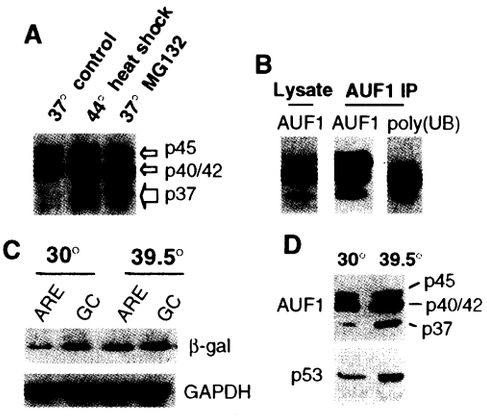

Fig. 4. (**A**) Stabilization of AUF1 by proteasome inhibition. HeLa cells at 37°C were untreated or treated for 4 hours with 20 μM MG132 or heat-shocked for 3 hours at 44°C. Equal amounts of lysates were resolved by gel electrophoresis and immunoblotted for AUF1. (**B**) AUF1 was immunoprecipitated from HeLa cells and immunoblotted with antibodies to ubiquitin (Zymed). (**C**) Mouse *ts*85 cells were transfected with plasmids encoding reporter β-gal GC-control or ARE-mRNAs for 36 hours, then maintained at nonrestrictive (30°C) or restrictive temperature (39.5°C) for 8 hours to inactivate the ubiquitin-activating enzyme E1. Total RNA was isolated and equal amounts used for Northern mRNA analysis. (**D**) Immunoblot analysis was performed using equal amounts of whole-cell lysates and antisera to AUF1 or p53 protein (p53 antibody; Santa Cruz sc99).

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12. Constructs contained the SV40 early promoter, a cap-independent 5′UTR (adenovirus tripartite leader) or a cap-dependent leader (*22*), the *lacZ* coding region, 3′UTRs containing the GM-CSF ARE or a mutated control ARE interspersed with G and C residues (GC control), and the SV40 splice-polyadenylation signal. Constructions are available upon request. HeLa cells were transfected in triplicate using Lipofectamine (Gibco-BRL), maintained at 37°C or heat-shocked at 44°C for the times indicated. Messenger RNA half-life analysis was carried out (*9*) with actinomycin D (5 μg/ml) to inhibit transcription. Total cell RNA was purified with Trizol reagent (Gibco-BRL), resolved by formaldehyde-agarose gel electrophoresis, and hybridized on membranes to ^{32}P-labeled probes. HeLa cells were labeled with 100 μCi of [^{35}S]methionine for 4 hours in Dulbecco's modified Eagle's medium without methionine containing 2% bovine serum. Cells were lysed in 1% NP-40, 150 mM NaCl, 20 mM Hepes (pH 7.5), and 2.5 mM EDTA with protease inhibitors (Boehringer-Mannheim Complete), resolved by SDS-PAGE, and visualized by fluorography. β-Galactosidase was immunoprecipitated from equal amounts of protein with antibodies to β-gal (5′-3′ Inc.). Results were quantitated by digital densitometry and are typical of three independent experiments. For indirect immunofluorescence, polyclonal antisera to all AUF1 isoforms (*3*), mouse monoclonal antibody (mAb) to hsp70 (W-27, Santa Cruz), or mouse α-tubulin mAb (Sigma) were reacted on cover slips. Secondary antibodies were sheep antibody to rabbit immunoglobulin G conjugated with fluorescein (AUF1) and sheep antibody to mouse immunoglobulin G conjugated with rhodamine (hsp70 or α-tubulin). Secondary antibody reacted in the absence of primary antibody showed no staining. Images were acquired on a Zeiss Axiophot photomicroscope.
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15. [^{35}S]Methionine-labeled HeLa cell lysates (*12*) from control cells or cells heat-shocked for 4 hours were clarified by centrifugation at 10,000*g* for 15 min at 4°C. AUF1 was immunoprecipitated from equal amounts of protein, resolved by SDS-PAGE, and then fluorographed. AUF1 was immunoprecipitated from equal amounts of unlabeled cell extracts treated with 2 μg of RNase A for 30 min at 30°C, followed by SDS-PAGE and immunoblotting with antibodies specific to hsp70, hsc70, or hsp105 (StressGen), eIF4G, PABP, or AUF1. Blots were developed with enhanced chemical luminescence (Amersham). Data represent typical results of three independent experiments. Polysomes were isolated by lysis of HeLa cells (*12*) (with or without RNase treatment) and centrifuged at 3000*g* for 2 min at 4°C, then supernatant was centrifuged at 430,000*g* for 30 min at 4°C in a Beckman TL-100 ultracentrifuge. The polyribosome pellet was collected and resolved by SDS-PAGE.
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23. We thank R. Morimoto for heat shock reagents, M. Rechsteiner for mouse *ts*85 cells, G. Dreyfuss for antiserum to PABP, N. Sonenberg for antiserum to eIF4G, and C. Daly, J. deDiego, and S. Srivastava for advice. Supported by NIH grants CA42357 (R.J.S.) and CA52443 (G.B.) and a fellowship from the Fundación Ramón Areces (R.C.).

