FRQ oscillation. Conversely, step-downs from 28° to 21°C should reset the clock to the time that corresponds to the high point of the FRQ cycle, about CT12; however, results showed that the resetting behavior was weaker than that seen for step-ups (Fig. 1B). Although the new phases were set to close to the high point of the FRQ oscillation, they were nonetheless somewhat dependent on the old phase, which was consistent with previous studies (*9, 10*). Temperature step-downs are not just a mirror image of step-ups. Consistent with most circadian systems (*8–10*), steps from extreme temperatures to temperatures within the permissive range reset the clock to CT0 and about CT12, respectively (Fig. 1C).

We were intrigued by this asymmetrical nature of the response to symmetrical temperature steps, and we reasoned that because the magnitude of the reciprocal temperature steps was the same, some insight into why the responses were different could come from understanding the kinetics with which *frq* expression changes after a temperature shift and from understanding at what stage, translational or transcriptional, this regulation of *frq* and FRQ expression was controlled. To this end, Northern blot and protein immunoblot analyses were used to monitor the changes in the expression of FRQ and *frq* after the temperature steps (Figs. 2 and 3). Two different initial time points about half a circadian cycle apart were chosen for these experiments: DD12 (CT0, subjective dawn), where FRQ levels are low and on the rise, and DD22 (CT13, subjective evening), where FRQ levels are high and decreasing (*17*). Given the observed differences in the amount of FRQ as a function of temperature (Fig. 1) (*17*), we expected that, after the temperature steps, the amount of FRQ would either increase (for step-ups) or decrease (for step-downs) to the level that was appropriate for the new temperature. For a temperature step-up from 21° to 28°C, judging from the overt rhythm (Fig. 1, B and C), we predicted that the clock would treat both time points as the trough of the molecular oscillation and that FRQ would increase with kinetics, independent of the previous phase. In contrast, after step-downs from 28° to 21°C, we predicted that FRQ would decrease and then restart the cycle at a much lower level.

These predictions were, in fact, the results obtained (Fig. 2) at the level of translation products. After temperature step-ups at either time, FRQ increased rapidly, peaking about 6 hours later and then decreasing (Fig. 2A); the kinetics of the response of FRQ were almost identical at the two different phases. This is in agreement with the overt rhythm data; the clock was reset to close to the phase that corresponded to the low point of the FRQ oscillation, independent of the previous phase. This response was also fast: only 1 hour after the step-up, the amount of FRQ was already higher than the peak FRQ level

