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12. Volunteers were immunized as with gp160 in a phase I vaccine trial organized by the NIAID AIDS Vaccine Clinical Trials Network. The design of the trial, including provisions for informed consent, will be described elsewhere (R. Dolin *et al.*, in preparation).
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17. A fragment of gp41 consisting of residues 546 to 645 of the envelope protein of the BRU isolate was expressed as a fusion protein in *Escherichia coli* using a glutathione S-transferase fusion protein expression vector (Glutagene, Medos Co., Victoria, Australia). Affinity-purified fusion protein was used to pulse B-LCL at a concentration of 60 μg/ml.
18. The use of vaccinia virus vectors for the expression of the HIV-1 *env* gene has been described [S. Chakrabarti, M. Robert-Guroff, F. Wong-Staal, R. C. Gallo, B. Moss, *Nature* **320**, 535 (1986)]. We used the vectors vPE7 and vPE16 which contain the *env* gene of IIIb-derived BH8 clone of HIV. Control infections were carried out with the vaccinia vector vSC8 (vac), which lacks the HIV *env* gene. Target cells were infected with recombinant vaccinia viruses at a multiplicity of infection of 10 for 12 to 16 hours at 37°C prior to labeling with ^{51}Cr and use in the cytolytic assay. Strain specificity was investigated using a vaccinia vector carrying the *env* gene from the MN strain (*6*).
19. Using a vaccinia vector carrying a truncated form of the HIV *env* gene with a stop codon at the end of the gp120 coding sequence, we showed that infected B-LCL that produced and secreted a large amount of gp120 did not process the protein for recognition by $CD4^+$, gp120-specific CTLs. Rather, attachment to a membrane anchor sequence was critical for delivery of gp120 to the compartment where this processing occurs [M. Polydefkis *et al.*, *J. Exp. Med.* **171**, 875 (1990)].
20. Three days after activation with PHA, targets were mock-infected or infected with HIV. Infections were done by resuspending the target clones in cell-free supernatants of supT1 cells infected 7 days previously with HIV-1 (IIIb isolate). After 4 hours at 37°C, cells were washed and then cultured in interleukin-2 (IL-2)–containing media. Infected cells were used as targets in CTL assays 4 days after exposure to HIV. At this time, infected cells showed no evidence of cytopathic effects and were uniformly positive for gag expression.
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23. Day 7 PBMC cultures positive for gp160-specific CTL activity were restimulated with irradiated (5000 R), gp160-pulsed autologous monocytes and cultured for an additional week in the presence of IL-2. Responding cells were then cloned by limiting dilution following stimulation with irradiated gp160-pulsed autologous monocytes or with PHA and irradiated allogeneic PBMC. Clones were screened for gp160-specific cytolytic activity as described above. Procedures for the characterization and long-term culture of human T cell clones have been previously described (*13*).
24. Cytolytic activity was measured as described (*13*) except that the assay period was 8 hours. All determinations were performed in quadruplicate. Data are expressed in the form of the mean + SEM of the % specific lysis determined as described [R. F. Siliciano, A. D. Keegen, R. Z. Dintzis, H. M. Dintzis, H. S. Shin, *J. Immunol.* **135**, 906 (1985)]. For all CTL assays, the SEM of the percent specific lysis was generally <5%.
25. We thank the SAVE study volunteers for their essential contributions, C. Hilton and E. Ellerbeck for coordinating the collection of samples, D. Pardoll, G. Ada, and D. Fearon for a critical reading of the manuscript, C. Montell and M. Strand for providing gp41 fusion proteins, P. Earl and B. Moss for providing vaccinia vectors, and S. Stern of the NIAID AIDS Research and Reference Reagent Program. Supported by National Institute of Allergy and Infections Diseases grant AI28108 and NIH contract NO1AI62515, PHS grant 5T32CA09243 (R.O.), and a short-term training grant from the National Institutes of Health (NIH 5T35HL07606) and by a Henry Strong Denison Award (to M.P.).

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Light Pulses That Shift Rhythms Induce Gene Expression in the Suprachiasmatic Nucleus

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Lighting cycles synchronize (entrain) mammalian circadian rhythms by altering activity of cells in the suprachiasmatic nucleus (SCN) of the hypothalamus, a circadian pacemaker. Exposure of hamsters and rats to light pulses at those phases of the circadian rhythm during which light can shift the rhythm caused increased immunoreactivity for the product of the immediate-early gene c-*fos* in cells in the region of the SCN that receives retinal fibers. Light pulses also increased messenger RNA for the Fos protein and for the immediate-early protein NGFI-A in the rat SCN. Similar increases in mRNA for NGFI-A were seen in the SCN of hamsters. Thus cells in this portion of the SCN undergo alterations in gene expression in response to retinal illumination, but only at times in the circadian cycle when light is capable of influencing entrainment.

