Purpose. Mutations at various loci on the rhodopsin gene have been shown to cause autosomal dominant retinitis pigmentosa (ADRP). One of the most common is a point mutation (P23H) near the N-terminus of the protein. The authors have studied the effects of light deprivation on the rate of degeneration in pigmented transgenic mice expressing the P23H mutation as well as two additional mutations near the N-terminus of opsin (V20G, P27L).

Methods. Transgenic and normal littermates were reared in darkness or in cyclic light (~7 foot-candle) for periods of 2, 4, or 6 months. Retinal structure and function were evaluated by electroretinography, retinal densitometry, light microscopy, and TUNEL labeling.

Results. Retinas of normal animals, whether reared in darkness or in cyclic light, had no structural or functional abnormalities. The rate of photoreceptor degeneration in dark-reared transgenic mice was significantly slower than in transgenic mice raised under cyclic light conditions. Differences between the two groups of animals were evident in the retinal histology, the electroretinographically determined sensitivity to photic stimulation, and the rhodopsin levels in the retina. TUNEL labeling of retinal wholemounts showed that cyclic light-reared animals had a threefold higher incidence of photoreceptor cell death than their dark-reared counterparts; the density of apoptotic cells was greatest in the inferior retina, the region most severely affected in patients with the P23H mutation. In comparison, photoreceptor cell death was more uniformly distributed across the retina in dark-reared transgenic mice.

Conclusions. These findings suggest that light activation of rhodopsin contributes to the severity of the degenerative disease resulting from the P23H opsin mutation, and they raise the possibility that minimizing exposure to light may help to prolong useful vision of patients with this form of retinitis pigmentosa. Invest Ophthalmol Vis Sci. 1996;37:775–782.
occurring form of ADRP and involves substituting histidine for proline at position 23 (P23H). The other two, the substitution of glycine for valine at position 20 (V20G) and leucine for proline at position 27 (P27L), have not been detected in patients with retinal disease but were introduced to facilitate antibody recognition of the mutant protein. Mice bearing the transgene are referred to as VPP to identify the amino acids changed in the transgene product. We studied VPP mice that express equal amounts of mutant and wild-type transcripts. Like humans with the P23H mutation, VPP mice exhibit slowly progressive photoreceptor degeneration, with early onset decline in the rod-mediated electroretinogram (ERG); initial sparing of cone photoreceptors, which become involved only at later stages of the disease; significantly delayed rate of dark adaptation; and sensitivity losses that can be ascribed solely to the lowered rhodopsin content of the rod photoreceptors, i.e., a reduced probability of quantum absorption. Indeed, the many similarities in the pathophysiology of the disease suggest that the VPP mouse is a useful model for humans with this form of ADRP.

In the current study, litters of normal and VPP mice were reared in either complete darkness or under typical laboratory cyclic light conditions, and they were tested at different ages with a battery of experimental procedures to document the histologic, electrophysiological, and photochemical properties of the outer retina. We found that rearing the VPP mice in complete darkness significantly retarded the process of photoreceptor degeneration.

MATERIALS AND METHODS

Transgenic Mice

The mice were derived from matings of normal mice to mice heterozygous for the VPP transgene, all on a C57BL/6 background. Because the transgene is passed in a Mendelian fashion, approximately half the offspring were normal and half were transgenic. Normal and VPP mice were distinguished by polymerase chain reaction amplification with a set of primers (AGACTGACAGGGAGGAAATCCAGA and GCGTTGATCACAGCATCTGA) producing a 1317-bp fragment. In the normal mouse, this fragment is cut twice with the restriction enzyme NcoI and gives rise to 689, 431-, and 197-bp bands on an agarose gel. In transgenic mice, the deletion of one of the NcoI sites results in the generation of two bands (886 and 431 bp).

Pregnant females were placed into one of two light conditions, and the offspring were raised from birth in that environment. Cyclic light-reared mice were raised under a 12-hour light–12-hour dark cycle; cage illumination was ~7 foot-candles during the light cycle. Dark-reared mice were raised from birth in complete darkness in a separate room within the animal facility. For dark-reared mice, husbandry was performed under dim long-wavelength illumination. Except for the lighting conditions, cyclic light-reared and dark-reared mice were not treated differently, and no differences were detected in the body weight or general appearance of mice raised in the two environments. Several litters of mice were dark reared on separate occasions; the results obtained from these litters were in general agreement and were pooled for statistical purposes. The cyclic light-reared group was composed of mice gathered from a greater number of litters and tested within a 1-year period.

