Downregulation of cGMP Phosphodiesterase Induced by Expression of GTPase-Deficient Cone Transducin in Mouse Rod Photoreceptors

Carol J. Raport,* Janis Lem,† Clint Makino,‡ Ching-Kang Chen,* Cindy L. Fitch,* Ann Hobson,* Denis Baylor,‡ Melvin I. Simon,‡ and James B. Hurley*

Purpose. Photoexcitation of vertebrate retinal rod photoreceptors stimulates GTP binding to the transducin α subunit. Like other GTP-binding proteins, transducin restores itself to an inactive form by hydrolyzing its bound GTP. The role of GTP hydrolysis in phototransduction was investigated.

Methods. A mutant form of cone transducin α deficient in its ability to hydrolyze bound GTP was expressed in mouse rod photoreceptors.

Results. Expression of the mutant cone transducin α at levels threefold to sixfold higher than endogenous rod transducin α led to a specific depletion of the transducin target, cGMP phosphodiesterase, and a decrease in the cGMP level. Suction electrode recordings revealed abnormally prolonged flash responses, decreased maximal response amplitudes, and a shift in the stimulus-response relation to higher flash strengths.

Conclusions. Rods expressing high levels of GTPase-deficient cone transducin α have reduced levels of phosphodiesterase catalytic subunits and cGMP. These changes are associated with prolonged flash responses, reduced dark current, and decreased sensitivity to light. Invest Ophthalmol Vis Sci. 1994;35:2932-2947.

Mutations in signal transduction genes have revealed important information about signalling pathways, such as the yeast pheromone response, Drosophila eye development, and Drosophila phototransduction.1-3 However, vertebrate phototransduction has not been the subject of genetic analyses. Mutant vertebrate photoreceptors have been impossible to analyze because all previously identified mutations in photoreceptor genes promote photoreceptor degeneration.5-6 Information about the vertebrate phototransduction pathway comes only from biochemical and physiological studies.

Light triggers hyperpolarization of vertebrate retinal photoreceptors by stimulating a biochemical cascade that culminates in hydrolysis of the intracellular second messenger cGMP.7 In the excitation phase of the light response, photoactivated rhodopsin promotes GTP binding to the α subunit of the heterotrimeric G-protein, transducin (Tα). Tα-GTP then relieves inhibitory restraints on cGMP phosphodiesterase (PDE) catalytic subunits, PDEα and PDEβ, imposed by the PDEγ subunit. The ensuing hydrolysis of intracellular cGMP closes cGMP-gated cation channels in the photoreceptor outer segment plasma membrane.

This light-stimulated enzymatic cascade is present in outer segments of both rods and cones. Each type of photoreceptor contains distinct forms of transducin α and β subunits,8-10 as well as many other phototransduction enzymes.

Several reactions contribute to the recovery phase of the photoresponse.7-11-12 One is the hydrolysis of GTP bound to Tα. Tα-GDP formed in this reaction reassociates with Tβγ and releases PDEγ to quench
PDE activity. Calorimetric measurements suggest that GTP may be hydrolyzed within a second after it binds to Ta.19 This rate is consistent with a role for GTP hydrolysis in recovery after photoexcitation.14 That GTP hydrolysis is essential for recovery was shown by the ability of a nonhydrolyzable GTP analogue to block recovery when dialyzed into a rod cell.15,16 Another report, however, suggested that light-stimulated PDE activity deactivates even when a nonhydrolyzable GTP analogue is present.17

To investigate further the role of GTP hydrolysis in phototransduction, we expressed a GTPase-deficient form of Ta in mouse rod photoreceptors in vivo. Certain mutations in a related GTP-binding protein, ras, slow hydrolysis of GTP bound to ras.18 One such mutation, Q61L, occurs in a region highly conserved in all GTP-binding proteins. A mutation at the corresponding glutamine in the α subunit of Gs, the adenylate cyclase stimulatory G-protein, slows GTP hydrolysis and causes persistent activation of adenylate cyclase.19,20 The corresponding glutamine in human rod Ta is Q200 and in human cone Ta it is Q204. In this study, we examined biochemical and physiological consequences of expressing in mouse rods Ta subunits in which this glutamine has been changed to leucine. We found that the presence of abnormally high levels of the GTPase-deficient cone Ta led to the specific loss of PDE catalytic subunits in mouse rods. Electrophysiological analyses of these altered photoreceptors revealed altered response kinetics and sensitivity.

METHODS

Point Mutations and DNA Constructs

Point mutations were introduced into human rod and cone Ta cDNAs by the method of Kunkel.21 Human Ta cDNAs were cloned into the EcoRI site of pBluescript (Stratagene). For rod transducin, the primer was 5'-ATGTGGGAGGGCT*GAGATCCGAGAG-3' to convert Q200 to L. For cone transducin, the primer was 5'-ATGTGGGCGGGCT*GCGCTCGGAGCC-3' to convert Q204 to L.

DNA for transgenic mouse production was constructed in pBluescript vectors. Human cDNAs for either wild-type or mutant rod or cone Ta (EcoRI fragment) were joined to a 0.6 kb fragment containing a small intron and polyA signal from the 3' untranslated portion of the mouse mP1 gene.93 The joined transducin-mP1 fragment was then linked to the 4.4 kb mouse rhodopsin promoter fragment94 in plasmid pRh0-1. The rhodopsin promoter is fused at a position that is 72 nucleotides downstream of the transcription start site as described.95 The number of nucleotides from the 5' end of the rod and cone transducin cDNA fragments to their respective initiator ATGs was 102 and 222. The plasmid constructs were confirmed by sequencing across each junction. Large-scale plasmid preparations were purified twice on CsCl gradients before use in transgenic mouse production.

Transgenic Mice

Transgenic mice were produced as described by Lem et al22 and were raised under a normal 12 hour/12 hour light–dark cycle. The presence of the transgene was tested by Southern blot and/or polymerase chain reaction (PCR) using genomic DNA.23 Southern blots were used to identify founders and to confirm the size of the inserted DNA. PCR analyses were performed on founder and progeny genomic DNA using a mixture of primers to amplify the transgene and to amplify the endogenous rhodopsin gene as an internal control. One primer, Rh-1, was common for both fragments. Its sequence corresponds to nucleotides −220 to −199 relative to the rhodopsin transcription start site. The Rh-2 primer sequence corresponds to nucleotides +121 to +142 relative to the rhodopsin transcription start site. Rh-2 together with Rh-1 amplified rhodopsin DNA. The transgene was amplified with Rh-1 and Tr-1. The transgene-specific primer, Tr-1, corresponded to nucleotides +121 to +139 (relative to the translation start site) of the human rod Ta cDNA22 and +135 to +151 of the human cone Ta cDNA. It was degenerate in one position to allow amplification of both human rod and cone Ta transgenes. The endogenous mouse Ta gene was not amplified.

