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23. At face value, $P < 0.05$ is not stringent enough for this study because for ~100 markers one expects at least five positives to occur by chance alone. Similarly, at the $P < 0.01$ level, a few false positives could occur. Thus, we mainly describe results that (i) were significant at $P < 0.001$, or (ii) were significant at $P < 0.01$ and were confirmed by both statistical tests. Other associations are suggestive, pending further analysis with additional markers.
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38. We thank J. Peterson and M. Cote for technical expertise, P. Neumann for valuable assistance and comments on mapping the epilepsy genes, E. Lander and S. Lincoln for advice and data analysis using MAPMAKER/QTL, and an anonymous reviewer for several helpful suggestions. This research was submitted to Boston College in partial fulfillment of the M.S. degree (M.L.R.) and was supported by PHS grants to T.N.S. (NS 23355) and NIH grants to J.M.C. (R35CA44385). W.N.F. is a special fellow of the Leukemia Society of America.

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Alteration of the Phase and Period of a Circadian Oscillator by a Reversible Transcription Inhibitor

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A function for transcription in the mechanism of a circadian oscillator was investigated with the reversible transcription inhibitor 5,6-dichloro-1-β-D-ribobenzimidazole (DRB). Two-hour treatments with DRB shifted the phase of the circadian rhythm of the isolated eye of *Aplysia*, and continuous treatments of DRB lengthened the free running period of this rhythm. Camptothecin, an inhibitor of transcription that is structurally unrelated to DRB, had similar effects on the circadian rhythm. These results suggest that transcription may be part of the circadian oscillating mechanism.

A REMARKABLE PROPERTY OF CELLS is their ability to generate endogenous rhythms with periods close to 24 hours. For example, a small piece of a pineal organ exhibits a circadian rhythm of melatonin secretion (*1*). Elucidation of the oscillator mechanism responsible for such rhythms requires identification of its components and determination of the regulatory processes by which these components interact.

Progress is being made in the identification of putative components of circadian oscillators. Researchers have identified a number of proteins by tracing environmental entrainment information to the oscillator (*2*), by tracing output pathways back to the oscillator (*3*), and by looking for proteins that are rhythmically synthesized (*4*). Moreover, genes that alter circadian rhythms have been identified (*5*). Some candidates for the cellular regulatory processes that link the putative oscillator components are protein phosphorylation (*6, 7*), Ca^{2+} regulation (*7, 8*), and protein synthesis (*9*). Protein synthesis is the most widely implicated regulatory process. For example, several inhibitors of protein synthesis shift the phase and change the period of the circadian rhythm associated with the isolated eye of *Aplysia* (*10, 11*). Furthermore, inhibitors of protein synthesis block some effects of entraining agents on the *Aplysia* eye rhythm (*2*). These entraining agents also change the synthesis of a number of eye proteins (*2*). Experimental results that implicate translation are also consistent with the participation of transcription in the circadian oscillator mechanism.

The results of a number of recent studies indicate that changes in transcription regulate circadian rhythms (*12*). In addition, transcription inhibitors abolish circadian rhythms, but these findings were difficult to interpret because irreversible transcription inhibitors were used in these experiments (*13*). We have investigated the effects of a reversible inhibitor of transcription, 5,6-dichloro-1-β-D-ribobenzimidazole (DRB), on the circadian rhythm of spontaneous nerve impulses from the isolated eye of *Aplysia*. DRB inhibits the synthesis of heterogeneous nuclear RNA at the level of transcription initiation by interfering with the RNA polymerase II function (*14*).

Isolated *Aplysia* eyes were treated with DRB for 2 hours during circadian time (CT) 06 to 08 hours, and a dose-dependent shift in the phase of the circadian rhythm was observed (*15*) (Fig. 1A). Treatment with DRB at 10^{-7} M did not produce phase shifts, whereas 10^{-6} M and 10^{-5} M DRB produced delay phase shifts of 1.8 ± 0.2 hours ($n = 4$) and 4.4 ± 0.4 hours ($n = 4$), respectively. To examine the sensitivity of the rhythm to DRB throughout a circadian cycle, we generated a phase response curve (PRC) by treating isolated eyes with DRB (10^{-4} M) for 2 hours at different phases of the rhythm. The effects of DRB on the rhythm were phase-dependent (Fig. 1B).