Electroretinography

Electroretinograms were recorded from anesthetized dark-adapted mice aged 2, 4, and 6 months using procedures described previously. Mice were transported to the recording room in a light-tight box and were dark adapted overnight. At each recording session, a dark-adapted luminance–response function was obtained for a stimulus range of ~3.13 to 0.85 log cd sec/m². Stimuli were presented in increasing order, and the responses to two successive flashes were averaged. A 30-second interflash interval was used for the lower stimuli; a 1-minute interval was used for the more intense stimuli. At the end of the recording session, the mice were killed by cervical dislocation, and the eyes were processed for histology, rhodopsin measurements, or biochemical assays. Procedures for animal care and experimentation were in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Histology

Enucleated eyes were opened at the ora serrata and placed in 0.1 M phosphate buffer (pH 7.4) containing 2% formaldehyde, 2.5% glutaraldehyde, and 0.0025% CaCl₂ at 4°C. After 2 hours in fixative, the anterior segments were discarded, and, after a buffer rinse, the tissues were postfixed in 1% OsO₄ in buffer containing 0.1 M cacodylate for 90 minutes. After dehydration through a graded ethanol series, eyecups were infiltrated overnight with a 1:1 mixture of Epon/Araldite; 1-μm sections were stained with azure-II–methylene blue. The sections were approximately along the horizontal meridian and passed through the optic nerve; the distribution of photoreceptor nuclei was nearly symmetric in the nasal and temporal regions of the retina. Photoreceptor nuclei were counted in a microscopic field that was centered at 300 μm from the edge of the optic nerve head and extended laterally across 70 μm of the outer nuclear layer.

Rhodopsin Densitometry

In situ measurements of rhodopsin density were obtained with a microscope-based fundus reflectome-
FIGURE 1. Histology of retinas of mice reared in darkness or in cyclic light. Micrographs of retinal sections from a 2-month-old normal mouse (A) and from VPP mice at 2 (C,D), 4 (E,F), and 6 (G,H) months of age; the animals were reared either in cyclic light (A,C,E,G) or in darkness (D,F,H). Note that the retinas of normal mice were not affected by the cyclic-light conditions (A). Retinal sections from VPP mice show progressive degenerative changes for both dark- and light reared animals (C to H), but, at every age, animals exposed to cyclic light showed more severe degeneration. Bar = 20 μm; * = outer nuclear layer (ONL).

(B) Number of photoreceptor nuclei plotted as a function of age. Data are expressed as percentages of the values obtained from normal littermates for cyclic light- and dark-reared mice; each data point represents the mean (± SEM) of 3 to 6 measurements. At each age examined, both the thickness and cellular content of the ONL were significantly greater in dark-reared than in cyclic light-reared VPP mice.

that was adapted for transmission measurements through the flatmounted isolated retinas of pigmented animals; details of the rapid scan, computer-based instrument have been described previously. Briefly, absorbance difference spectra were derived from transmissivity data recorded at 26 wavelengths ranging from 410 to 700 nm. The density differences (ΔDx) represent the wavelength variation in retinal transmissivity between scans recorded from a dark-adapted retina and again after the retina had been exposed for 2 minutes to an intense yellow light (Watten 16; 460 mW/mm²) that bleached virtually the full complement of available rhodopsin in the circular test area (1.8 mm²) of the measuring beam. It is important to note that the value of ΔDx (the λmax of the absorbance spectrum) does not represent the actual rhodopsin density for light passing axially through the rod outer segments. The values of ΔDx are diluted by that fraction of the light reaching the photocell that has passed through the interstices between photoreceptors (stray light) and, in the case of the isolated retina, by additional factors that contribute to reductions in the measured absorbance changes, namely disorientation of receptor outer segments with respect to the path of the incident light and the loss of outer segments during retinal detachment. Nevertheless, the technique has provided a reliable means by which to compare the kinetics and relative content of rhodopsin in normal and diseased retinas.

TUNEL Labeling

Retinas were fixed for 6 hours in 4% paraformaldehyde in 0.13 M phosphate buffer and processed as flatmounts using the in situ nick translation method for the demonstration of fragmented DNA. Labeled cells were mapped using a Zeiss microscope-based Eutectics Neuron Tracing System (Carl Zeiss, Thornwood, NY; Eutectic Electronics, Raleigh, NC). Counts of labeled cells were analyzed statistically using random effects models. Other retinas were prepared...
FIGURE 2. Electroretinographic data. (A) ERGs recorded from normal 2-month-old mice (upper row) and from VPP mice at the ages indicated (lower rows) after rearing in cyclic light (left waveforms) or in darkness (right waveforms). The cornea-negative a-wave was measured from the prestimulus baseline to the trough of the initial deflection (arrow). Bar = 200 µV and 100 msec. (B) Bar graphs of a-wave results obtained from normal mice reared in cyclic light (open bars) or darkness (filled bars); error bars = ±SEM. (C) Amplitude of the a-wave plotted as a function of age for the VPP mice. Data are expressed as percentages (± SEM) of the values obtained from normal littermates.