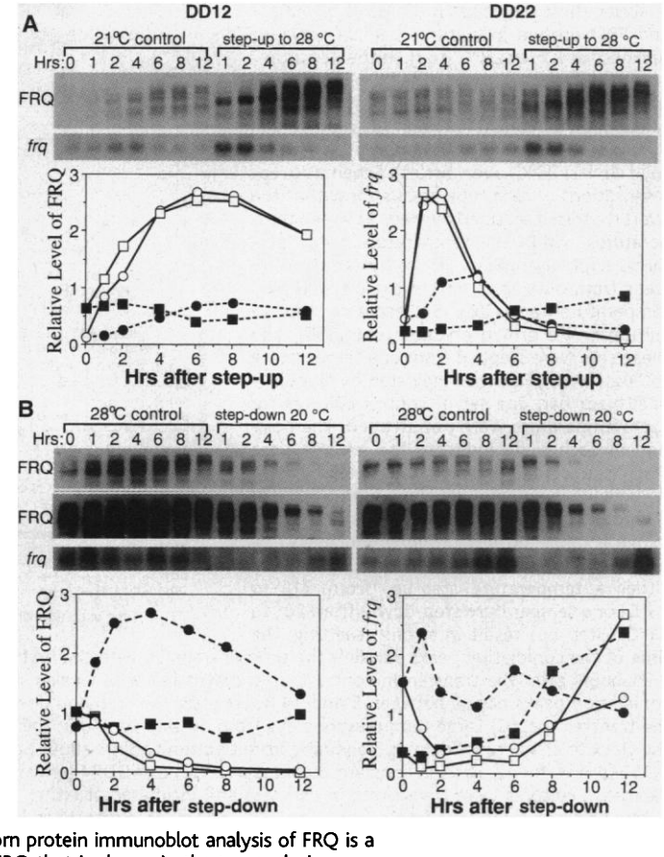

Fig. 2. Molecular cycles in *frq* RNA and FRQ respond quickly to shifts in the ambient temperature. Liquid cultures were grown in constant light at 25°C and then transferred into darkness at a control temperature of either (**A**) 21°C or (**B**) 28°C. At DD12 (circles) and DD22 (squares), about half of the cultures were either stepped up to 28°C (open symbols) (A) or stepped down to 20°C (open symbols) (B), the rest of the samples staying at the previous temperature as controls (solid symbols). Samples were collected and processed for protein immunoblot and Northern blot analyses (*31*) at the times indicated. In panel (A), temperature step-ups from 21° to 28°C result in increases in *frq* RNA and FRQ. In panel (B), temperature step-downs from 28° to 20°C result in a turnover of *frq* RNA and FRQ; the bottom protein immunoblot analysis of FRQ is a longer exposure of the FRQ that is shown in the top analysis.

at 21°C. The peak level of FRQ after the step-up was about three times the level of the control samples at 21°C, a difference similar to that shown in Fig. 1.

The response of the *frq* transcript to temperature step-ups was quite different. Initially, *frq* was rapidly induced to a level about 2.5 times that of the control at 21°C; however, this induction was only transient, and the amount of the *frq* transcript decreased after 2 hours. This response was independent of phase. These data, along with data from Fig. 1 and (*18*), demonstrated that transcriptional and posttranscriptional regulation contribute to resetting by step-ups. After an initial resetting of the clock, posttranscriptional regulation keeps FRQ oscillating around higher levels at higher temperatures. After a step-up, a similar amount of time (~6 hours) always elapses before the peak level of FRQ is reached. Together, the resetting kinetics and the internal dynamics of the feedback loop provide an explanation for the phase seen after resetting; FRQ amounts will increase for 6 to 8 hours, and therefore, the clock appears to be immediately reset to the phase that corresponds to a time of 6 to 8 hours before the peak in FRQ, CT0.

The results of step-down experiments were primarily opposite of the results of step-ups (Fig. 2B). The amounts of *frq* mRNA and FRQ decreased after the step-down, and the responses were similar at different times. FRQ became highly phosphorylated before its degradation (*17, 18*) and remained at a low level for about 8 hours before the initiation of a new cycle of synthesis at hour 12. Thus, after the step-down, the clock is reset to a new phase that is near the peak of the FRQ oscillation. In comparison, although the level of *frq* mRNA also decreased after the step-down, it reached the trough rather quickly (within 2 hours), and the magnitude of the decrease was not as great as with FRQ. Levels of *frq* mRNA remained low for several hours, started to increase at hour 8, and were comparable to the peak at 28°C by hour 12. For temperature step-downs then, the clock is reset by adjusting the amount of FRQ to a lower level that is appropriate for the lower temperature, a change reflecting both transcriptional and posttranscriptional effects.

Temperature, therefore, resets the *Neurospora* clock in part by changing the levels of *frq* and FRQ; as with light, the responses of these clock components were rapid (being visible within 15 min after a step) and proportional to the size of the step (*19*), just as the magnitude of phase shifts correlates with the size of the temperature step (*9, 10*). However, unlike resetting by light, in which an external factor triggers a cellular response outside of the clock to effect a change in phase, resetting by temperature appears to be the result of changes brought about directly within the oscillator. In the simplest case, there need be no independent temperature sensor to trigger a reaction; instead, the levels of clock components (*frq* and FRQ, for example) initially remain unchanged, but the relative levels are interpreted by the dynamics or "rules of the cycle" for the new temperature, and the levels respond accordingly by increasing or decreasing. Stated differently, the same absolute amount of a clock component, or ratio of components, corresponds to a different subjective time at different temperatures, and in terms of biological time, a step change in temperature that alters the relation between components has the same effect as an instantaneous step change in the amount of a clock component at a constant temperature. There are clear parallels between this line of thinking and the limit cycle view of circadian oscillators (*20*).