DAILY (CIRCADIAN) RHYTHMS ARE a pervasive feature of mammalian physiology and behavior (*1*). They are controlled by a system that includes a central pacemaker (or clock), which generates an endogenous near 24-hour periodicity, and an entrainment mechanism, which responds to environmental lighting cycles by adjusting the period of this rhythm to precisely 24 hours and synchronizing its phase to local time. The entrainment mechanism operates by producing daily phase shifts in the pacemaker, primarily in response to light exposure between dusk and dawn; the circadian system is relatively insensitive to light during the day (*2*).

In mammals, the dominant pacemaker for many daily rhythms is the suprachiasmatic nucleus (SCN), located in the anterior hypothalamus immediately dorsal to the optic chiasm (*3*) (Fig. 1A). Two visual projections reach the SCN, one originating in the retina (*4, 5*) and one originating in a retinorecipient area of the lateral geniculate nuclei [the intergeniculate leaflet (IGL) and adjacent

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parts of the ventral lateral geniculate nucleus (vLGN)] (*6*). These projections have overlapping terminal zones in and near the SCN; they convey the photic information that entrains the circadian system (*7*).

A good deal is known about the physiology of entrainment in mammals: photic stimuli or activation of the optic nerves alter firing and metabolic rates of SCN cells (*8–10*), and putative neurotransmitters have been identified that appear to be involved in conveying photic information to the SCN (*6, 10*). However, the cellular and molecular mechanisms by which light information causes phase shifts of the SCN pacemaker are not known.

Expression of the proto-oncogene c-*fos* is altered in mammalian neurons in response to a number of stimuli (*11–14*), and c-*fos* expression has been proposed to be a useful marker of neural activation (*13*). We examined whether either spontaneous or light-induced changes in cellular activity in the SCN were accompanied by altered expression of c-*fos* and another immediate-early gene, NGFI-A (*15*).

We housed male hamsters (*16*) in individual boxes under a light-dark 14:10 lighting cycle [14 hours of light (L) daily from General Electric Cool White fluorescent tubes (~30 lux), alternating with total darkness (D)]; male rats (*16*) were housed individually on open shelves in a room exposed to an LD 12:12 lighting schedule. For immunocytochemical studies, animals were removed from their cages and killed immediately with an overdose of barbiturate anesthetic (>250 mg per kilogram of body weight) at various times in their normal lighting cycle, or after a 48-hour exposure to continuous darkness (DD) (*17*). Some animals were first exposed to either 30 or 60 min of light (~30 lux) immediately before being killed. Brains were perfused with 4% paraformaldehyde and 70-μm-thick sections through the hypothalamus and thalamus were cut on a Vibratome. Tissue was reacted for Fos-like immunoreactivity (Fos-lir) with a modified avidin-biotin labeling method with diaminobenzidine as the chromogen (*11, 12*). Three different primary antibodies were used in dilutions from 1:1000 to 1:5000 (*18*).

No SCN cells showed Fos-lir in the brains of nine hamsters killed during the D phase of an LD cycle or at any phase tested in DD (Fig. 1C), although spontaneous Fos-lir was usually seen in cells in the paraventricular nucleus of the thalamus (PVTh). Of 12 hamsters killed immediately after a 30- or 60-min light pulse during the D phase, all showed robust nuclear staining in SCN cells. In the rostral SCN, stained cells were found at the dorsal and lateral borders of the SCN, but throughout most of the SCN there was a heavy concentration of labeled cells in the ventral and lateral SCN (Fig. 1B), with scattered cells extending dorsally as far as the PVTh and adjacent thalamic areas. The pattern of label in the SCN and in the immediately adjacent periventricular hypothalamus corresponded to the area identified as the terminal zone of the direct retinal projection to the hypothalamus with cholera toxin–conjugated horseradish peroxidase as a tracer for retinal afferents (*5*). We refer to this as the "RHT pattern." The distribution of responsive cells is also consistent with evidence that cells within and dorsal to the SCN respond neurophysiologically to photic stimuli (*9*).