Rh-1: 5'-GAATTCCCAAGAGGACTCTGGG-3'
Rh-2: 5'-GACGTTGAGAAAGGCACATAC-3'
Tr-1: 5'-CTTGACGATGGTGCTCTTT/CCC-3'

The Tr-1 oligonucleotide corresponds to bases +133 to +155 from the initiator ATG of Tca and +121 to +143 of TTr. Approximately 0.5 to 1 μg of genomic DNA was used in each 50 μl PCR reaction as well as 500 ng of each primer. Conditions for PCR were: 93°C 3 minutes, 93°C 1 minute, 59°C 45 seconds, 72°C 1 minute (×30), 72°C 10 minutes. The endogenous rhodopsin fragment was 360 bases in length, and the human rod and cone Tα fragments were 545 and 674 bases, respectively.

Methods for securing animal tissue were humane and complied with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Transgene RNA Detection

RNA from the transgene was identified by PCR on reverse-transcribed retina RNA. One adult mouse retina was dissected and gently homogenized using a Kontes disposable pestle in 100 μl cold 10 mM Tris pH 8.6/0.5% NP-40/0.14 M NaCl/1.5 mM MgCl2/20 mM NaCl. The number of nucleotides from the 5' end of the rod and cone transducin cDNA fragments to their respective initiator ATGs was 102 and 222. The plasmid constructs were confirmed by sequencing across each junction. Large-scale plasmid preparations were purified twice on CsCl gradients before use in transgenic mouse production.

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DTT/75 U RNasin (BRL). This was underlaid with 100 μl of the same buffer containing 25% sucrose, chilled 5 minutes on ice and spun 15 minutes in a microfuge at 4°C. Intact nuclei pelleted through the sucrose. An equal volume of 400 μg/ml proteinase K/0.2 M Tris pH 7.5/25 mM EDTA:0.3 M NaCl/2% SDS was added to the upper layer, and the mix was incubated 30 minutes at 37°C. This solution was extracted once with phenol–chloroform and once with chloroform alone, and then it was precipitated with 2.5 volumes ethanol. The pellet was resuspended in 20 μl dH₂O/0.2% SDS.

The pellet was resuspended in 20 μl dH₂O/0.2% SDS and precipitated again with 0.5 volume 5 M ammonium acetate and 2.5 volumes ethanol. No DNA was detected in these preparations. Using random hexamers and MMLV reverse transcriptase (BRL), 100 to 200 ng RNA was reverse transcribed. Half of this was used in PCR containing 500 ng each primer, 2.5 U taq polymerase (Perkin-Elmer/Cetus), and a final buffer concentration of 10 mM Tris pH 8.3/50 mM KC1/1.5 mM MgCl₂/0.001% gelatin. Both rod and cone Ta primer sets crossed an intron so that PCR products from genomic DNA contamination could be recognized. One primer (MP1PCR) came from exon 2 of the mPl gene and was common for both rod and cone transgenes. The other primer was specific for either rod Ta (RHTRPCR) or human cone Ta (RHTCPCR):

MP1PCR: 5'-ATGGTGATGAGGCGGCG-3'

RHTRPCR: 5'-CTCGAGCTCAACATGCG-3'

RHTCPCR: 5'-CTTGGACCTCAACATGCG-3'.

These produced a 316 bp fragment from rod Ta transgene RNA and a 574 bp fragment from cone Ta transgene RNA. Conditions for PCR cycles were: 93°C 3 minutes, 93°C 1 minute, 48°C 45 seconds, 72°C 1 minute (×35), 72°C 10 minutes.

Northern Analysis

Total RNA was isoalted from control and Δ66 mouse retinas as described in Transgene RNA Detection. Samples containing 4 μg of total RNA were separated on a 1.2% agarose formaldehyde gel and transferred to nitrocellulose (Schleicher and Schuell). Filters were prehybridized at 42°C for 4 hours as described previously22 using a random primer labeled 2 kb fragment of mouse PDEβ cDNA4 spanning nucleotides 35-2121. The gel was subsequently reprobed with human glyceraldehyde-3-phosphate dehydrogenase cDNA as a control for RNA loading.

Immunobots

Ta subunits and other retinal proteins were identified and quantitated by immunoblotting. Mouse retinas were homogenized by vortexing in 20 mM MOPS pH 7.2/2 mM MgCl₂/60 mM KC1/30 mM NaCl/1 mM DTT/100 μM PMSF (ROS buffer). Fifteen micrograms of retinal homogenate protein per lane were loaded, electrophoresed, and transferred to nitrocellulose for probing with antibody. Expressed human cone Ta protein was detected with antibody A1.1 (1:20 dilution of hybridoma culture supernatant), a mouse monoclonal antibody that recognizes human and bovine cone Ta but does not recognize mouse cone Ta. Other retinal proteins were detected with previously described antibodies: rod Ta8, T9, rhodopsin (4D2),27 PDE ("MOE," provided by B. Fung), arrestin (provided by K. Palczewski), recoverin,28 phosphodin (provided by R. Lee). Relative levels of retinal proteins were estimated by reflectance densitometry using a BioRad 620 Video Densitometer. The results should be taken only as estimates because the reflectance density may not be exactly proportional to the relative amounts of each protein. Using our electrophoresis system with 12% acrylamide and 0.32% bis acrylamide, the PDE catalytic subunits nearly always appear as a closely spaced doublet well resolved from previously observed proteolytic fragments of the PDE catalytic subunits.29 Attempts to identify the bands using an antibody thought to be specific for mouse PDEβ revealed high background levels and uninterpretable results in our experiments. Therefore, the results are based on the assumption that the upper band of the doublet is PDEα and the lower band of the doublet is PDEβ. The antibody used in these experiments, "MOE", was raised against bovine PDE, and it has been reported to react with all three types of PDE subunits, but it labels the bovine PDE α subunit more intensely than the PDEβ subunit (see Fig. 3C in ref. 30). The levels of transgene cone Ta compared to endogenous rod Ta were determined from standard curves on each blot using varying amounts of trpE–rod Ta or trpE–cone Ta fusion proteins as references.