RESULTS

Histology

Figure 1 shows representative cross-sections taken from a 2-month old normal mouse (Fig. 1A), and from VPP mice at ages 2 (Figs. 1C, 1D), 4 (Figs. 1E, 1F), and 6 (Figs. 1G, 1H) months; the animals were reared either under cyclic light conditions (Figs. 1A, 1C, 1E, 1G) or in darkness (Figs. 1D, 1F, 1H). The level of cyclic light to which the animals were exposed had no adverse effect on the morphology of normal (control) littermates (Fig. 1A), nor was an age-related structural change evident in sections from 4- and 6-month old normal mice (not shown). On the other hand, the sections taken from VPP mice illustrate the degenerative nature of the disease process. Irrespective of the lighting conditions under which the animals were raised, the VPP mouse retinas showed progressive thinning of the outer nuclear layer and a concomitant shortening of the photoreceptor outer segments (Figs. 1C to 1H); at each age tested, there was a significantly greater number of photoreceptors retained in the dark-reared animals than in their cyclic light-reared counterparts (Fig. 1B). In addition, the decrease in the length of the rod outer segments was more prominent in VPP mice raised in cyclic light than in darkness.

Electroretinography

Electroretinographic recordings from cyclic light and dark-reared VPP mice were consistent with the histologic findings. Recorded from the corneal surface, the a-wave component of the ERG waveform provides a useful index of the light-evoked activity of the rod photoreceptors. Figure 2A shows typical recordings obtained from normal mice and from VPP mice reared under cyclic light and in darkness for the various ages tested. As illustrated in Figure 2B, a-wave amplitudes were similar from normal mice reared in cyclic light or in darkness, and both showed a small but consistent decline with age. Although there was evidence of disease progression in both groups of VPP mice, the loss of electroretinal activity was initially less severe in the dark-reared animals, and the response amplitude was retained at a higher level throughout the 6-month experimental period. These observations are summarized in Figure 2C, which plots a-wave amplitudes recorded in response to the highest intensity stimulus flash (0.85 log cd sec/m²) from dark-reared and cyclic light-reared VPP mice. Each value is plotted as a percentage of the value obtained from the corresponding normal littermates tested in the same session. At this flash luminance, the ERG a-wave was larger in dark-reared mice than in their cyclic light-reared counterparts. A similar difference was noted at
Light Deprivation in a Transgenic Mouse Model of ADRP

FIGURE 3. Rhodopsin measurements in 2-month-old mice. Density difference spectra obtained from normal (diamonds) and transgenic (circles) mice reared in cyclic light (A) or in darkness (B). Data points indicate the mean (± SEM) for 5 to 10 mice. (C) Rhodopsin density, expressed relative to the value obtained from normal littermates.

Rhodopsin Densitometry

Rhodopsin measurements, made on light- and dark-reared normal and VPP mice also showed the beneficial effects of dark rearing. Figure 3 presents density difference spectra from the retinas of 2-month old normal and transgenic mice reared under cyclic light (Fig. 3A) or in darkness (Fig. 3B). The higher rhodopsin content in normal dark-reared animals compared to their cyclic light counterparts probably was caused by an increase in the length of the rod outer segments, which tend to elongate after prolonged periods of darkness. Nevertheless, when expressed as a percentage of the value measured in normal littermates, transgenic mice reared in darkness had a greater rhodopsin content than did mice reared in cyclic light (Fig. 3C). This result reflects the greater length and number of rod outer segments present in the retinas of dark-reared VPP mice (compare Figs. 1C and 1D). We were unable to obtain densitometry data from the retinas of 4- and 6-month-old cyclic light-reared VPP mice, in which the changes in absorbance were too small relative to the intrinsic noise of the photomultiplier system to provide reliable data.

TUNEL Labeling

Photoreceptor cell death occurs by apoptosis in a variety of hereditary retinal degenerations. Retinas from 1-month-old mice were processed by the TUNEL method to determine the regional distribution of apoptotic cells. As shown in Figures 4A and 4B, apoptotic nuclei were numerous in the VPP retinas, whereas each normal retina typically contained 10 or fewer TUNEL-positive nuclei (not shown). Cross-sections of TUNEL-processed VPP retinas that had been immunostained with anti-rhodopsin revealed that the apoptotic nuclei belonged to rod photoreceptors with rhodopsin-positive somata. It is noteworthy that the pattern of apoptosis was different for cyclic light- and dark-reared VPP mice. Compared to the relatively uniform distribution of TUNEL-positive nuclei in the retinas of dark-reared VPP mice (Fig. 4B), the retinas of mice reared in cyclic light contained significantly more apoptotic nuclei, particularly in the inferior region (Fig. 4A). For the cyclic light-reared animals, counts in the inferior retina averaged eight times higher than in the superior region; no differences were found in counts from the inferior and superior regions of the dark-reared retinas.