Fourteen hamsters killed after 30- or 60-min light pulses during the projected mid-D phase (subjective night) in DD all showed

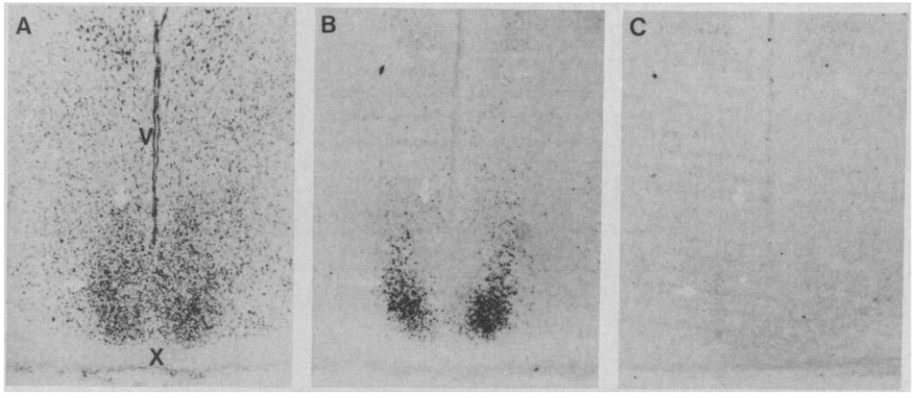

Fig. 1. (**A**) A coronal section through the hypothalamus of a hamster, stained with cresyl violet to show the location of the SCN V, third ventricle; and X, optic chiasm. The white arrows show the approximate dorsal and lateral borders of one SCN in (A) to (C); (**B**) A section through the SCN region of a hamster brain stained for Fos-lir with a rabbit antibody (Medac) (*18*) at a dilution of 1:5000. The hamster was killed 5 hours into the dark phase of the daily LD cycle, after 60 min of light exposure. (**C**) A section treated as in (B) from the brain of a hamster killed 4 hours into the dark phase without prior light exposure.

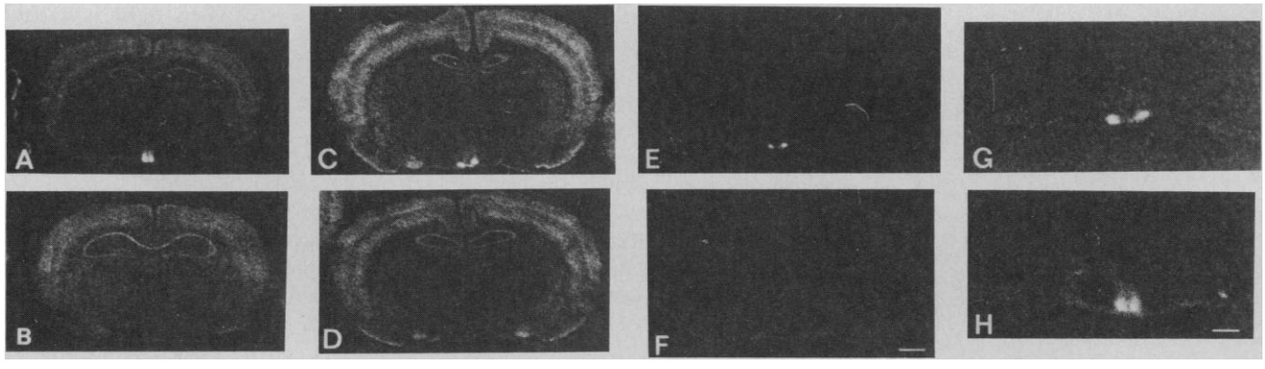

Fig. 2. Autoradiograms of sections of rat and hamster brains through the SCN from in situ hybridization studies of the Fos and NGFI-A mRNAs (*21*). All animals were killed during a standard LD cycle, either 4 hours after the lights were turned off (no light treatment) or 4.5 hours after the lights were turned off (30 min of light exposure). (**A**) Hamster brain showing NGFI-A mRNA after a 30-min light exposure. (**B**) Hamster brain showing NGFI-A mRNA in darkness. (**C**) Rat brain showing NGFI-A mRNA after a 30-min light exposure. (**D**) Rat brain showing NGFI-A mRNA in darkness. (**E**) Rat brain showing Fos mRNA after a 30-min light exposure. (**F**) Rat brain showing Fos mRNA in darkness. (**G**) Rat hypothalamus showing Fos mRNA after a 30-min light exposure; the anatomical distribution is similar for NGFI-A mRNA. (**H**) Hamster hypothalamus showing NGFI-A mRNA after a 30-min light exposure. The scale bar in (F) applies to (A) through (F), 1 mm; scale bar in (H) applies to (G) and (H), 0.5 mm.

similar patterns of heavy nuclear label in retinorecipient portions of the SCN. Of 12 hamsters killed during the projected L phase (subjective day) immediately after a similar light pulse, 1 had a substantial number of stained cells in the SCN, 3 had a few stained cells in and near the rostral SCN, and 8 had no stained SCN cells; none showed Fos-lir in the RHT pattern. These results suggest that the increases in Fos-lir after light exposure during the subjective night (Fig. 1B) are specific to the time of day during which light can phase shift rhythms.