Immunocytochemistry

Mouse eyes were enucleated under room illumination, pierced at the ora serrata, incubated in PBS with 4% paraformaldehyde at room temperature 4 hours, sunk in PBS/30% sucrose overnight at 4°C, and frozen in OCT on dry ice; 8 to 10 μm sections were mounted on gelatin-coated slides and stored at -20°C. Human cone Ta was localized using affinity-purified antibody 1611 detected using a horseradish peroxidase-linked secondary antibody system from Zymed.

Trypsin Sensitivity of Expressed Ta

Two to four mouse retinas were homogenized by vortexing in ROS buffer and were exposed to room light. Thirty-microgram aliquots were adjusted to 100 μM GTP or 100 μM GTPγS and spun for 10 minutes in a
FIGURE 1. Transgene construct. The 4.4 kb mouse rhodopsin promoter fragment was linked to human transducin cDNA (rod transducin, 1.1 kb; cone transducin, 1.8 kb). The 0.6 kb mouse protamine I fragment, containing an intron, was used as a transcription terminator.

Microfuge, and the supernatants were saved. Supernatants containing GTP-eluted Tα were treated with 5 μg trypsin for 8 minutes at room temperature, and then 25 μg soybean trypsin inhibitor was added. Control samples were not treated with trypsin. Each reaction was then subjected to immunoblot analysis. Human cone Tα fragments were identified using the A1.1 antibody.

Phosphodiesterase Activity

Mice were dark adapted overnight (approximately 15 hours). Retinas were dissected and homogenized by vortexing in ROS buffer in darkness under infrared lights. Homogenates from at least two littermate transgenic mice were always pooled to minimize individual differences. Nontransgenic littermates were used as controls. Homogenates were stored on ice at 4°C and used within 3 days.

A 3H-cGMP based PDE assay was scaled down for mouse retina homogenates. In darkness, a stock containing enough homogenate for several time points (5 μg protein and 5 μl per time point) made up in 80 mM Tris pH 8/4 mM MgCl₂ was added to an equal volume of a stock solution of 3.6 mM cGMP/450 μM GTP/450 μM ATP/10 mM CaCl₂. The stock solution also contained 300,000 DPM 3H-cGMP (Amersham) and 1000 DPM 14C-GMP (to monitor recovery) per time point (Amersham). The assay was initiated with a light flash that bleached 0.008% of the rhodopsin (determined spectrophotometrically using bovine rod outer segments). Aliquots were removed at specified times and heated at 100°C for 5 minutes to stop the reaction. 5' GMP product was converted to guanosine by adding 10 μl 1 mg/ml snake venom (Ophiophagus hannah, Sigma, St. Louis, MO) to each tube and incubating it for 15 minutes at 30°C. Samples were loaded onto DEAE-Sephadex columns, and guanosine was eluted in 2.5 ml dH₂O and quantitated by scintillation counting. Dark activities were typically 15% of the maximal light-stimulated activities of control retinas and were subtracted to give the results shown in Figure 4. Dark activities were unaffected by any of the transgene derived Tαs.

To determine the light sensitivity of PDE activation, light flashes of increasing intensity and increasing duration up to 10 seconds were used to initiate a 3-minute assay (range, 0.00016% to 1.6% rhodopsin bleach).

Trypsin-Activated Phosphodiesterase Activity

Retinas were dissected and homogenized by vortexing in ROS buffer. Homogenates (2 μg protein) were treated with 0.1 μg trypsin for 3 minutes at room temperature, then 0.5 μg soybean trypsin inhibitor was added. PDE activity was measured in 80 mM Tris pH 8/4 mM MgCl₂/7.2 mM cGMP/10 mM CaCl₂/300,000 DPM 3H-cGMP/1,000 DPM 14C-GMP. The reaction mixture was incubated for 3 minutes at 30°C then stopped by heating for 3 minutes at 100°C. The products were separated on DEAE-Sephadex columns and analyzed as above.

Measurements of cGMP Levels

Cyclic GMP in light-adapted mouse retinas was extracted by boiling in 0.1 N HCl as described by Farber and Lolley. We found no significant effect of dark adaptation on the cGMP content of nontransgenic mouse retinas. An aliquot of each extract was taken before boiling to measure the protein concentration by the method of Lowry using BSA as the standard. The amount of cGMP in the extract was determined by radioimmunoassay using a kit purchased from Amersham.

Guanylyl Cyclase Assays

Mouse retinal extracts were made from mice dark adapted overnight. Dissected retinas were frozen at −70°C. Frozen retinas were homogenized after adding 63 ml of 2 X GC buffer (GC buffer: 50 mM MOPS...
FIGURE 2. Expression of human cone Ta in retinal photoreceptors. Frozen sections of mouse eyes were probed with an affinity-purified, anti-peptide antibody specific for human cone Ta. Sections from the same retinas treated in the same way but without primary antibody showed no staining. (A) Transgenic mouse retina from line 3-16 expressing wild-type cone Ta. (B) Normal mouse retina. OS, outer segment; IS, inner segment; ONL, outer nuclear layer. Similar patterns of expression were detected in retinas of A47 and A66 mice, although staining at levels less intense than in outer segments was also observed in the cell bodies and synaptic pedicles of A47 and A66 rods.
GTPase-Deficient Cone Transducin in Mouse Rods

Normal

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<tr>
<td>97</td>
<td>66</td>
<td>43</td>
<td>31</td>
<td>22</td>
<td>118</td>
</tr>
</tbody>
</table>

GTPase-deficient

Trypsin

- + + - + +

GTPyS

- + - - + -

GTP

- - + - - +

FIGURE 3. Trypsin sensitivity of wild-type and mutant transducin. Supernatants from homogenates of mouse retinas expressing normal (lanes 1 to 3) and Q204L (lanes 4 to 6) human cone Ta subunits were treated with trypsin in the presence of either GTPyS (lanes 2 and 5) or GTP (lanes 3 and 6). Lanes 1 and 4 show supernatants not exposed to trypsin. The resulting fragments were visualized on a Western blot using the cone Ta-specific antibody A11. Endogenous mouse cone Ta is not recognized by this antibody. The pattern of tryptic digestion of Q204L human cone Ta in the presence of GTP resembles the pattern of digestion of normal human cone Ta in the presence of GTPyS. This suggests that Q204L cone Ta hydrolyze bound GTP at a reduced rate.

