HRG4 (UNC119) Mutation Found in Cone–Rod Dystrophy Causes Retinal Degeneration in a Transgenic Model

Akira Kobayashi,1 Tomomi Higashide,1 Duco Hamasaki,1 Shinya Kubota,1 Hitoshi Sakuma,1 Weijun An,1 Takuro Fujimaki,1 Margaret J. McLaren,2 Richard G. Welleber,3 and George Inana1

PURPOSE. To investigate the function and pathogenicity of HRG4, a photoreceptor synaptic protein homologous to the Caenorhabditis elegans neuroprotein UNC119.

METHODS. HRG4 was screened for mutations in patients with various retinopathies, and a transgenic mouse model was constructed and analyzed based on a mutation found.

RESULTS. A heterozygous premature termination codon mutation was found in a 57-year-old woman with late-onset cone–rod dystrophy. In some transgenic mice carrying the identical mutation, age-dependent fundus lesions developed accompanied by electroretinographic changes consistent with defects in photoreceptor synaptic transmission (depressed b-wave, normal c-wave), and retinal degeneration occurred with marked synaptic and possible transsynaptic degeneration.

CONCLUSIONS. HRG4, the only synaptic protein known to be highly enriched in photoreceptor ribbon synapses, is now shown to be pathogenic when mutated. (Invest Ophtalmol Vis Sci. 2000; 41:3268–3277)

Isolation and study of genes expressed in the retina have resulted in identification of a number of pathogenic causes of retinal degeneration and furthering of our understanding of retinal biology.1–7 Because only approximately one fourth of all cases of inherited retinal degeneration can be attributed to known causative genes at the present time, we have been isolating new retinal genes using a subtractive cDNA cloning approach8 and have been studying them to identify new pathogenic causes of retinal degeneration and to elucidate their function in the retina.9 Human retinal gene 4 (HRG4) is a novel photoreceptor-expressed gene that was isolated by this strategy.10 The expression of HRG4 was highest in the retina among the 14 different tissues examined and showed temporal and spatial correlation with photoreceptor development and function. The 240-amino-acid encoded protein showed a two-domain structure consisting of the proximal one quarter—rich in proline and glycine, predicted to interact with other proteins, and moderately conserved (67%) between the human and rat—and the distal three quarters, which is 100% conserved between the two species. Initially, it showed no homology to any known sequence.

While the initial report of HRG4 was in press, a novel Caenorhabditis elegans gene, unc-119, was reported, which had been isolated on the basis of a mutation causing abnormal coordination and feeding due to defective chemosensation.11 Comparison of the two sequences revealed that HRG4 showed a 57% homology with unc-119. Subsequent immunofluorescence microscopy and immunocytochemistry localized HRG4 in the rod and cone photoreceptor synapses, establishing it as the first synaptic protein enriched in the photoreceptors.12 The HRG4 gene has been mapped to chromosome 17q11.2 and shown to consist of five exons and a promoter containing GC boxes.13

To investigate the function and pathogenic potential of HRG4, we screened the HRG4 gene for mutations in patients with various retinal degenerations, constructed a transgenic mouse model that expresses a discovered mutation, and studied its phenotype. A heterozygous premature termination codon mutation in HRG4 was uncovered in a patient with late-onset cone–rod dystrophy, and transgenic mice carrying the same mutation showed a spectrum of electroretinographic abnormality of b-wave depression and evidence of retinal degeneration, demonstrating the pathogenic potential of HRG4 and providing evidence of its involvement in neurotransmission.

METHODS

Patient Study

Informed consent was obtained from blood donors in accordance with the institutional human subject study protocol and the tenets of the Declaration of Helsinki. Clinical evaluation of
the patients consisted of a complete ophthalmic examination, Goldmann perimetry, and International Society for Clinical Electrophysiology of Vision (ISCEV) standard Ganzfeld electroretinography.14–16 Mutational analysis of the HRG4 gene was performed on the patients’ DNA by denaturing gradient gel electrophoresis, as previously described.17 The five exons of the HRG4 gene were amplified from the DNA by polymerase chain reaction (PCR), with primers from the flanking intronic sequences shown in our recent publication.13 The mutations were identified by direct dideoxy-chain–termination sequencing of the DNA.