This suggestion was confirmed by the results of studies of animals killed during the L phase of their normal LD cycle. Of six hamsters killed throughout the L phase, beginning 4 hours after the lights were turned on, three had labeled cells at the rostral edge of the SCN and a few scattered cells around the SCN, and none showed Fos-lir in the RHT pattern characteristic of animals exposed to light at night. We evaluated the effects of light onset in a stable lighting cycle with eight hamsters in two photoperiods (LD 14:10 and 18:6). Increased Fos-lir was observed in the SCN of two of four hamsters killed in each photoperiod approximately 1 hour after light onset. By contrast with the middle of the subjective night, light at dawn is not universally effective in inducing Fos-lir. Because of individual differences in patterns of entrainment, light at dawn may or may not exert phase-shifting effects in different animals (*1, 2*).

Similar results were found in studies with rats. Two rats killed during the dark phase showed little or no Fos-lir in SCN cells, but 60 to 90 min of light exposure during the dark phase produced strong Fos-lir in SCN cells in two rats. The pattern of staining resembled the anatomical pattern of RHT innervation in the rat, which is different from that found in hamsters (*5*). Photic regulation of Fos-lir in the rat SCN has also been reported with different antibodies to Fos (*19*).

The IGL and adjacent parts of the vLGN have also been implicated as part of the entrainment pathway in hamsters (*7*). Cells in this region showed no Fos-lir in either species in animals killed in the dark. In ten hamsters and two rats that received light pulses and showed clear increases in Fos-lir in the SCN, we examined the geniculate area in detail, using sheep antibody. Five hamsters had strong label in the IGL and the external portion of the vLGN, and five had scattered labeled cells in the same regions; both rats showed strong label in the IGL. The dorsal LGN, which is also innervated by retinal ganglion cells, did not show increased Fos-lir in response to these light pulses.

Fos-lir is increased in other systems by de novo protein synthesis after a rapid increase in mRNA transcription (*14*), so we tried to determine whether the mRNA for Fos is also increased by nighttime light exposure by using an in situ hybridization method. In addition, we examined the expression of a second immediate-early gene product, NGFI-A (*15*), which has been shown to be closely associated with the induction of long-term potentiation in the hippocampus (*20*). Rats and hamsters were removed from their cages and rapidly decapitated 4 to 5 hours into the D phase, either without manipulation of lighting or immediately after a 30-min light pulse. Their brains were dissected, blocked, and frozen on dry ice until analyzed for levels of Fos and NGFI-A mRNA (*21*).

Autoradiograms showed an absence of mRNAs for both Fos and NGFI-A in the SCN of five hamsters and four rats killed in darkness during the D phase (Fig. 2, B, D, and F). Exposure to light for 30 min in the D phase caused dramatic increases in the mRNA for Fos (Fig. 2E) and NGFI-A (Fig. 2C) in the SCN of all four rats tested and for NGFI-A in all five hamsters (Fig. 2A). The oligonucleotide probe based on rat Fos failed to recognize hamster Fos mRNA, possibly because of a sequence difference between the rat and hamster c-*fos* genes. However, light-induced increases in Fos mRNA have been found in the hamster SCN with the use of a different probe (*22*). The patterns of autoradiographic label in the SCN differed in the two species, and these differences paralleled those for Fos-lir and for the patterns of RHT innervation in the SCN (Fig. 2, G and H).

The increase in Fos-lir observed in cells in the retinorecipient area of the SCN does not simply reflect increased metabolic activity. SCN metabolic activity, as measured by the 2-deoxyglucose uptake method, peaks during the subjective day (*23*); similarly, firing rates of SCN neurons in rats and hamsters are highest during the middle of the subjective day (*24*), yet there was neither a spontaneous nor a light-evoked increase in Fos-lir in the retinorecipient zone of the hamster SCN during the subjective day or the L phase.

The protein products of c-*fos* and related immediate-early genes may function as tertiary cellular messengers that interact with other gene products to alter DNA transcription (*13, 25*); thus, Fos-induced changes in DNA transcription in the SCN and IGL could have a role in phase-shifting the circadian pacemaker. The finding that photic stimuli selectively increase c-*fos* expression only at times when light can shift rhythms provides a model for studying the role of cellular factors in the regulation of c-*fos* and other immediate-early genes, as well as for the further analysis of the circadian entrainment pathway.