(pH 7.4) 100 mM KCl, 8 mM NaCl, 10 mM MgCl₂, 5 mM 2-mercaptoethanol, 4 mM cGMP 10 μM dipyrindamol, and 10 μM zaprinast) by 45 seconds of vortexing, 10 seconds of centrifugation in a microfuge, 2 minutes of bath sonication, then freezing on dry ice and thawing. These steps were repeated three times for each retinal extract. Such extensive homogenization was required to obtain consistent results. Reaction mixtures for guanylyl cyclase assays contained 1 X GC buffer, 1 mM GTP, 1 μCi [α-32P]GTP, 0.1 μCi [3H] cGMP, 2 mM Ca²⁺/EGTA buffer, and retinal homogenates (12.5 μl of homogenate contained 50 μg total protein by Bradford assay). Reaction mixtures were prepared and incubated for 8 minutes at 30°C under infrared illumination. Reactions were quenched by heating to 95°C for 2 minutes then stored on ice. Each sample was centrifuged at 10,000 rpm for 5 minutes, then 8 μl aliquots were loaded onto a plastic Polygram PEI-cellulose-coated thin layer chromatography plate (Alltech), then developed in 0.2 M LiCl. Spots containing cGMP were excised and dried, and 3H and 32P were eluted in 2 M LiCl by gentle shaking overnight and were counted. Initial amounts of [3H] and [32P] were determined from samples in which the retinal homogenate was inactivated by boiling before incubation. Samples with high free Ca²⁺ contained an additional 2.3 μl
of 50 mM CaCl2 per reaction. Less than 10% of cGMP was hydrolyzed during the reaction. Each complete set of assays used two retinas from a single mouse. All measurements within an assay set were performed in triplicate. Each complete assay shown was performed in duplicate.

**Electrical Recordings From Single Cells**

In preparation for electrophysiological measurements, mice were dark adapted for a minimum of 1.5 hours. In experiments using Δ66 mice, they were dark adapted for at least 12 hours. After CO2 narcosis was induced, mice were decapitated and pithed. Eyes were excised and retinas removed into oxygenated Leibovitz’s L-15 medium (Gibco, Grand Island, NY). To remove as much vitreous as possible, the retinas were mounted vitreal side down on a glass capillary array (8 or 50 μm capillary diameter, Gallileo Electro-optics, Sturbridge, MA) with gentle suction, and a double-edged razor blade was slid underneath. Tissue preparation and electrical recording methods were similar to those of Baylor et al. A piece of retina was removed, chopped into small pieces, and placed on the polystyrene-coated floor of an experimental chamber. The chamber was perfused continuously with a bicarbonate-buffered medium saturated with 95% O2/5% CO2 pH 7.4. Per fusate was heated to bring the chamber to 35°C to 38.5°C. The outer segment of an intact rod was pulled into a suction electrode, and the current was recorded with a current-to-voltage converter. The majority of recordings were from rods attached to a small piece of tissue. However, a few isolated rods were also used. Recordings were low-pass filtered at 30 Hz (3 dB) with an eight-pole Bessel filter and were digitized at 200 Hz. The times to peak of the electrical responses were not corrected for the 17 msec delay introduced by the low-pass filter. Flashes at 500 nm and steady, background light at 520 nm wavelengths were obtained from a shuttered incandescent source and narrow band interference filters.

Electrodes were filled with a solution containing (mM): Na+, 141; K+, 3.6; Mg++, 2.4; Ca++, 1.2; Cl−, 143.3; succinate, 3; l-glutamate, 3; HEPES buffer, 3; EDTA, 0.02; glucose, 10; BME vitamin supplement (Sigma or Gibco); BME amino acid supplement (Sigma). Perfusion medium was bicarbonate buffered; 20 mM NaHCO3 replaced an equimolar amount of NaCl. In some experiments, succinate and glutamate were omitted.

**RESULTS**

**Production of Transgenic Mouse Lines**

To express human Ta subunits in mouse rod photoreceptors, we joined cDNAs encoding normal and mutant forms of either human rod Ta or human cone Ta22 to a 4.4 kb mouse rhodopsin promoter fragment containing the mouse rhodopsin transcription initiation site24 as shown in Figure 1. Point mutations were introduced into Ta cDNAs at sites expected to slow the rate of GTP hydrolysis without blocking GTP binding or phosphodiesterase activation. We changed Q200 in human rod Ta and Q204 in human cone Ta.
TABLE 1. Summary of Mouse Lines Derived From Injection of Human Rod (hTr) and Cone (hTc) Transducin α Transgenes

<table>
<thead>
<tr>
<th>Number</th>
<th>hTr</th>
<th>hTc.Q200L</th>
<th>hTc</th>
<th>hTcQ204L</th>
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<tbody>
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<td>Mice produced from injected eggs</td>
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<td>105</td>
<td>27</td>
<td>219</td>
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<td>Express transgene RNA</td>
<td>4</td>
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Expression was measured by assaying mouse retinas for RNA and protein derived from the transgene. mRNA was detected by reverse transcription followed by PCR amplification using transgene-specific primers. Transgene-derived protein was detected by immunoblotting and by immunocytochemistry using specific antibodies.

Human rod Ta mRNA transcripts were detected in retinas from four mouse lines, and Q200L human rod Ta mRNA transcripts were detected in one line (Table 1). No available antibody probes distinguish human rod Ta (hTr) from mouse rod Ta because they are 98% identical. We were, therefore, unable to confirm the presence of either normal human rod Ta (hTr) or mutant human rod Ta (hTrQ200L) protein in these lines.