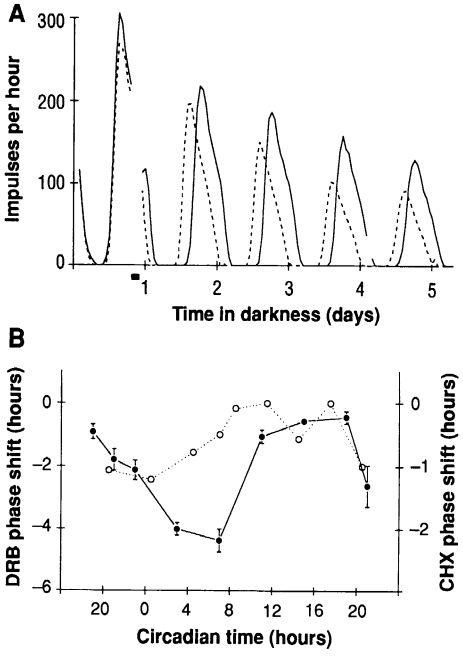

Fig. 1. Effect of DRB on the phase of the ocular circadian rhythm. (**A**) A delay shift in the phase of the rhythm was produced by a 2-hour treatment with DRB (10^{-4} M) during CT 06 to 08 hours, shown by the dark bar under the x axis (dashed line, control; solid line, DRB). The frequency of spontaneous optic nerve impulses from two isolated eyes of the same animal is plotted as a function of the time the eyes were in constant darkness. (**B**) Phase shifts of the rhythm are plotted as a function of the time of exposure of isolated eyes to DRB (closed circles). The error bars represent SEMs. The number of eyes exposed at different phases to DRB were the following: six (CT 18 to 20), five (CT 20 to 22), four (CT 22 to 24), four (CT 02 to 04), four (CT 06 to 08), four (CT 10 to 12), four (CT 14 to 16), five (CT 18 to 20, second cycle), and four (CT 20 to 22, second cycle). Data for the phase shifts of the rhythm produced by 1-hour CHX treatments (open circles) were derived from (*11*).

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Treatments with DRB for 2 hours produced delay phase shifts when administered over the interval CT 20 to 12 hours but had no effect on the rhythm when given over the interval CT 12 to 20 hours. The lack of an effect of DRB at some phases of the rhythm was not a result of a differential inhibition of transcription. Treatment with DRB inhibited total RNA synthesis in the eye as measured by its effect on the incorporation of [^{3}H]uridine into trichloroacetic acid (TCA)–precipitable material (*16*). Treatment with DRB (10^{-4} M) had similar effects on the incorporation of [^{3}H]uridine when DRB produced phase shifts (CT 02 to 04 hours, 72 ± 3% inhibition, n = 4) and when it did not produce phase shifts (CT 14 to 16 hours, 75 ± 3% inhibition, n = 4). The frequency of spontaneous nerve impulses was inhibited 56 ± 5% (n = 21) during exposure to 10^{-4} M DRB but not during exposure to 10^{-5} M DRB (9 ± 11%, n = 5).

The effect of different concentrations of DRB on [^{3}H]uridine incorporation was correlated with the effect of DRB on the rhythm. Treatment with DRB inhibited incorporation by 72 ± 3% at 10^{-4} M, 40 ± 4% at 10^{-5} M, and 10 ± 2% at 10^{-6} M. Because DRB is reported to affect only RNA polymerase II, it should inhibit only mRNA synthesis (*14*). Thus, the magnitudes of inhibition by DRB that we observed probably underestimate its effect on mRNA synthesis because we only assessed its effects on total RNA synthesis. Incorporation of [^{3}H]uridine increased to 77 ± 7% (n = 4) and 99 ± 4% (n = 3) of control values 3 and 5 hours after removal of DRB (10^{-4} M), respectively. Treatment with DRB appeared to have no effect on translation as measured by [^{3}H]leucine incorporation during 2-hour DRB treatments (*17*).