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Regulation of Mammalian Circadian Behavior by Non-rod, Non-cone, Ocular Photoreceptors

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Circadian rhythms of mammals are entrained by light to follow the daily solar cycle (photoentrainment). To determine whether retinal rods and cones are required for this response, the effects of light on the regulation of circadian wheel-running behavior were examined in mice lacking these photoreceptors. Mice without cones (*cl*) or without both rods and cones (*rdta/cl*) showed unattenuated phase-shifting responses to light. Removal of the eyes abolishes this behavior. Thus, neither rods nor cones are required for photoentrainment, and the murine eye contains additional photoreceptors that regulate the circadian clock.

Mammals use light both to generate a visual image of their environment and to provide time-of-day information. Internal circadian time is synchronized (entrained) with the solar day by light-induced resetting (photoentrainment) mechanisms, which correct for deviations in the period and phase of the endogenous clock (*1*). Eye loss in both human and nonhuman mammals abolishes photoentrainment, demonstrating that the eyes provide the primary source of light information to the clock (*2*). However, the retinal projections that convey light information to the visual and circadian centers

of the brain are quite distinct (*3*), and visual blindness due to partial loss of rod and cone photoreceptors is not necessarily associated with an attenuation of circadian responses to light (*2*). Collectively, these findings have led to speculation that (i) the mammalian circadian system can maintain normal photosensitivity with only small numbers of rods or cones; and (ii) the eye contains unrecognized photoreceptors that mediate, or help mediate, the effects of light on the circadian system (*4*). In the absence of an experimental model completely lacking rods and cones, distinguishing between these alternatives has been problematic.

Two mouse models have been used previously to examine the impact of rod photoreceptor loss on circadian physiology: (i) mice homozygous for *retinal degeneration* (*rd/rd*) gradually lose all rod photoreceptors but retain normal circadian responses to light (*5*); and (ii) transgenic mice (*rdta*) undergo specific ablation of rod photoreceptors during early development (*6*) and are also circadian photosensitive. The responses of *rdta* mice were about twice as great as those of wild-type and *rd/rd* mice of the same genetic background (*7*). Loss of the eyes in both *rd/rd* and *rdta* mice abolished the effects of light on the circadian system. Collectively, these results showed that rods are not required for circadian photoentrainment and that the photoreceptors mediating these responses are ocular.

Both *rd/rd* and *rdta* mice sustain a secondary degeneration of cone photoreceptors. However, limited numbers of cones remain into old age (*6*, *8*), making them strong candidates for the regulation of temporal physiology. The murine retina contains two populations of cones, sensitive in the green [maximum wavelength (λ_{max}) = 508 nm] (*9*) and ultraviolet (UV) (λ_{max} = 359 nm) (*10*). Both cone classes have been implicated in photoentrainment by action spectrum studies (*11*). Moreover, the identification of a fully functional "green" photopigment (λ_{max} = 534 nm) within the eyes of the blind mole rat (*Spalax ehrenbergi*) provides indirect evidence for the involvement of cones in circadian regulation (*12*).