Our explanations provide a basis for understanding the direction and the magnitude of shifts caused by small temperature steps, but they can also speak to the action of large shifts from outside to within the physiological range. [Recent careful studies have considered the reverse of this, resetting by heat shock pulses (*21*).] All clocks operate within only a part of the physiological temperature range for the growth of an organism (*8, 18, 22*), and an additional characteristic of circadian systems is their ability to use single large temperature steps from outside to within the permissive temperature range as zeitgebers (*1, 8–10, 16, 22*). Thus, steps from temperatures above the permissive range to temperatures within it are interpreted as dusk (light to dark), setting the clock to about CT12, and steps from temperatures below the permissive range to temperatures within it are interpreted as dawn (*9, 10, 16, 22, 23*). To explore the molecular basis of this response, we monitored levels of *frq* mRNA and FRQ by following steps from a nonpermissive temperature to within the permissive temperature range (Fig. 3). At low temperatures, *frq* mRNA accumulated while FRQ dropped to low levels (Fig. 3A) (*19, 23*), producing levels typical of the late night during the circadian cycle (*17, 23*). On transfer from 12° to 25°C, RNA levels began to fall within 4 hours, and FRQ rose to a peak at ~8 hours after the transfer (Fig. 3A), which was a response of FRQ that would be anticipated in the normal cycle if the clock was held at CT22 to CT0, given a strong pulse of light, and then released. Conversely, when held at 38°C (above the permissive limit), *frq* mRNA and highly phosphorylated FRQ accumulated to moderate to high levels (Fig. 3B) (*19*) as normally seen late in the subjective day (Fig. 1) or during prolonged exposure to constant bright light (*3, 19*). On transfer to the permissive range, RNA and protein levels fell in concert as if the clock had been held at CT12 and then released. After 4 hours, the *frq* transcript began to reappear, peaking at ~16 hours after the transfer [as it would normally peak at ~16 hours after subjective dusk (*24*)], and FRQ peaked 4 hours later at hour 20. Thus, although we can only conjecture the molecular basis of the forces that drive *frq* mRNA and FRQ to the levels seen at the temperature extremes, it appears that phases of the oscillator following steps into the permissive range simply derive from the static amounts of *frq* mRNA and FRQ protein seen before the step.

The notion that light is the single most important time cue for clock resetting for all organisms is prominent in the literature, although only a few systematic studies have compared light and temperature (*7, 8, 9, 13, 14, 25, 26*). To better understand the relative strength and relation between the two entraining factors, we designed experiments in which we forced light and temperature to compete with each other and found, surprisingly, that temperature can be a stronger entraining factor than light. First, because both high temperature and light can elevate FRQ expression, cultures were grown in constant saturating bright light (LL) (>1000 lux) at temperatures ranging from 4° to 30°C and then transferred into darkness (DD) at 30°C (Fig. 4A). The cultures were thus presented with opposing phase cues: transfer from light to dark (LD) sets the clock to dusk (CT12), whereas a cold to warm transfer sets the clock to dawn (CT0). If light is always dominant over temperature, the phase of the clock should always be set to about CT12 (dusk); as predicted, the controls (30°C LL to 30°C DD) were set to about CT12 (the center of the first conidial band occurring ~10 hours after the

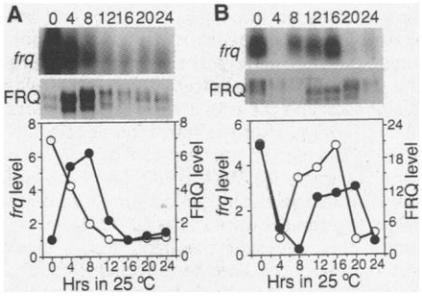

Fig. 3. Extended exposure to temperatures beyond the range for rhythmicity sets the amounts of *frq* mRNA and FRQ at characteristic levels. *Neurospora* liquid cultures were grown at 25°C LL (*31*) and then transferred into darkness at (**A**) 12°C or (**B**) 38°C for 12 hours before stepping to 25°C DD at 0 hours. Cultures were harvested either immediately or at 4-hour intervals for a full day and processed for Northern blot (top) and protein immunoblot (middle) analyses (*31*) [*frq* mRNA (open circles); FRQ (solid circles)]. Experiments in which the cultures were incubated at 12° and 38°C for 24 hours produced equivalent results.

LD transition). However, when cultures grown at 18°C LL (well within the physiological range) were stepped to 30°C, the rhythm was substantially delayed, which is consistent with the clock being set to about CT4. Further decreases in the LL growth temperature to 14°C or below (*27*) produced further delays to phases near CT23.5 (about subjective dawn). Thus, consistent with other suggestions (*14, 25*), physiologically natural temperature differences can be dominant over transitions between saturating light and total darkness.