We next tested the effect of continuous treatments of DRB on the ocular circadian rhythm. Continuous exposure of *Aplysia* eyes to DRB at 10^{-7} M had no effect on the period of the rhythm (0.2 ± 0.1 hour, n = 4), but DRB at 10^{-6} M lengthened the period of the rhythm by 1.6 ± 0.1 hours (n = 4) (Fig. 2A) (*18*). A higher dose of DRB (10^{-5} M) further lengthened the period of the rhythm (8.0 ± 0.7 hours, n = 5) (Fig. 2B). Exposure to 5×10^{-5} M DRB appeared to abolish the rhythm after one broad cycle (Fig. 2C). The eyes with no apparent rhythms produced spontaneous spikes with normal amplitudes, but the frequency of these spikes was greatly reduced.

To test whether the effect of DRB on the rhythm was a result of its inhibition of transcription and not a nonspecific effect, we explored the effects of camptothecin, a transcription inhibitor that functions by inhibiting topoisomerase I (*19*). Camptothecin, like DRB, inhibited [^{3}H]uridine incorporation and produced delay phase shifts in the rhythm (*20*). Continuous treatment of eyes with camptothecin (2×10^{-8} M) produced a small but significant lengthening of the period of the rhythm (0.50 ± 0.05 hour, n = 7). The effects of camptothecin on the rhythm were apparent over a very narrow range of doses and appeared to be more variable than those produced by DRB. For example, pulse treatments of 4×10^{-7} M camptothecin shifted the phase of the rhythm, but 2×10^{-7} M camptothecin appeared to have no effect.

Our results indicate that DRB acts on the ocular circadian rhythm through a primary effect on transcription. One can examine the temporal relation between the sensitivity of the circadian system to inhibitors of translation and transcription by comparing a PRC obtained with 1-hour treatments of *Aplysia* eyes with the translation inhibitor cycloheximide (CHX) (*11*) and with the PRC reported here for DRB (Fig. 1B). The beginning of the intervals of sensitivity to DRB (CT 20 to 22 hours) and CHX (CT 20 to 21 hours) appear to coincide.

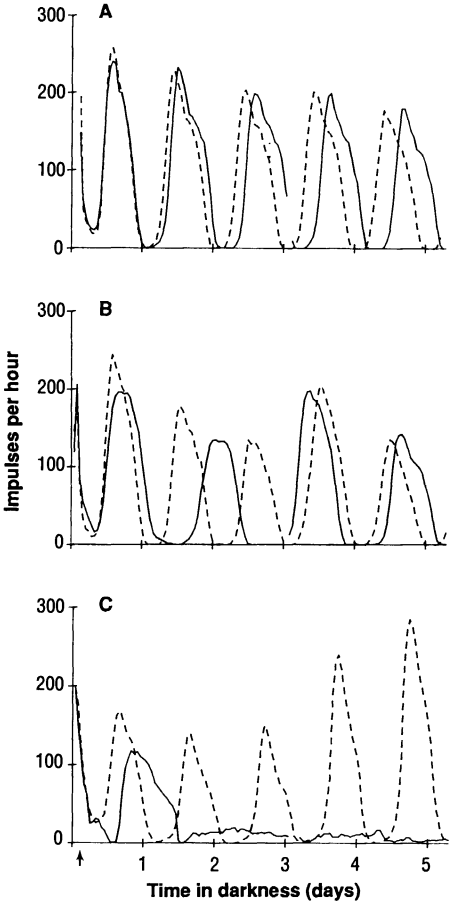

Fig. 2. Effect of continuous treatments of DRB on the periods of the rhythm (dashed line, control; solid line, DRB). Exposure of eyes to DRB began at the time shown by the arrow at the bottom of the figure. Concentrations of DRB were (**A**) 10^{-6} M, (**B**) 10^{-5} M, and (**C**) 5×10^{-5} M.