DISCUSSION

Results of this study indicate that light deprivation slows the progressive retinal degeneration caused by the mutant VPP transgene. Compared with transgenic animals reared in cyclic light, animals reared in the...
dark had greater survival of photoreceptor cells, rhodopsin content of the retina, and preservation of retinal function. It is important, however, to recognize that rearing the transgenic mice in darkness did not prevent photoreceptor degeneration. The age-related decline in the number of outer nuclear layer cells in the dark-reared animals indicates that light deprivation retarded, but did not arrest, the degenerative process (Fig. 1B). The progressive shortening of the rod outer segments suggests that the mutant opsin may cause an imbalance between the relative rates of outer segment disk synthesis and shedding. In the cyclic light-reared animals, the additional influence of a light-entrained daily rhythm may exacerbate this imbalance, thereby accelerating the degenerative process.

TUNEL staining revealed that apoptosis is a prominent feature of photoreceptor cell death in VPP mice, a feature shared with other rodent models of retinal degeneration. Although apoptotic figures were found in both cyclic light- and dark-reared VPP mice, the total number was much greater in light-reared animals, particularly in the inferior retina. This observation appears to provide an additional point of similarity between the VPP mouse and humans bearing the P23H mutation, in whom disease severity is typically greatest in the inferior retina. Because the inferior retina receives more illumination when the light...
source is overhead, these results support the contention that light exposure accelerates the rate of photoreceptor degeneration.

The hypothesis that light deprivation might prove beneficial in preserving vision in patients with RP was proposed more than 20 years ago. However, the results of a 5-year clinical trial of two patients with RP of undetermined genotype were disappointing; compared with the fellow eye, there was no greater retention of vision in the eye that had been occluded for 6 to 8 hours/day throughout the trial period. Nevertheless, there was evidence to suggest that the course of retinal degeneration could be influenced by retinal illumination in the P23H form of autosomal dominant RP (ADRP). For example, Heckenlively and colleagues found that patients with P23H whose employment history revealed exposure to high light levels (e.g., welders), experienced more severe degenerative changes than did age-matched patients with the same genetic mutation. Findings consistent with this observation were reported recently for transgenic mice expressing a human P23H mutation. Despite the rapid retinal degeneration observed in this mouse model, the decline in the electroretinal response was retarded significantly when the animals were raised in darkness.

It is important to stress that the beneficial effects of light deprivation do not seem to apply to all forms of genetically mediated retinal degenerations. Although dark rearing tended to slow the retinal degeneration in albino Royal College of Surgeons rats, it had no apparent effect on pigmented Royal College of Surgeons rats, nor did it alter the degenerative process in various naturally occurring models of retinal disease, e.g., the rds mouse, the "nervous" (nr/nr) mutant mouse, and the vitiligo mouse (C57BL/6-mi/mi). Similarly, dark rearing had no effect on disease progression in transgenic mice expressing either a point deletion (I-155/6-del) in the mouse opsin gene or a point mutation (K296E) in the human opsin gene. Thus, there appears to be some feature of the P23H mutation that renders the photoreceptors more sensitive to photic exposure.

In conclusion, we have shown that the rate of retinal degeneration induced by the expression of a mutant rhodopsin transgene is faster in mice reared under cyclic light than in complete darkness. Although the degree of intervention that we imposed on the VPP mice may be inappropriate for humans, some patients with RP may benefit from either uniconcular occlusion or an overall decrease in light exposure. We suggest that minimizing photic exposure for patients with autosomal dominant RP caused by the P23H mutation may extend their years of useful vision.

**Key Words**
apoptosis, dark rearing, electroretinography, retinitis pigmentosa, rhodopsin, transgenic mice

**Acknowledgments**
The authors thank R. Molday for the gift of anti-rhodopsin, P. Rhode, D. Possin, J. Chang, and I. Klock for technical help, M. Emond for biostatistical advice, C. Stephens for photographic assistance, and J. Zakevics for retinal histology and assistance throughout the course of the study. M. R. Al-Ubaidi, K. R. Alexander, D. Pepperberg, and G. A. Fishman provided valuable comments on the manuscript.

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