To determine the impact of cone photoreceptor loss on photoentrainment, we used mice in which cone photoreceptors were ablated by the introduction of a synthetic transgene (*cl*) (*13*). This construct consists of a portion of the human red cone opsin promoter, attached to an attenuated diphtheria toxin gene (*14*). The retinas of these mice have normal numbers of rods and a substantially reduced number of UV cones (>95% lost) and appear to lack green cones (<1% remain in some retinas) (*13*). Our molecular (Fig. 1) (*15*) and immunocytochemical analysis (*16*) of the *cl* retina confirms these findings. Despite this massive insult to cone photoreceptors, *cl* mice showed unattenuated circadian responses to monochromatic 509-nm light (Fig. 2A). Bilateral enucleation abolished the ability of *cl* mice to entrain to a 12 hour light:12 hour dark cycle and to phase shift their circadian locomotor rhythm in response to a light pulse (*17*). In view of the loss of green cones, these data suggest that green cone photoreceptors are not required for photoentrainment. Moreover, the insensitivity of UV cones to 509-nm light (*10*) suggests that a non-cone photoreceptor is involved in this process. As rods remain unaffected in *cl* mice, under these circumstances, rods might mediate photoentrainment. Although previous studies with both *rd/rd* and the *rdta* mouse models indicate that rod photoreceptors are not required for circadian photoentrainment (*5*, *7*), our results might reflect redundancy of photoreceptor inputs to the clock, with both rod and cone photoreceptors providing photic input to the

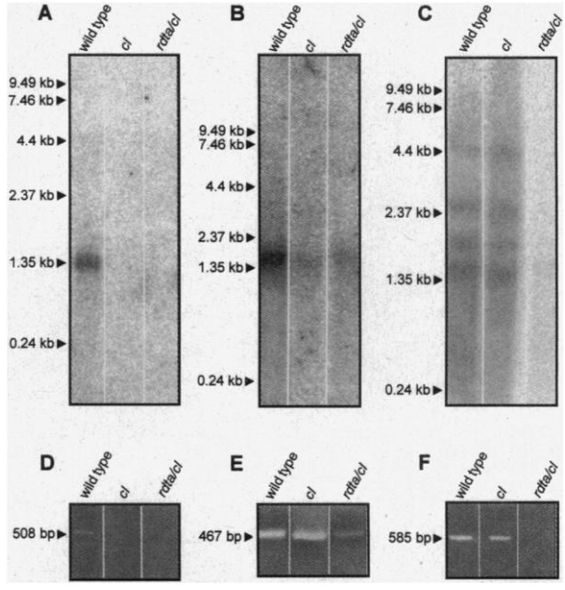

Fig. 1. Effect of transgenic ablation on the expression of photoreceptor genes in *cl* and *rdta/cl* mice. Northern blot (**A** through **C**) and RT-PCR (**D** through **F**) detection of mRNA-encoding green cone opsin (A and D), UV cone opsin (B and E), and rod opsin (C and F) in wild-type and transgenic retinas (*15*). Introduction of the *cl* transgene rendered green cone opsin mRNA undetectable by Northern blot (A) in either *cl* or *rdta/cl* genotypes. RT-PCR techniques also failed to amplify a band visible on an ethidium bromide–stained agarose gel in either genotype (D). The effect of the *cl* transgene on UV cones was less marked, with UV cone opsin mRNA detectable in both *cl* and *rdta/cl* mice by Northern blotting (B) and RT-PCR techniques (E). Rod photoreceptors were unaffected by the *cl* transgene. By contrast, the *rdta/cl* retina contained no rod opsin transcript (C and F); bp, base pairs.