**Construction of the Transgene**

A hybrid mouse–rat HRG4 gene with a nonsense codon mutation was designed as the transgene. Because of the unavailability of a full-length mouse cDNA clone, the hybrid gene was constructed to maintain the size of the expressed message. The mouse HRG4 gene (MRG4) was cloned from a Lambda Fix II mouse genomic library, and an exon 1 region just upstream of the translational initiation codon to codon 57 was PCR-amplified with a termination codon mutation inserted at codon 57 and PstI sites at the ends. This fragment was combined with PstI-digested rat HRG4 cDNA19 to obtain a hybrid cDNA. The ability of the hybrid HRG4 cDNA to express the truncated 56-amino-acid MRG4 protein was tested by cloning into the 55-amino-acid MRG4 protein was tested by cloning into the PstI site of the bovine opsin promoter.18 The 2.2-kbp bovine rhodopsin promoter,18 to

**Detection of Transgene Expression**

**Production of Transgenic Mice**

Production of transgenic mice was performed at the Transgenic Facility of the University of Miami School of Medicine. All procedures using mice were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The transgene was injected into approximately 100 fertilized eggs (C57Bl6/J/SJL) each time (5–10 ng/egg, four injections). Seven transgenic founders were obtained. Two of the founders (numbers 456 and 919) were sterile. To establish independent transgenic lines and to breed out the transgenic genotype was achieved in all the transgenic animals.

**Detection of Transgenic Mice**

**PCR Amplification**. Two PCR primers were made, one (BRP-2, 5’-agggccatcagctgccagc-3’) in the bovine rhodopsin promoter and another (10R-13, 5’-ggccagctgctgcgctg-3’) in the RRG4 cDNA region of the transgene, which can amplify a transgene-specific fragment. Genomic DNA obtained from tail biopsies of mice was subjected to PCR amplification with these primers, and a 551-bp product was detected in the transgenic animals.

**Genomic Southern Blot Analysis**. Ten micrograms of mouse genomic DNA from the tail biopsies was digested with EcoRI or BamHI, electrophoresed, and blotted. The blot was hybridized with a 32P-labeled EcoRI fragment of the transgene as the probe, washed, and autoradiographed.

**Detection and Quantitation of Transgene Expression**

**Northern Blot Analysis**. Retinas were dissected from the transgenic and nontransgenic mice and used for RNA extraction by the guanidium thiocyanate method.20 Five micrograms of RNA was electrophoresed in a formaldehyde gel and blotted.21 The blots were hybridized with a transgene-specific probe (PCR product of primers 5’-ACGAGTCGTC-CAGCGGAGACG-3’ at the transcription start site of the bovine opsin gene and 5’-GGGGCGGGAACCCGGGAG-3’ at the ligation site of the bovine opsin promoter to the mutated mouse MRG4 sequence—i.e., sequence expressed only from the transgene) and endogenous MRG4-specific probe (PCR product of primers 5’-CCCTTTCCCTGGCTCCAGC-3’ at the transcriptional start site of MRG4 and 5’-CCTCGCGGGGCGCCA-GATCCTC-3’ at the end of the 5’ untranslated region not contained in the transgene—i.e., sequence expressed only from the endogenous MRG4 gene). The blot was also hybridized with actin to determine the quantity and quality of RNA present in each lane. The hybridized signals were quantitated by densitometry.

**Quantitative RT-PCR Analysis**. To determine the regional expression of the transgene in the mouse retina, primers were produced to detect the transgene transcript by reverse transcription–polymerase chain reaction (RT-PCR): TGRT-2 (5’-ctcagagactcccggtt-3’) in the bovine rhodopsin promoter region after the transcriptional start site and 10R-15 in the RRG4 cDNA as described earlier, yielding a product of 239 bp. Two primers were also produced to detect the endogenous MRG4 expression by RT-PCR: MRG4F-1 (5’-gaaggtgagaaagggccggc-3’) and MRG4B-1 (5’-aggggacagcagtgct-3’) in the coding sequence of the MRG4 transcript corresponding to a region that straddles exons 1 and 2, yielding a 234-bp product. The transgene plasmid was used as a control template for the 239-bp product. One microgram of RNA from the four retinal quadrants of transgenic mice was subjected to RT, and the product was divided into 12 to 18 equal portions. The samples were PCR-amplified for a varying number of cycles (26, 28, 30, 32, 34 and 36 cycles) with primers to detect both the transgene and the endogenous MRG4 transcript in the transgenic mouse retina. β-Actin was also PCR amplified and examined to confirm the quality and quantity of RNA used for the analysis.