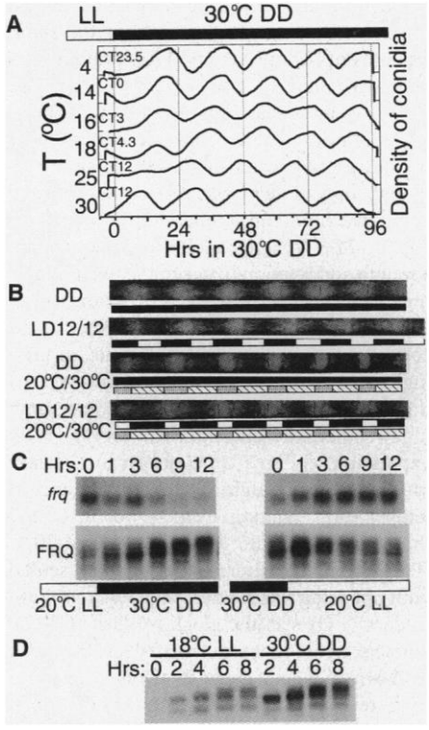

Fig. 4. Temperature can be a stronger entrainment factor than light. **(A)** When rhythmicity is initiated by a single environmental transition, temperature cues can take precedence over light. Race tube cultures grown in constant light at different temperatures (from 4° to 30°C) were transferred into 30°C DD (*31*), and the phase of the subsequent rhythms was monitored. Densitometric scans of the race tube images are shown to better compare the relative phases, and the actual steady-state phases of the rhythms after the transition as calculated (*16*) are shown on the top left of each rhythm's trace. **(B)** With conflicting light and temperature cycles, the conidiation rhythm can follow the temperature cycle. LD 12/12 cycles (white bars represent the light period; black bars represent the dark period) and 20°/30°C temperature cycles (stippled bars represent the cool period; hatched bars represent the warm period) were used. **(C)** Comparison of *frq* mRNA and FRQ induction after light and temperature shifts. **(D)** Side-by-side comparison of the amount of FRQ induced by a shift from dark to light conditions or from cool to warm conditions; a step elevation in temperature can produce stronger induction than a step from darkness to light. The cultures were grown in constant darkness at 18°C. At 0 hours, they were transferred either to 18°C LL or to 30°C DD, and samples were harvested and prepared for protein immunoblot analysis at the hours shown.

We then asked how temperature cycles along with LD cycles affect the phase of the overt rhythm (Fig. 4B). The controls were LD cycles at constant temperature (25°C) and temperature cycles (20° to 30°C) in constant darkness. As expected (*9, 10, 26*), conidiation occurred mostly during the dark period in an LD 12/12 cycle (12 hours of light and 12 hours of darkness) and in the low-temperature period of a 20°/30°C temperature cycle (12 hours at 20°C and 12 hours at 30°C). Temperature cycles yielded rhythms having greater synchrony than those seen in LD cycles; almost no conidiation occurred during the high-temperature period (Fig. 4B). To allow light and temperature to compete, we introduced reversed LD-temperature cycles (light and low temperature to dark and high temperature), reasoning that if LD cycles determined phase, we would see conidiation occurring mostly during the dark phase. Instead, conidiation occurred mostly during the light and 20°C period (Fig. 4B); the 20°/30°C cycle was more effective in determining the phase of the overt rhythm than the light to dark cycle. The 10°C span for a temperature cycle is not exceptional; such differences are encountered almost daily in the spring and fall in temperate regions of the world. Temperature can thus be a stronger environmental time cue for clock resetting than light.

An examination of the molecular correlates of rhythmicity confirmed this result (Fig. 4C) and further demonstrated the importance of the amount of FRQ protein to the overall determination of phase. Transfer from cool light to warm darkness produced a steady increase in the amount of FRQ but (after a transient increase) produced a decrease in *frq* transcript amounts. Conversely, although the reverse transfer (30°C DD to 20° LL) elicited the expected transient increase in the *frq* transcript, it ultimately produced a decrease in FRQ. The relative effects of light versus temperature are clearly seen in Fig. 4D; light induced the *frq* transcript, thereby promoting FRQ synthesis (*3, 17*), but the inherent temperature-responsive posttranscriptional controls on FRQ translation (Fig. 1A) (*18*) produced an effect that was twice as strong, substantially increasing the amount of FRQ present. These results complemented the overt rhythm data (Fig. 4, A and B), demonstrating for this oscillator that temperature, by influencing the amount of FRQ, could play a dominant role in setting the phase of the clock.