Human cone Ta mRNA transcripts were detected in three mouse lines, and Q204L cone Ta transcripts were detected in seven lines. Expression of normal and mutant human cone Ta transgenes was further analyzed using an antibody that recognizes human cone Ta but not mouse cone Ta. Immunocytochemical analyses revealed that the expressed protein concentrated in rod outer segments (Fig. 2). Three lines that carry the human cone Ta transgene (3–11, 3–16, 3–19) and two lines that carry the Q204L cone Ta transgene (Δ47, Δ66) express the protein in all rod photoreceptor cells with none of the apparent gradients or patches of expression within the retina that had been reported. Other lines did show uneven or patchy expression. Human Q204L cone Ta (hTcQ204L) was approximately twofold to threefold more abundant than endogenous mouse rod Ta in Δ47 retinas and fourfold to sixfold more abundant than endogenous mouse rod Ta in Δ66 retinas. These estimates were based on quantitative immunoblots using bacterially expressed human rod and cone Ta-trpE fusion proteins as standards (data not shown). Levels of human cone Ta protein in all other lines were less than or nearly equal to the level of endogenous mouse rod Ta. Our studies focused on Δ47 and Δ66 retinas because expression was highest in these retinas and because they showed the most pronounced biochemical and physiological abnormalities. There were no obvious signs of retinal degeneration when retinal sections from transgenic mice up to 5 months of age were examined by light microscopy.

Characterization of Expressed Cone Ta

To confirm that the mutant cone Ta expressed in Δ47 and Δ66 retinas is deficient in its ability to hydrolyze bound GTP, we made use of the observation that the sensitivity of Ta to proteolysis by trypsin is altered when hydrolysis of bound GTP is blocked. Transducin was eluted from mouse retinas by incubation of retina homogenates with either GTP or GTPγS followed by centrifugation. The resulting supernatants containing Ta were then treated with trypsin, and the fragments of human cone Ta were analyzed by immunoblotting using an antibody that recognizes only human cone Ta (Fig. 3). Intact 40 kD human cone Ta was present in untreated mouse retina homogenates expressing normal human cone Ta (lane 1). Incubating these homogenates with trypsin generated 39 kD and 35 kD immunoreactive fragments when GTPγS was included in the reaction (lane 2) and a 26 kD peptide when GTP was included in the reaction (lane 3). Homogenates of retinas expressing Q204L human cone Ta differed only in the GTP–trypsin reaction (compare lanes 3 and 6). In the presence of GTP, mutant cone Ta behaved as if bound to a nonhydrolyzable GTP analog, confirming its inability to hydrolyze bound GTP.

Phosphodiesterase Activity in Transgenic Mouse Retinas

Because the Q204L mutation was chosen to slow hydrolysis of GTP bound to Ta, we expected that Q204L human cone Ta in Δ47 and Δ66 rods would prolong PDE activity stimulated by a light flash. We measured PDE activity in homogenates of whole retinas from dark-adapted control, Δ47, and Δ66 mice by assaying the extent of cGMP hydrolysis after light flashes of varying strength (Fig. 4A). Pools of homogenized dark-adapted retinas from two or three mice were used in each set of experiments. PDE activity in the dark was unaffected by expression of any of the Ta transgenes. The intensity of the stimulus was varied to bleach from 0.00016% to 1.6% rhodopsin, as shown in Figure 4A. Unexpectedly, Δ47 retina homogenates had only ~40% of the control PDE activity at each light intensity, and Δ66 retinas had no measurable PDE activity (Fig. 4A).

Kinetics of PDE activation and inactivation were measured after an intermediate light flash that
FIGURE 5. Levels of phototransduction proteins. Immunoblots were used to detect a variety of phototransduction proteins in normal and transgenic retinas. Retinal homogenates (15 μg total protein) from control mice and from Δ47 and Δ66 mice were electrophoresed, blotted, and probed. The resulting blots were scanned by reflectance densitometry to estimate relative levels of proteins. (A) Western blots probed for human cone Ta (hTca), phosphodiesterase α, and β subunits (PDEαβ) and rhodopsin (BHO). (B) Reflectance density scans of immunoblots probed with antibodies against rhodopsin (RHO), rod Ta (Ta), Tβ (Tβ), PDEα (Pα), PDEβ (Pβ), PDEγ (Pγ), phosducin (PHOS), arrestin (ARR), recoverin (REC) in nontransgenic (white), Δ47 (hatched), and Δ66 (black) retinas. Values with error bars represent means ± SD relative to littermate controls for at least two blots. Values without error bars represent single determinations relative to littermate controls. The reflectance for each control was arbitrarily defined as 1.0. (C) Northern blot analysis of 4 μg total RNA from Δ66 and nontransgenic littermate retinas. The blot was probed using a fragment of PDEβ cDNA spanning nucleotides 35 to 2121. To monitor the quality and amounts of RNA loaded, the blot was reprobed with human glyceraldehyde-3-phosphate dehydrogenase cDNA.
bleached 0.008% rhodopsin (Fig. 4B). Dark-adapted whole retina homogenates were mixed with GTP and ATP to provide the factors required for PDE activation and inactivation. A burst of PDE activity followed the light flash in both nontransgenic control and Δ47 retina homogenates. This activity was completely inactivated within 2 minutes after the light flash. However, Δ47 homogenates hydrolyzed only 37% as much cGMP as control homogenates. Homogenates of Δ66 retinas hydrolyzed less than 2% of the cGMP hydrolyzed by control homogenates (Fig. 4B). PDE activities of transgenic retinas expressing normal human cone Ta were indistinguishable from controls (data not shown). However, levels of cone Ta expression in those retinas were lower and, therefore, were not directly comparable with retinas expressing Q204L cone Ta (data not shown).

Secondary Biochemical Changes in Retinas
The loss of PDE activity in Δ47 and Δ66 retinas could be caused by a deficiency in PDE catalytic subunits or in a component that regulates PDE activity. To determine if functional PDE catalytic subunits were present at normal levels, the photoreceptor PDE was fully activated by proteolytic degradation of its inhibitory subunit, PDEγ. Such treatment of Δ47 retina homogenates activated PDE to ~37% of the level achieved in retina homogenates from nontransgenic littermates (Table 2). No PDE activity could be detected after treatment of Δ66 retina homogenates.

Immunoblot analyses of retina homogenates revealed that the photoreceptor PDE catalytic subunits, PDEα and PDEβ, were significantly less abundant in Δ47 retinas than in nontransgenic littermate controls (Fig. 5A). PDEα was even less abundant and PDEβ nearly undetectable in Δ66 homogenates.