The close temporal relation between the PRCs for DRB and CHX indicates that the effects of transcription and translation are linked. Although the [^{3}H]leucine incorporation was not affected during DRB treatment (*17*), we found that the synthesis of at least five specific proteins was altered during 2-hour DRB treatments (*21*). Further support for a close coupling of transcription and translation in regulation of the rhythm comes from the appearance of phase shifts in the rhythm within a few hours after the end of 2-hour treatments with DRB (Fig. 1A). Therefore, DRB most likely alters the rhythm by a primary effect on transcription and a subsequent secondary effect on translation.

Our results indicate that a critical period for the transcription of specific genes involved in the generation of circadian rhythms occurs from CT 20 to 10 hours. Alternative functions of transcription in the circadian system are also consistent with our data. For example, transcription and translation may be necessary to maintain the supply of a protein component of the oscillator with activity that is rhythmically regulated in some way other than at the level of transcription or translation. Distinguishing between the alternative possibilities requires the identification and study of the relevant mRNAs and proteins. Although we have not defined the precise role of transcription and translation, our results demonstrate that at least one short-lived mRNA and protein are important for circadian timing over a particular time interval. Identification of specific proteins that are decreased by brief DRB treatments in the interval from CT 20 to 10 hours and mRNAs that are newly transcribed around CT 20 hours may help elucidate the nature of the circadian oscillator.

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15. Spontaneous optic nerve impulses were recorded from isolated experimental and control eyes of the same animals. *Aplysia californica* were entrained to cycles of 12 light hours and 12 dark hours before they were used in experiments. Eyes were isolated at CT 12 hours and kept at 15.5°C in constant darkness in buffered filtered seawater (BFSW) (10 ml). The last offset of light experienced by intact animals was at CT 12 hours. Thus, CT 24 or CT 00 hours is the projected time of light onset while the isolated eyes are maintained in constant dark conditions. The frequencies of nerve impulses were obtained with a personal computer that allowed us to count, print out, and plot data over the 5 days of experiments. Chemical treatments were administered while we kept the eyes in darkness by completely exchanging the solution surrounding the eyes. We terminated treatments by removing the experimental solution and rinsing the eyes with ten exchanges of BFSW over a 1-hour interval. Chemical treatments (2-hour), which were used to obtain the PRC, were administered during an interval that began 6 hours after eyes were isolated and extended for about one cycle from this point. Further details of experimental procedures and media have been described [A. Eskin, *J. Neurobiol.* **8**, 273 (1977)]. Five cycles of rhythms were recorded. We measured phase shifts by comparing the peaks of rhythms of matched experimental and control eyes during the fourth cycle of the rhythm. Phase shifts produced by DRB appeared stable; the value obtained for the third, fourth, and fifth cycles were not significantly different from one another. For example, for DRB given at CT 02 to 04 hours, the phase shifts for the third, fourth, and fifth cycles were 3.6 ± 0.2 hours (±SEM), 4.0 ± 0.2 hours, and 3.5 ± 0.4 hours (n = 4), respectively.
16. To measure the effect of various treatments on transcription, we determined the incorporation of [^{3}H]uridine (ICN Radiochemicals, Irvine, CA) into TCA-precipitable material. Groups of three control and three contralateral experimental eyes were used for each experiment. The experimental group was treated with the drug 15 min before being exposed to [^{3}H]uridine (40 μCi/ml) and then exposed to the drug and [^{3}H]uridine for 2 hours. Except as noted in the text, exposure of eyes to [^{3}H]uridine began at CT 06 hours. At the end of experiments, eyes were rinsed with ice-cold BFSW and homogenized on ice in a tris-HCl buffer that contained EDTA, EGTA, protease inhibitors, urea, CHAPS, and β-mercaptoethanol. Details of this buffer and the method of TCA precipitation have been described (*2*). Incorporation of [^{3}H]uridine into total RNA (TCA-precipitable material) was corrected for an effect of DRB on the uptake of uridine as described [D. Granick, *J. Cell Biol.* **65**, 398 (1975); I. Tamm, R. Hand, L. A. Caliguiri, *ibid.* **69**, 229 (1976)]. In this procedure, the ratio of experimental to control TCA-precipitable radioactivity was divided by the ratio of experimental to control total radioactivity of the homogenized samples.
17. We measured the effect of DRB on protein synthesis by determining its effect on the incorporation of [^{3}H]leucine (ICN Radiochemicals) into TCA-precipitable material. Experimental eyes were exposed for 2 hours to [^{3}H]leucine (40 μCi/ml) and 10^{-4} M DRB, and then the TCA-precipitable radioactivity was measured as described (*16*). The [^{3}H]leucine incorporation in DRB-treated and control eyes differed by 8 ± 12%, n = 4.
18. To measure the effect of DRB on the period of the rhythm, we exposed experimental eyes continuously to DRB a few hours after the eyes were isolated. The period difference between experimental and matched control eyes averaged 2.1 ± 0.8 hours (cycle 1 to 2), 1.4 ± 0.2 hours (cycle 2 to 3), 1.1 ± 0.2 hours (cycle 3 to 4), and 1.7 ± 0.1 hours (cycle 4 to 5) (n = 4). The average free-running periods of the control eyes during the same cycles were 21.5, 24.4, 23.8, and 23.2 hours, respectively.
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20. Camptothecin was initially dissolved in dimethyl sulfoxide (DMSO). The final concentration of DMSO (0.01%) did not affect the ocular circadian rhythm. Incorporation of [^{3}H]uridine into TCA-precipitable material, measured as described (*16*), was inhibited by the following amounts by camptothecin: 58 ± 5% (4×10^{-7} M, n = 4) and 33 ± 14% (2×10^{-8} M, n = 3). Camptothecin at a concentration of 4×10^{-7} M (CT 06 to 08 hours) produced delay phase shifts of 2.2 ± 0.8 hours, n = 5.
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Concurrent Processing and Complexity of Temporally Encoded Neuronal Messages in Visual Perception