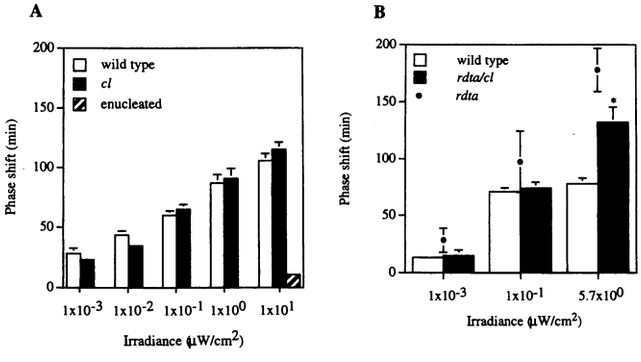

Fig. 2. Irradiance-dependent phase shifts of circadian locomotor activity (*17*). (**A**) Phase shifts of locomotor activity in *cl* mice. Phase shifts (mean ± SEM) of wild-type and *cl* mice, after exposure to a defined irradiance, 15-min monochromatic light (509 nm) pulse delivered at CT16 (n = 6 to 15 animals per genotype at each irradiance). There were no significant differences between *cl* or wild-type mice at irradiances that produce either saturating or subsaturating phase shifts [two-way analysis of variance (ANOVA): $P > 0.05$]. (**B**) Phase shifts of locomotor activity in *rdta/cl* transgenic mice. Phase shifts (mean ± SEM) of wild-type and *rdta/cl* mice, after exposure to a 15-min monochromatic light (509 nm) pulse delivered at CT16 (n = 5 to 7 animals per genotype at each irradiance). Both genotypes showed an irradiance-dependent increase in the amplitude of phase shifts. However, at an irradiance of 5.7 μW/cm², phase shifts were significantly enhanced in *rdta/cl* mice, compared with wild-type mice (two-way ANOVA: $P < 0.001$; post hoc Student–Newman-Keuls tests comparing genotypes at each irradiance: *, $P < 0.05$). For further discussion, see (*23*).

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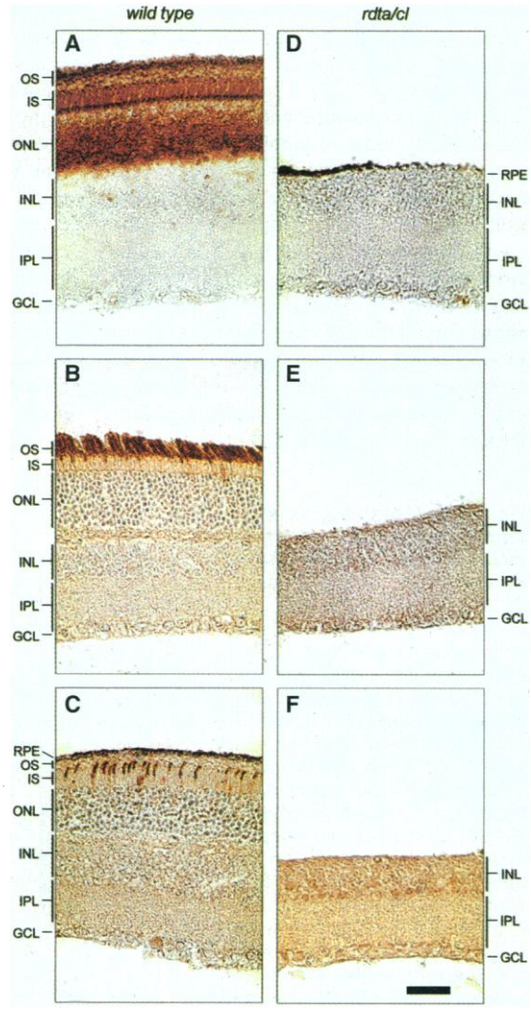

Fig. 3. Histological analysis of serial sections from wild-type (**A** through **C**) and *rdta/cl* (**D** through **F**) retinas (*15*). Immunocytochemical staining failed to identify rod or cone photoreceptors in the retinas of *rdta/cl* mice. Tissue was fixed with Bouins (75% picric acid, 25% formalin, and 5% acetic acid) for 24 hours and paraffin-embedded, and 8-μm sections were treated with antibodies recognizing rod (A and D), rod and green cone (B and E), and UV cone (C and F) photoreceptors. Visualization was accomplished with ABC methods (Vectastain Elite, Vector Labs). GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer; ONL, outer nuclear layer; IS, inner segments; OS, outer segments; and RPE, retinal pigment epithelium. Scale bar, 40 μm.