To determine if the presence of Q204L human cone Ta expression in Δ47 and Δ66 retinas affected other photoreceptor proteins, immunoblots of control, Δ47, and Δ66 retina homogenates were probed with antibodies that recognize a variety of other photoreceptor proteins. Relative amounts of each protein were estimated by reflectance densitometry (Fig. 5B). Rhodopsin, PDEγ, endogenous rod Ta and Tβ, recoverin, phosducin, and arrestin were all present at normal levels in Δ47 and Δ66 retinas. Only PDE catalytic subunits were affected.

The reduced concentration of PDE catalytic subunits in mouse rods expressing GTPase-deficient cone Ta could be caused by a decreased rate of transcription of PDE catalytic subunit genes, a decreased stability of PDE catalytic subunit mRNA, a decreased rate of translation of PDE catalytic subunit transcripts, or a decreased stability of PDE catalytic subunits. To address these possibilities, we analyzed total RNA prepared from Δ66 and nontransgenic littermates by Northern Blot analysis using a PDEβ subunit gene fragment as a probe (Fig. 5C). There appeared to be no significant reduction in the amount of PDEβ mRNA transcript in Δ66 retinas. These results indicate that downregulation of PDE catalytic subunits in Δ66 retinas is caused either by reduced translation or reduced stability of the PDE catalytic subunits.

The loss of PDE activity in Δ47 and Δ66 retinas might be expected to elevate the intracellular cGMP concentration. However, quantitation of retinal cGMP by radioimmunoassay revealed that the cGMP content was approximately 40% to 50% normal in Δ47 and Δ66 retinas (Table 3). These findings raised the possibility that, in addition to a reduction of cGMP hydrolysis activity in these retinas, cGMP synthesis might also be reduced. However, guanylyl cyclase assays revealed that Ca2+-sensitive guanylyl cyclase activity was normal in Δ47 retinas and reduced to 60% of normal in Δ66 retinas (Fig. 6).

Single Cell Recordings From Normal Mouse Rod Photoreceptors
Light responses of rods from nontransgenic mice (Fig. 7A) were similar to those of rods from other mammalian species. Flashes gave rise to outward photocurrents that increased in amplitude with flash strength. For 50 rods from 14 mice, the saturating response was 6.0 ± 2.5 pA (mean ± SD). The largest saturating response (Imax) observed was 11 pA. In both

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**TABLE 2. Trypsin Activation of PDE From Control Mouse Retinas and From Retinas Expressing GTPase-deficient Human Cone Transducin α**

<table>
<thead>
<tr>
<th>Source of Retina Homogenate</th>
<th>Phosphodiesterase Activity (relative to nontransgenic littermate control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Δ47</td>
<td>0.367 ± 0.09 (n = 3)</td>
</tr>
<tr>
<td>Δ66</td>
<td>0.004 ± 0.006 (n = 2)</td>
</tr>
</tbody>
</table>

The trypsin activated PDE activity of control retinas was 5.9 ± 1.3 nmol cGMP/min per μg of mouse retinal homogenate protein.

**TABLE 3. cGMP Content Relative to That of Nontransgenic Retinas**

<table>
<thead>
<tr>
<th>Source of Retina Homogenate</th>
<th>cGMP Content of Retina (relative to nontransgenic littermate control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Δ47</td>
<td>0.46 ± 0.10 (n = 4)</td>
</tr>
<tr>
<td>Δ66</td>
<td>0.43 ± 0.04 (n = 4)</td>
</tr>
</tbody>
</table>

The concentration of cGMP in control retinas was 50 ± 9 pmol/μg protein (n = 8).
FIGURE 6. Guanylyl cyclase activity. Calcium-sensitive guanylyl cyclase activities in homogenates of Δ47 (hatched), Δ66 (black), and nontransgenic (white) retinas were assayed in the presence of high (1.5 μM) or low (2 nM) concentrations of free Ca²⁺. Activities are presented as per cent of maximal wild-type activity at low concentrations of Ca²⁺. The wild-type guanylyl cyclase activity at 2 nM free Ca²⁺ was 2.71 ± 0.48 nmol cGMP min⁻¹μg⁻¹ protein.

nontransgenic and low-expression transgenic mouse rods, the recovery to bright flashes was marked by a persistent current that stepped between discrete levels separated by about 0.5 to 1.0 pA. These “steps” often lasted for many seconds. Their superposition gave rise to the plateau at the end of the averaged flash response to saturating flashes shown in Figure 7A.

The amplitude of the single photon response was estimated by dividing the peak ensemble variance of the dim flash response by the mean response amplitude. For 17 rods from six nontransgenic mice, this gave a single photon response of 0.6 ± 0.2 pA, or about 0.09 rmax. The ratio of the flash sensitivity (the dim flash response amplitude divided by flash strength) to the single photon response gave a mean collecting area of 0.25 μm² for 10 of these rods. This is similar to the collecting area of 0.28 μm² estimated from measurements of the rod dimensions (see ref. 34).

As in rods from other species, the relation between response amplitude and flash strength took the form of a saturating exponential:

\[ \frac{r}{r_{\text{max}}} = 1 - \exp(-k_i) \]

where \( r \) is the amplitude of the response, \( r_{\text{max}} \) is the maximal response, \( k \) is a constant, and \( i \) is the flash strength. The flash strength that gave a half-maximal response, \( i_o \), was 46 ± 31 photons μm⁻² (500 nm wavelength; \( n = 50 \) rods from 14 mice) (Fig. 7B). This corresponds to the photoexcitation of about 13 rhodopsin molecules, assuming a collecting area of 0.28 μm².

At subsaturating intensities, increasing flash strength shortened the time to peak response (\( t_p \)) in 44 of 50 rods. The half-maximal response peaked within 130 ± 30 msec (\( n = 49 \) rods from 14 mice). The integration time (\( T_i \)), defined as the half-maximal response area divided by the peak amplitude, was 270 ± 140 msec (\( n = 50 \) rods from 14 mice). Larger \( T_i \) correlated with smaller \( i_o \) values.

Adaptation of mouse rod photoreceptors to background light was tested by superimposing test flashes on background lights of different intensities. The observed decrease in flash sensitivity with increasing background intensity was fitted by the Weber-Fechner relation:

\[ \frac{S_F}{S_F'} = \frac{1}{1 + L_B/L_o} \]

where \( S_F' \) is the flash sensitivity in darkness, \( I_B \) is the background intensity, and \( I_o \) is the background at which flash sensitivity declined to half that in darkness. The mean \( I_o \) was 106 ± 117 photons μm⁻² sec⁻¹ (500 nm; \( n = 6 \) rods from 5 mice).