The ability of organisms to be entrained by environmental signals is a fundamental property of circadian clocks. We found that temperature resets the *Neurospora* circadian cycle by changing the set points and internal dynamics of the feedback loop, in response to which the levels of *frq* and FRQ change. FRQ oscillated around a higher level at higher temperatures as a result of posttranscriptional regulation, and temperature changes resulted in adjustments of *frq* mRNA and FRQ to levels required by the new temperature; responses were rapid and proportional but phase independent, in agreement with physiological studies (*9, 10*). Our work concentrated on steps as being ecologically relevant, but temperature pulses can be viewed simply as two juxtaposed steps (*7, 8*); during a pulse, the levels of *frq* mRNA and FRQ change in response to the new set points defined by the new temperature, so that on return to the ambient temperature, the oscillator will have moved to a different subjective time. Although we began the study of temperature effects with a mind-set that viewed temperature steps as akin to light treatments that would change the levels of clock components like FRQ, thereby resetting the oscillator, we came to understand that this sequence is in some sense reversed. It is instead, in broad outline, the relation among the components rather than their absolute amounts that is instantaneously changed, and the amounts of components like *frq* RNA and FRQ then respond according to the time represented by the new relation. Hence, the phase to which the clock is set after a temperature step appears to be primarily the phase that corresponds to the amounts of clock components existing at the time when the step occurs, but these amounts are interpreted in terms of the new temperature, as predicted in the limit cycle view of the clock pioneered by Winfree (*20*). Given that small-amplitude temperature cycles [1.5°C in *Drosophila* (*15*) and 1° to 2°C in lizards (*14*) and *Neurospora* (*16*)] entrain circadian oscillators and that daily temperature cycles in healthy human adults range from 1° to 1.5°C, with a low point just before dawn (*28*), it seems plausible that a benefit of the human body temperature cycle could be the daily nonphotic resynchronization of the body's cell and tissue autonomous clocks. Additionally, parallels between mammals and *Neurospora* in the effects of light (*6*) and in the phase of cycling of putative clock components *per1*, *per2*, and *per3* in darkness (*6, 29*) suggest that, like FRQ, the low point of human brain PER proteins could occur just before dawn at the time roughly corresponding to the temperature nadir. In nature, the asynchrony between light and temperature cycles may be significant for organisms living at temperate latitudes. In the spring and fall, for instance, daily oscillations in ambient temperature often span the lower boundary of the physiological range for rhythmicity (*Neurospora*, ~15°C; *Gonyaulax* and several plant species, ~12°C; Dro-

sophilids, 8° to 15°C; lizards, 10°C) (*8, 14, 22*), so that, under these conditions, the dominant entraining cue could be the temperature cycle. Data presented here provide insight on how physiologically and ecologically relevant temperature steps and pulses act to reset a day-phase circadian oscillator. More generally, the data provide another example in which highly conserved and plainly adaptive behaviors of a circadian system can be understood in terms of the straightforward responses of clock components to factors in the environment of the organism.