Densitometric analysis of the RT-PCR products was performed, the result was plotted, and the linear phase of the PCR-amplification was identified and used to compare the levels of the transgene and the endogenous MRG4 transcripts. The linear phase of the reaction was used because, with excess substrates and primers, the extent of the reaction in this phase depends solely on the amount of reverse transcribed mRNA present.

**Western Blot Analysis**. Protein was extracted from the transgenic and nontransgenic retinas (whole and quadrants) and analyzed (100 μg) by Western blot analysis with the RRG4 antibody.12 Approximate quantitation was performed by densitometry of the chemiluminescent bands.
Fundus Examination
Animals were anesthetized with a mixture of ketamine, xylazine and urethane; the pupils were dilated with phenylephrine and atropine; and fundus photographs were taken (model RC2 camera; Kowa, Tokyo, Japan).

Electroretinography
Methods for recording ERGs from mice have been reported earlier. In brief, mice were anesthetized and the pupils were dilated as for the fundus examination. The ERGs were recorded between a wick Ag-AgCl electrode placed on the cornea and a reference electrode (a 30-gauge hypodermic needle) placed subcutaneously on the head. The animal was grounded by an electrode placed subcutaneously in the neck region. The responses were fed to a preamplifier (A39; Tektronix, Beaverton, OR) with the half-amplitude bandpass set at DC to 10 kHz (DC recordings) or at 0.1 Hz to 10 kHz (AC recordings). The output of the preamplifier was displayed on an oscilloscope and fed to a signal averaging program (M100; Biopac; Goleta, CA). The light for the stimulus was obtained from a quartz halogen bulb. The lamp filament was brought into focus in the plane of a shutter (Uniblitz; Vincent, Rochester, NY), and another lens focused the filament onto the tip of a fiber optic bundle. The other end of the fiber optic bundle was brought into the Faraday cage, and the tip was placed 1 to 2 mm from the cornea.

The stimulus intensity was measured with a photometer (UDT Instruments, Orlando, FL) with the detector placed at the position of the cornea. The maximum stimulus luminance was $1.59 \times 10^3$ candelas [cd]/m$^2$, and neutral density (ND) filters were used to attenuate the full-intensity stimulus. The stimulus intensity was increased in 0.5 log unit steps, and two responses were averaged at the lower stimulus intensities (ND $= 6.0–3.5$). Only one response was recorded at the higher stimulus intensities (ND $= 3.0–0$). The implicit time of the b-waves was measured in 10 transgenic mice from line 452 and in 10 control animals. The measurements were made on the electroretinograms (ERGs) elicited by the full-intensity stimulus.

Light and Electron Microscopy
Age-matched transgenic and nontransgenic control mice were examined at ages 9 to 30 months. Mice were killed and eyes were enucleated and immersed in fixative containing 2.0% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4. For light microscopy, eyes were embedded in paraffin and examined in histologic sections stained with hematoxylin and cosin. For electron microscopy, blocks of tissue, fixed as described, were prepared from affected retinal regions of transgenic mice and comparable regions in control animals, then postfixed in buffered 1% OsO$_4$, dehydrated through a graded series of alcohols, and embedded in epoxy resin, by using standard procedures. Ultrathin sections stained with uranyl acetate and lead citrate were examined in an electron microscope (JEOL, Peabody, MA).

RESULTS
Gene Screening
One hundred thirty-eight patients with retinopathies, including 34 autosomal dominant retinitis pigmentosa, 43 autosomal recessive retinitis pigmentosa, 20 cone–rod dystrophy, 11 Usher syndrome, and others, including Leber congenital amaurosis, Stargardt disease, Best disease, and congenital stationary night blindness, were screened for mutations in the HRG4 gene. A patient with a heterozygous A-to-T transition in codon 57, changing a lysine to a premature termination codon, was identified (Fig. 1A). The position of this mutation was precisely at the border between the putative proximal and distal domains of the 240-amino-acid HRG4 protein. This mutation was not present in 100 unaffected individuals. A polymorphic variant (C to T) was also identified in the 3' noncoding region.