circadian system. Hence, the absence of either cell type might be compensated for by the presence of the other. To resolve this issue, we generated mice that carry lesions to both rod and cone photoreceptors by introduction of the *cl* transgene into mice heterozygous for the rodless (*rdta*) transgene. Immunocytochemical and mRNA analyses of *rdta/cl* mouse retinas (*15*) indicate that both rod and green cone photoreceptors and their associated photopigments are eliminated from the retinas of these mice (Figs. 1 and 3). Despite the absence of rods and green-sensitive cones, *rdta/cl* mice show unattenuated circadian phase shifts in response to a 15-min monochromatic light (509 nm) pulse of varying irradiance (Fig. 2B).

These results demonstrate that the mammalian eye contains non-rod, non-cone photoreceptors capable of regulating circadian behavioral responses to light. Published data suggest strongly that these receptors use a vitamin A–based photopigment (*11*, *18*). Nonetheless, their molecular basis has been the subject of considerable recent speculation (*19*–*22*). The *rdta/cl* model provides an opportunity to address this issue by determining the spectral sensitivity of these uncharacterized photoreceptors.

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15. Total RNA was extracted from (n = 5 to 10) retinas from wild-type, *cl*, and *rdta/cl* mice with the Promega (Southampton, UK) SV RNA Isolation kit. For Northern (RNA) blot analysis, 5 μg of total RNA from each genotype was size-separated on a 1.2% agarose-formaldehyde electrophoresis gel and blotted overnight onto Hybond N+ membrane (Amersham). Three blots were hybridized with ^{32}P-labeled probes specific for mouse rod opsin {cDNA probe after W. Baehr *et al.* [*FEBS Lett.* **238**, 253 (1988)]} and green and UV cone opsins {cRNA probes after M. von Schantz *et al.* [*Mol. Brain Res.* **770**, 131 (1997)]} and, subsequently, with a ^{32}P-labeled cDNA probe for glyceraldehyde phosphate dehydrogenase. Two micrograms of total RNA from each genotype was used for reverse transcriptase–polymerase chain reaction (RT-PCR) analysis. PCR amplification was then undertaken with primers designed against the published sequences for mouse rod opsin, green cone opsin, UV cone opsin, and tubulin (positive control) over 35 cycles under optimized conditions. Appropriate negative controls (total RNA without RT step) were included for all genotypes. Southern (DNA) blotting of the gel shown in Fig. 1D and hybridization with an appropriate radiolabeled DNA probe resulted in a barely detectable band in both genotypes. The exquisitely sensitive nature of RT-PCR, our failure to find green cone opsin protein (see Fig. 3) or green cone cyclic guanosine monophosphate gamma phosphodiesterase mRNA with RT-PCR (*16*), and the results of R. J. Lucas, M. S. Freedman, M. Muñoz, J. Garcia-Fernández, and R. G. Foster [*Science* **284**, 505 (1999)] argue that these levels of message are not physiologically significant. Immunocytochemical analysis used polyclonal antisera (*14*) directed against the human blue cone pigment (1:8000 dilution) and human red-green pigments (1:8000 dilution) and a monoclonal antibody against rat rod opsin (1:20,000 dilution) [D. Hicks and R. S. Molday, *Exp. Eye Res.* **42**, 55 (1986)].
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23. The simplest explanation for this enhanced response is that it reflects the presence of the *rdta* transgene. Previous experiments with *rdta* mice have suggested that the ablation of rod photoreceptors induced by this construct early in development results in an increase in the amplitude of phase shifts in response to high irradiance light. Our results in *rdta/cl* mice fall within the range of the published phase shifts for *rdta* mice (indicated on Fig. 2B) exposed to this irradiance. For a full discussion of the *rdta* phenotype, see (*7*).

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