References and Notes

1. J. C. Dunlap, *Science* **280**, 1548 (1998); *Annu. Rev. Genet.* **17**, 579 (1996).
2. T. Kondo *et al.*, *Science* **266**, 1233 (1994); A. J. Millar, I. A. Carré, C. A. Strayer, N.-H. Chua, S. A. Kay, *ibid.* **267**, 1161 (1995); L. A. Sawyer *et al.*, *ibid.* **278**, 2117 (1997); J. Loros, *Semin. Neurosci.* **7**, 3 (1995); A. Sehgal, *ibid.*, p. 27; P. Hardin and K. Siwicki, *ibid.*, p. 15; D. Bell-Pedersen, N. Garceau, J. J. Loros, *J. Genet.* **75**, 387 (1996); J. C. Hall, *Trends Neurosci.* **18**, 230 (1995); M. W. Young *et al.*, *Cold Spring Harbor Symp. Quant. Biol.* **61**, 279 (1996); M. Rosbash *et al.*, *ibid.*, p. 265; P. Y. King *et al.*, *Cell* **89**, 641 (1997).
3. S. C. Crosthwaite, J. J. Loros, J. C. Dunlap, *Cell* **81**, 1003 (1995).
4. M. Hunter-Ensor, A. Ousley, A. Sehgal, *ibid.* **84**, 677 (1996); H. Zeng, Z. Qian, M. Myers, M. Rosbash, *Nature* **380**, 129 (1996); C. Lee, V. Parikh, T. Itsukaichi, K. Bae, I. Edery, *Science* **271**, 1740 (1996).
5. M. P. Myers, K. Wager-Smith, A. Rothenfluh-Hilfiker, M. W. Young, *Science* **271**, 1736 (1996).
6. Y. Shigeyoshi *et al.*, *Cell* **91**, 1043 (1997); U. Albrecht, Z. S. Sun, G. Eichele, C. C. Lee, *ibid.*, p. 1055; L. P. Shearman, M. J. Zylka, D. R. Weaver, L. F. Kolakowski Jr., S. M. Reppert, *Neuron* **19**, 1261 (1997).
7. C. S. Pittendrigh and V. G. Bruce, in *Photoperiodism and Related Phenomena in Plants and Animals*, R. B. Withrow, Ed. (American Association for the Advancement of Science, Washington, DC, 1959), pp. 475–505; C. S. Pittendrigh, *Cold Spring Harbor Symp. Quant. Biol.* **25**, 159 (1960).
8. V. G. Bruce, *Cold Spring Harbor Symp. Quant. Biol.* **25**, 29 (1960); B. Sweeney and J. W. Hastings, *ibid.*, p. 87; W. F. Zimmerman, C. S. Pittendrigh, T. Pavlidis, *J. Insect Physiol.* **14**, 669 (1968); E. Bünning, *The Physiological Clock* (Springer-Verlag, New York, ed. 3, revised, 1973).
9. V. D. Gooch, R. A. Wehseler, C. G. Gross, *J. Biol. Rhythms* **9**, 83 (1994).
10. S. Dharmananda, thesis, University of California, Santa Cruz (1980); H. Nakashima, *J. Interdiscip. Cycle Res.* **18**, 1 (1987).
11. I. Edery, J. E. Rutila, M. Rosbash, *Science* **263**, 237 (1994).
12. R. K. Barrett and J. S. Takahashi, *J. Neurosci.* **15**, 5681 (1995); M. Zatz, G. Lange, M. Rollag, *Am. J. Physiol.* **266**, R50 (1994); C. Heintzen *et al.*, *Plant J.* **5**, 799 (1994).
13. C. H. Johnson, *An Atlas of Phase Response Curves for Circadian and Circatidal Rhythms* (Department of Biology, Vanderbilt University, Nashville, TN, 1990).
14. H. Underwood and M. Calaban, *J. Biol. Rhythms* **2**, 179 (1987); K. Hoffman, *Z. Vgl. Physiol.* **62**, 93 (1969).
15. D. A. Wheeler, M. J. Hamblen-Coyle, M. S. Dushay, J. C. Hall, *J. Biol. Rhythms* **8**, 67 (1993).
16. C. Francis and M. L. Sargent, *Plant Physiol.* **64**, 1000 (1979).
17. N. Y. Garceau, Y. Liu, J. J. Loros, J. C. Dunlap, *Cell* **87**, 469 (1997).
18. Y. Liu, N. Y. Garceau, J. J. Loros, J. C. Dunlap, *ibid.*, p. 477.
19. Y. Liu, M. Merrow, J. Loros, J. C. Dunlap, data are not shown.
20. A. T. Winfree, *The Geometry of Biological Time*, vol. 8 of *Biomathematics* (Springer-Verlag, New York, 1980); M. Menaker *et al.*, Eds., *Biochronometry* (National Academy of Sciences, Washington, DC, 1971), pp. 81–106; E. L. Petersen, *J. Theor. Biol.* **84**, 281 (1980); V. D. Gooch, in *Temporal Order*, L. Rensing and N. I. Jaeger, Eds. (Springer-Verlag, New York, 1985), p. 232; P. L. Lakin-Thomas, S. Brody, G. G. Coté, *J. Biol. Rhythms* **6**, 281 (1991).
21. D. Sidote, J. Majercak, V. Parikh, I. Edery, *Mol. Cell. Biol.* **18**, 2004 (1998).
22. E. Bünning, *Jahrb. Wiss. Bot.* **81**, 411 (1935); D. Njus, L. McMurry, J. W. Hastings, *J. Comp. Physiol. B* **117**, 335 (1977).
23. S. K. Crosthwaite, J. C. Dunlap, J. J. Loros, *Science* **276**, 763 (1997).
24. B. D. Aronson, K. A. Johnson, J. J. Loros, J. C. Dunlap, *ibid.* **263**, 1578 (1994).
25. S. A. Finlayson, I. Lee, P. W. Morgan, *Plant Physiol.* **116**, 17 (1998).
26. L. Rensing, *Chronobiol. Int.* **6**, 297 (1989).
27. The further delay of phases with decreasing temperature is not due to a long time being needed for warming of the culture; warming of the race tubes from 4° to 30°C occurs in less than 20 min. To confirm this, in another set of experiments, we first transferred the cultures to 30°C LL for 20 min before the light was turned off, and the same amount of phase delay was observed (*19*).
28. C. A. Czeisler, thesis, Stanford University (1978); J. Aschoff and R. Wever, in *Handbook of Behavioral Neurobiology*, J. Aschoff, Ed. (Plenum, New York, 1981), vol. 4, pp. 311–336.
29. H. Tei *et al.*, *Nature* **389**, 512 (1997); Z. S. Sun *et al.*, *Cell* **90**, 1003 (1997).
30. Cultures of *bdA* were inoculated into 14 sets of six race tubes each prepared as previously described (*18, 24*). The cultures were grown in constant light for ~24 hours and then were transferred into constant darkness either at 21°C (seven sets) or 28°C (seven sets). After 48 hours and at five 4-hour intervals thereafter, groups of race tubes were reciprocally shifted from 21° to 28°C or vice versa; for two control sets, there was no temperature step given after they were transferred into darkness. As a result of the difference in temperature, there were slight differences in the period length between the two sets: the average period length was 22.3 hours at 21°C and 20.5 hours at 28°C.
31. The *bdA* (wild-type clock) strain was used in all the experiments described in this study. Conditions used for liquid culture experiments were as described (*3, 24*). For the experiment shown in Fig. 1A, after the cultures were grown in LL at 25°C for a few hours, they were transferred from L to D and from 25°C to either 21° or 28°C at hour 0. Thirteen hours later and, subsequently, at 5- to 6-hour intervals, samples were collected and used as a source for RNA and protein (*3, 17, 23, 24*). Equal amounts of total RNA (40 μg) or protein (100 μg) were loaded onto agarose or acrylamide gels for electrophoresis as previously described (*3, 18, 23*), and the gels were blotted and probed as appropriate either with a *frq* RNA-specific probe (*3*) or with an antibody to FRQ (*17*). Equal loading among lanes was confirmed by probing the RNA blot with a ribosomal DNA probe (*3*) and by staining the protein blot with amido black (*18*). After developing the blots, densitometry was performed (*17, 18*).