![Figure 1](https://via.placeholder.com/150)

**FIGURE 1.** Patient study. (A) Direct sequencing of exon 1 of the patient’s HRG4 gene, detected to contain a mutation by denaturing gradient gel electrophoresis, showing a heterozygous A-to-T transition in codon 57, resulting in a change of lysine to a premature termination codon. (B) Fundus photograph of the patient with cone–rod dystrophy at age 57 showing macular atrophy. (C) Visual fields of the patient showing bilateral pericentral ring scotomas. (D) ERG showing subnormal rod and cone responses with prolonged cone b-wave implicit times to 30.3-Hz flicker and single photopic flash (length of arrows). Note severe loss of oscillatory potentials (OPs).
of the gene (position 778 in the cDNA sequence) at a frequency of 29%. The patient, who had had symptoms of poor night vision, defective color vision, and light sensitivity from age 40, at age 57 years had reduced visual acuity (20/40), myopia, macular atrophy, pericentral ring scotomas, and an electroretinogram (ERG) consistent with cone–rod dystrophy (Figs. 1B through 1D). A 36-year-old daughter carrying the same mutation had a history of seeing bright flashes from a young age, as was also experienced by her mother (proband), but does not yet demonstrate clear-cut ERG abnormalities, probably because of her young age for a late-onset disease. An older son and a daughter who do not carry the mutation have normal vision.

**Design and Production of Transgenic Mice**

To confirm the pathogenicity of the premature termination codon mutation in HRG4 uncovered in the patient, an animal model was constructed. Because a transgenic model expressing the mutant allele in the presence of the normal endogenous gene would be the appropriate model for the heterogeneous mutation observed in the patient, a transgenic mouse model was constructed in which the same 56-amino-acid truncated mouse HRG4 protein was expressed in the mouse retina. Because of unavailability of a full-length mouse HRG4 cDNA, a mouse–rat hybrid cDNA containing a nonsense codon at codon 57 in the mouse sequence was constructed to maintain the full length of the message and inserted into the gBR200-lacF vector containing the 2.2-kbp bovine rhodopsin promoter. Although the natural HRG4 promoter has GC boxes, suggesting that the gene may be expressed in other tissues besides photoreceptors, the rhodopsin promoter was used in this transgenic model, because our goal was to study the effect of the mutant HRG4 in the retina. Expression of the truncated HRG4 from the hybrid mouse–rat cDNA was confirmed by insertion into the prokaryotic expression vector pGEX (Pharmacia Biotech) and expression in vitro (data not shown).

The transgene construct was injected into fertilized mouse eggs at four different times, 100 eggs each. The four injections yielded 20, 26, 32, and 17 pups. The transgenic animals were identified by tail biopsies and DNA analysis. The DNA analysis consisted of both a PCR amplification assay to identify products unique to the transgenic animals (Fig. 2A) and a genomic Southern blot analysis of the mouse DNA to identify fragments also unique to the transgenic animals (Fig. 2B). From the four groups of pups, seven transgenic founders were identified. To produce independent lines of transgenic animals and to breed out the rd gene that was present in the mice used for the injection, the transgenic founders were initially mated with a normal strain (C57B6J), and their transgenic offspring were mated with each other. The presence of the rd allele was detected by PCR amplification of the mutated portion of the cyclic guanosine monophosphate-phosphodiesterase β-subunit gene and restriction digestion assay as described. Achievement of rd heterozygosity or complete elimination of the rd genotype was confirmed in the transgenic animals. Two of the original seven founders were sterile and could not be propagated. The remaining five lines were subjected to further analysis.