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The intrinsic neuronal code that carries visual information and the perceptual mechanism for decoding that information are not known. However, multivariate statistics and information theory show that neurons in four visual areas simultaneously carry multiple, stimulus-related messages by utilizing multiplexed temporal codes. The complexity of these temporal messages increases progressively across the visual system, yet the temporal codes overlap in time. Thus, visual perception may depend on the concurrent processing of multiplexed temporal messages from all visual areas.

THE ABILITY OF MONKEYS TO RECOGnize pictures depends on a large number of cortical and subcortical brain regions (*1*). To understand how neurons within these regions contribute to visual perception, we must learn what codes they use to transmit information. Despite many years of studying visual neurons, researchers have failed to define this intrinsic code. In the classical hypothesis, neurons encode information about images only in the number of action potentials (spikes) in their responses by using a mean firing rate or strength code (*2*). However, neurons in four brain regions spanning the visual system in the monkey also carry information about images in the distribution of spikes within the response (*3, 4, 5*). This temporal code is two to three times more efficient than the strength code in representing visual information. Furthermore, the proportion of the information carried by the temporal modulation increases in regions more distant from the retina. These findings show that temporal modulation that is dependent on the stimulus is a common neuronal mechanism, and we hypothesize that it may be important for visual perception.

We recorded electrophysiologically from the following: ganglion cell fibers (RET), which carry visual information out of the retina; neurons in the parvocellular division of the lateral geniculate nucleus (LGN), which receive input from the retina and which project to the primary visual cortex (V1); complex cells in layers two and three of V1, which receive input from layer four of V1 and project to later visual areas; and neurons in the inferior temporal cortex (IT), which receive input from earlier visual areas and project beyond the visual system to the limbic system (*6*). Awake monkeys were trained to fixate on a small spot while stimuli were presented on a video monitor. The stimuli used in these experiments (Fig. 1) consisted of a complete set of two-dimensional black-and-white pictures based on Walsh functions (*7*). Neuronal responses were quantified by the Karhunen-Loève transform (KLT), which is similar in principle to a Fourier transform but does not use sine waves as its basis. Instead, the basis of the KLT is a set of waves of excitation and inhibition, called principal components (Fig. 2, ϕ_0 through ϕ_3), that must be computed separately for each neuron. Because no principal component can be represented by a sum of the others, each can be interpreted as a separate element in a temporal code.

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