Single Cell Recordings From Transgenic Mouse Rods

In transgenic mouse lines where human Ta was expressed at levels equal to or lower than endogenous mouse rod Ta, photoreceptor responses were indistinguishable from those of nontransgenic controls. A summary of \( i_o \), integration time, and time to peak for all mouse lines is shown in Table 4.

Some rods from Δ47 mice failed to respond to unattenuated flashes (flash strength > 8 × 10⁴ photons μm⁻² at 500 nm). For those giving responses, \( i_o \) was 87 ± 39 photons μm⁻² (\( n = 8 \) rods from three mice) (see Table 4). The stimulus-response relation, though shifted to higher flash strength, still took the form of equation 1 as shown in Figure 7B. No change in \( T_i \) or in the rate of decay of the response to dim flashes was observed. However, the reduction of \( t_p \) that occurred with increasing flash strength in nontransgenic rods did not occur in Δ47 rods.

Rods from Δ66 mice were even less sensitive to light. Many more rods from these retinas failed to respond to unattenuated flashes. In the rods that responded to light, \( r_{\text{max}} \) was small. Responses larger than 4 pA were never observed (\( n = 5 \) rods). The \( i_o \) for these rods was 1780 photons μm⁻², 40-fold higher than that of nontransgenic control rods (Table 4). Flash responses of Δ66 rods were markedly prolonged compared to nontransgenic controls (Fig. 7A). Although \( t_o \) was unaffected, \( T_i \) increased about tenfold to 2980 ± 1620 msec.

The size of the dark current observed in Δ47 and Δ66 rods was highly variable. For rods that gave light responses, the dark current in Δ47 rods was not significantly different from that of nontransgenic controls, but the dark current in Δ66 rods was smaller.
FIGURE 7. Photocurrents in rod cells. (A) Response kinetics. Averaged response families of mouse rods with stimulus monitor shown at the bottom. Flash strengths were increased by factors of two in the left and middle panels and by factors of four in the right panel. Left panel: responses from a control rod. Maximal response amplitude was 8.7 pA. For the half-maximal response, $t_p$ was 150 msec and $T_e$ was 260 msec. Middle panel: responses from a Δ47 rod. Maximal response was 7.1 pA although two rods were inside the electrode. For the half-maximal response, $t_p$ was 100 msec and $T_e$ was 240 msec. Right panel: responses from a Δ66 rod. The maximal response was 2.3 pA. The half-maximal response had a $t_p$ of 150 msec and a $T_e$ of 4290 msec. (B) Stimulus–response relations. Intensity response relations of control and transgenic mouse rods. Flash intensity is plotted on the abscissa (log scale, 500 nm wavelength). Symbols plot results from rods in panel A as labeled. Continuous lines show the fits to the equation 1 with $i_o$ values of 27, 155, and 1950 photons$\cdot$um$^{-2}$ for control, Δ47, and Δ66, respectively.

DISCUSSION

It has been reported that GTP hydrolysis is essential for recovery of the dark state of a photoreceptor after photoexcitation. Recovery was blocked in those experiments by dialyzing a hydrolysis-resistant GTP analogue into photoreceptors. The purpose of our study was to extend that observation by using molecular genetics to slow hydrolysis of only those GTP molecules bound to $\alpha$.

We expressed $\alpha$ subunits in mouse rod photoreceptors by introducing into the mouse genome normal and mutant forms of human rod and cone $\alpha$ cDNA linked to a 4.4 kb fragment of the mouse opsin promoter. Expression of human cone $\alpha$ in rod photoreceptors of transgenic retinas was confirmed by immunoblot and immunocytochemical analyses using antibodies that recognize only human cone $\alpha$. Immunocytochemical analyses revealed that expressed cone $\alpha$ accumulated in the outer segments of rod photoreceptors. This suggests that any signals present in $\alpha$ subunits required for transport to the outer segment may be similar in rods and cones.

Our studies focused on retinas from Δ47 and Δ66
The experiments in Figure 3 demonstrated that the expression of Q204L human cone Ta protein at levels significantly above the level of endogenous rod Ta protein.

Based on the known effect of certain mutations in other GTP-binding proteins, such as ras and Gsa, the Q204L mutation was expected to slow hydrolysis of GTP bound to expressed Ta. The data shown in Figure 3 are consistent with the Q204L mutation having this effect on cone Ta. These results also demonstrate that the expressed cone transducin folds properly. The experiments in Figure 3 demonstrated that the conformation-dependent trypsin sensitivity of Q204L human cone Ta resembles that of normal human cone Ta transgene were also established, none of them expressed human cone Ta protein at levels significantly above the level of endogenous rod Ta protein.

Expression of Q204L human cone Ta in mouse rod photoreceptors had the unexpected effect of lowering the concentration of photoreceptor PDE catalytic subunits in the retina. Of the nine photoreceptor proteins examined, only PDE α and β catalytic subunits were affected. Rhodopsin, PDEγ, arrestin, phosducin, recoverin, and the endogenous rod Ta and Tβ subunits were present at normal levels. These results do not exclude the possibility that other phototransduction enzymes not examined might also be affected in Δ47 and Δ66 retinas. Downregulation of PDE catalytic subunits must occur at the level of either protein synthesis or protein stability because PDEβ mRNA levels appear to be normal in Δ66 mouse retinas. The depletion of PDE catalytic subunits could reflect an exaggerated normal adaptation process or an increased rate of degradation of active PDE catalytic subunits. It has been shown elsewhere that persistent activation leads to accelerated degradation of some G-protein α subunits. Further analyses of the mechanism of PDEα and PDEβ downregulation in Δ47 and Δ66 retinas will be necessary to resolve these questions.