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RNA-Mediated Trans-Activation of Transcription from a Viral RNA

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The red clover necrotic mosaic virus genome is composed of two single-stranded RNA components, RNA-1 and RNA-2. The viral capsid protein is translated from a subgenomic RNA (sgRNA) that is transcribed from genomic RNA-1. Here, a 34-nucleotide sequence in RNA-2 is shown to be required for transcription of sgRNA. Mutations that prevent base-pairing between the RNA-1 subgenomic promoter and the 34-nucleotide trans-activator prevent expression of a reporter gene. A model is proposed in which direct binding of RNA-2 to RNA-1 trans-activates sgRNA synthesis. This RNA-mediated regulation of transcription is unusual among RNA viruses, which typically rely on protein regulators.

RNA performs many of the functions that were once thought to be restricted to proteins. RNA molecules perform various enzymatic reactions in addition to catalyzing peptide bond formation (*1*). Given this diversity of functions, it is not surprising that gene expression can be regulated posttranscriptionally by the structure or stability of an mRNA (*2*). Noncoding RNAs as well as the 3′ untranslated regions (3′ UTRs) of cellular mRNAs function as trans-acting regulators of cell division and differentiation (*3*). In the nematode *Caenorhabditis elegans*, the small noncoding lin-4 RNAs alter the stability or translatability (or both) of lin-14 mRNAs by interacting with their 3′ UTRs (*4*). However, RNA-mediated regulation of transcription from an RNA molecule has not been observed.

Red clover necrotic mosaic *Dianthovirus* (RCNMV) contains two RNA components, a polycistronic RNA-1, which encodes the viral polymerase and capsid protein (CP), and RNA-2, which encodes the viral movement protein

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