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16. The sources for the animals used were as follows: hamsters (*Mesocricetus auratus*), Lak:LVG, SYR, Charles River, Montreal, Quebec, and Canadian Hybrid Farms, Centreville, Nova Scotia; rats (*Rattus norvegicus*), Sprague-Dawley, Canadian Hybrid Farms.
17. Animals killed in darkness were exposed to a dim red light (<1 lux) filtered through a Kodak DBX-2 safelight filter for ~30 s just before death.
18. A rabbit antibody (Medac, Hamburg) was raised against a β-galactosidase–Fos fusion protein expressed in bacteria with a Fos insert corresponding to amino acids 151 to 292 of the mouse Fos protein [B. Verrier, D. Müller, R. Bravo, R. Müller, *EMBO J.* **5**, 913 (1986)]. An affinity-purified sheep antibody [OA-11-823; Cambridge Research Biochemicals (CRB), Cambridge] was raised against a synthetic peptide corresponding to amino acids 2 to 16 in the NH_2-terminal region, a sequence that is conserved in the mouse and human Fos molecule. Immunoblot analysis (CRB technical data sheet for batch 03078, 10 October 1989) indicates that it recognizes Fos (62 kD) and several Fos-related antigens (at 48/49 and 70 kD). Incubation of the sheep antibody with the synthetic peptide antigen

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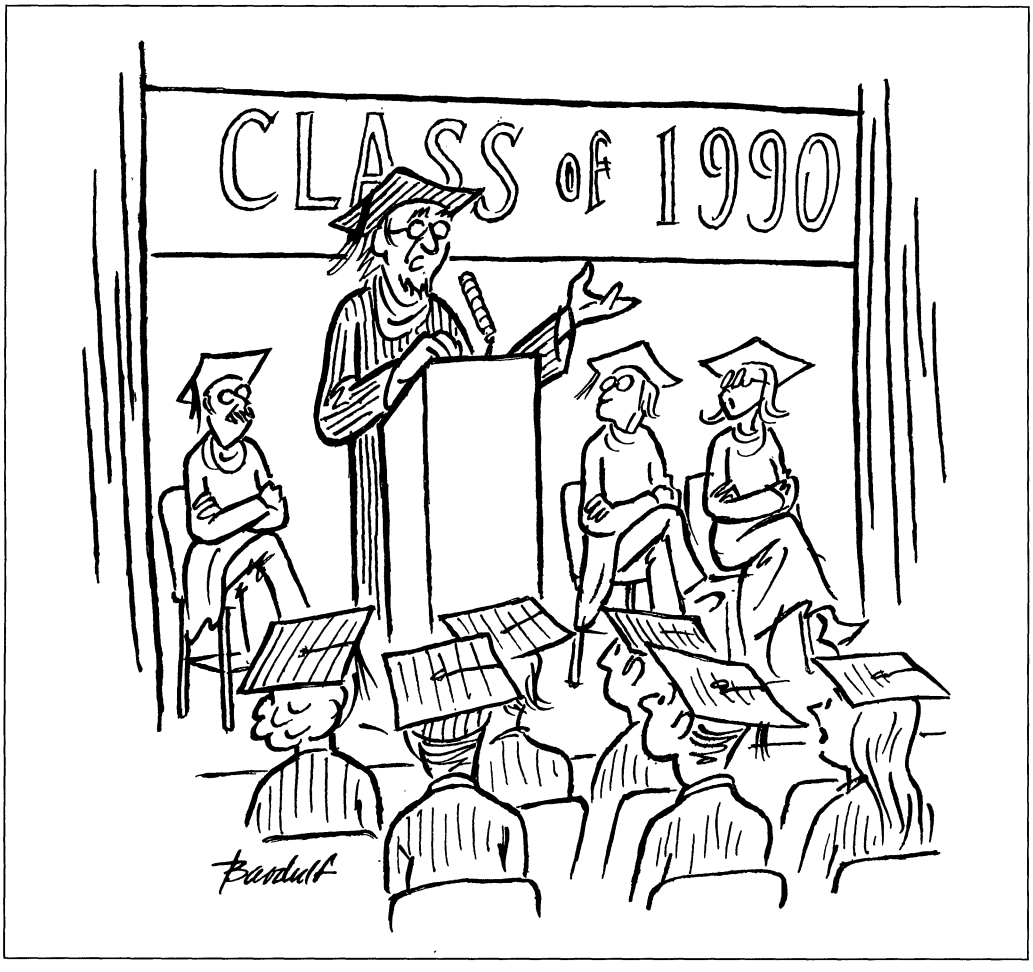

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