Despite the reduced PDE activity in Δ47 and Δ66 retinas, the cGMP content of these retinas was lower than in normal mouse retinas. This result was unexpected because it had been shown previously that a defective PDE β subunit gene in rd/rd mutant mice elevates retinal cGMP levels and ultimately causes photoreceptor degeneration. However, another report has suggested that the reduced cGMP content of heterozygous rd/+ mouse retinas may be caused by a reduction in cGMP binding sites associated with partial loss of PDE catalytic subunits. The loss of cGMP binding sites may also be partially responsible for the reduced levels of cGMP in Δ47 and Δ66 retinas. Ca²⁺-sensitive guanylyl cyclase activity was normal in Δ47 and 60% of normal in Δ66 retina homogenates. The slightly reduced level of guanylyl cyclase activity does not appear to be sufficient to account for the reduced levels of cGMP found in Δ66 retinas that also lack nearly 100% of normal PDE activity. The guanylyl cyclase activity shown in Figure 6 most likely derives primarily from photoreceptors because only the photoreceptor guanylyl cyclase has been reported to show this type of Ca²⁺-sensitivity. Furthermore, a previous study showed that most of the guanylyl cyclase activity in vertebrate retinas comes from photoreceptor outer segments. These findings suggest that a different factor, such as altered intracellular Ca²⁺ levels, may be responsible for reduced levels of cGMP in Δ66 retinas. No obvious signs of photoreceptor cell death were detected in Δ47 or Δ66 mice even as old as 5 months, although preliminary electron-microscopic analyses

### Table 4. Effects of Human Ta Expression on the Flash Response

<table>
<thead>
<tr>
<th>Mouse Type</th>
<th>$i_0$ (photon/μm²)</th>
<th>$T_1$ (ms)</th>
<th>$t_p$ (ms)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>46 ± 31 (50)</td>
<td>270 ± 140 (50)</td>
<td>130 ± 30 (49)</td>
</tr>
<tr>
<td>hTr</td>
<td>34 ± 21 (35)</td>
<td>280 ± 140 (35)</td>
<td>140 ± 40 (35)</td>
</tr>
<tr>
<td>hTrQ200L</td>
<td>48 ± 16 (13)</td>
<td>180 ± 50 (13)</td>
<td>120 ± 20 (12)</td>
</tr>
<tr>
<td>hTc</td>
<td>26 ± 10 (7)</td>
<td>470 ± 180 (7)</td>
<td>150 ± 20 (7)</td>
</tr>
<tr>
<td>hTcQ204L</td>
<td>22 ± 20 (6)</td>
<td>400 ± 270 (6)</td>
<td>180 ± 50 (6)</td>
</tr>
<tr>
<td>Δ47</td>
<td>87 ± 39 (8)</td>
<td>200 ± 40 (8)</td>
<td>110 ± 20 (8)</td>
</tr>
<tr>
<td>Δ66</td>
<td>1780 ± 1630 (7)</td>
<td>2980 ± 1620 (5)</td>
<td>160 ± 20 (7)</td>
</tr>
</tbody>
</table>

Values are mean ± SD (n values in parentheses). $i_0$ = the flash strength giving rise to a half-maximal response; $T_1$ = the integration time for the half-maximal response; $t_p$ = the time to peak for the half-maximal response.
revealed a smaller number of rods per retina and an occasional rod with minor abnormalities in disc morphology (J. Flannery, personal communication). Photoreceptor degeneration in rd/rd retinas is complete within 20 to 28 days of birth based on the disappearance of opsin mRNA.51 Our observation that cGMP levels are lower than normal in Δ47 and Δ66 retinas, despite significant loss of PDE activity, distinguishes Δ47 and Δ66 mice from rd/rd mice.

Although PDE was depleted in Δ47 mouse photoreceptors, the kinetics of PDE activation and deactivation after a light flash appeared normal. The sensitivity of the Δ47 rods was several-fold lower than normal, presumably because of reduced PDE catalytic power within the Δ47 rods. The kinetics of the light-induced current changes in Δ47 mouse rods appeared normal. The electrical recordings most directly monitor the kinetics of cGMP-mediated changes in plasma membrane cation channel activity. However, because rod cGMP levels are determined in part by transducin-activated PDE activity, it is surprising that expression of a persistently active form of cone Ta did not alter the response kinetics of Δ47 rods. At least two explanations could account for the observed kinetics. One is that PDE, stimulated by persistently active Ta, may be inactivated by a process independent of GTP hydrolysis. The existence of such a mechanism has been suggested by Erickson et al.17 Another possibility is that human cone Ta is less effective than mouse rod Ta at stimulating mouse rod PDE. If so, then most of the PDE activity we detect in Δ47 rods derives from stimulation by normal endogenous mouse rod Ta. The persistent responses observed from some Δ66 rods suggests that the high concentration of mutant cone Ta in those rods may force the interaction of the GTPase-deficient cone Ta with the mouse rod PDE. The efficiency with which Q204L human cone Ta stimulates mouse rod PDE has not been determined. Gillespie and Beavo52 have shown that rod Ta activates cone PDE more efficiently than it activates rod PDE. Difficulties in purifying cone Ta from retinas and difficulties in expressing Ta subunits in Escherichia coli and Sf9 cells have prevented us from making similar measurements using human cone Ta.

In Δ66 mouse retinas, PDE activity was so low that the kinetics of activation and deactivation could not be measured biochemically. Physiological recordings revealed that in the few rods that gave light responses, the flash sensitivity was reduced forty-fold. The photoreceptor response was significantly prolonged. As noted above, this result is consistent with the residual photoreceptor in these cells being mediated by overexpressed GTPase-deficient human cone Ta rather than the endogenous mouse rod Ta.

We described in this report an initial characterization of mouse retinas that express a GTPase-deficient form of cone Ta in their rods. Interestingly, the rod cells compensated for the presence of GTPase-deficient cone Ta by downregulating the Ta target, the cGMP phosphodiesterase. The effects we observed may be caused either by the misexpression of cone Ta, the overexpression of a Ta subunit, the expression of a GTPase-deficient Ta subunit, or by a combination of these factors.

Unlike photoreceptors of rd/rd mice, which have reduced PDE activity because of a defect in their PDEβ subunit gene, Δ47 and Δ66 rods show no obvious signs of cell death. A significant decrease in the cGMP content of Δ47 and Δ66 retinas suggests that reduced activity of guanylyl cyclase may have prevented photoreceptor degeneration. Aside from heterozygous rd/+ mice,49 Δ47 and Δ66 mice are the only other vertebrate phototransduction mutants that have viable photoreceptors. These mutant retinas can now be further characterized biochemically and electrophysiologically and may serve as useful tools for the study of phototransduction mechanisms.

Key Words
transducin, phototransduction, photoreceptors, rods and cones, cyclic GMP

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References