**Analysis of Transgenic Mice**

**Evidence of Transgene Expression.** The expression of the transgene in the retina of the transgenic animals was confirmed by Northern blot analysis. Line 452 expressed the transgene message at the highest level at 172% of the endogenous message (Fig. 3A), but the transgene protein was only 8% of total MRG4 by Western blot analysis (Fig. 3B), indicating there was no overexpression of the transgene product. The transgene expression in the other four lines (914, 1441, 1446, 1453) ranged from 0% to 78% of the endogenous gene (Fig. 3A), and no significant level of the transgene protein could be detected (Fig. 3B). The expression of the transgene did not appear to change significantly with age. To determine the level of transgene expression in the different regions of the retina, the retina from a line 452 transgenic mouse was divided into four quadrants and subjected to quantitative reverse transcription-PCR (RT-PCR) analysis in which the levels of the endogenous MRG4 gene, transgene, and actin transcripts were assayed. Comparison of the actin-normalized values from the linear portion of each of the assays indicated the transgene expression to be highest in the superior temporal quadrant with the expression arbitrarily being 1.0 in the superior temporal quadrant, followed by 0.72 in the inferior temporal quadrant, 0.23 in the superior nasal, and 0 in the inferior nasal (data not shown). The highest level of the transgene protein in the superior temporal quadrant was also confirmed by Western blot analysis (data not shown).

**Funduscopic Examination.** The fundus of transgenic and normal mice was examined by indirect ophthalmoscopy and photography (RC2 camera; Kowa). The fundus of transgenic animals demonstrated variable changes. These ranged from an essentially normal fundus to a few localized white dots and streaks, to multiple flecks, to outright degenerative changes (Fig. 4). The localized white lesions usually started in the superior temporal region of the retina, consistent with the highest expression of the transgene in this region. More generalized degenerative changes were seen in the older transgenic animals. The abnormalities were observed in line 452 mice, which expressed the highest level of the transgene, but not in the other lines. Normal or nontransgenic siblings of various ages (6–22 months) showed no abnormality in the fundus on examination.

**ERG.** Transgenic and normal mice were subjected to ERG analysis. Because the severity of the fundus abnormality correlated with the age of the transgenic animals in line 452 mice, which expressed the highest level of the transgene, the ERG of 452 transgenic mice less than 1 year of age (11 animals) and 1 year of age or more (23 animals) was compared with normal. A significant reduction of the b-wave was observed in the older transgenic animals (P < 0.001) compared with age-matched nontransgenic mice, whereas the younger transgenic mice did not show a statistically significant reduction (Fig. 5A), confirming the age-dependent nature of the ERG abnormality. The b-wave is generated by activated bipolar cells as a result of synaptic transmission at the photoreceptor ribbon synapse. The b-wave reduction increased with the intensity of the stimulus and ranged from 71% to 47% of the corresponding normal average value. The absence of a b-wave gain at the higher stimulus intensities seemed to suggest the possibility of an impairment in the cone system.

As another measure of photoreceptor function, the ERG c-wave was measured and compared with the b-wave. The ERG c-wave originates in the apical membrane of the retinal pigment epithelium as a result of a photoreceptor hyperpolarization-induced decrease in subretinal K+.

Plotted of the c-/b-
wave ratio revealed that this ratio was higher in the transgenic mice than in control animals (Fig. 5B). This was because the b-wave was reduced, whereas the c-wave remained intact in the transgenic mice. Thus, the result was consistent with an abnormality in the synaptic transmission from the photoreceptors to the secondary neurons. For the line 452 transgenic mice, the mean ± SD of the b-wave implicit time was 133.4 ± 12.7 msec, and that for the control animals was 126.2 ± 13.3 msec. This difference was not significant ($P = 0.27$). No ERG abnormality could be demonstrated in the other four transgenic lines, expressing the transgene at lower levels.

**Histopathology**

**Light Microscopy.** A spectrum of pathologic changes was observed in the line 452 transgenic retinas, depending on the age of the animal and the severity of the observed funduscopic and ERG abnormalities. In the younger transgenic mice with mild b-wave reduction and a few fundus lesions, the outer nuclear layer appeared more disorganized and loose than that of age-matched nontransgenic animals, with numerous photoreceptor nuclei appearing to migrate into the inner segment and outer plexiform layers (Figs. 6A, 6B). In older transgenic mice with multiple flecks or degenerative changes in the fun-
dus and significant reduction in the b-wave, the retina showed evidence of outright degeneration, with thinning of the outer nuclear layer down to four to five rows of nuclei, compared with approximately 10 rows seen in age-matched nontransgenic mice (Fig. 6C). There was also extensive vacuolation and pyknosis in the inner nuclear layer and an overall reduction in the number of nuclei present in this layer (Figs. 6C, 6E). Within the outer plexiform layer, the site of localization of HRG4, extensive vacuolation was apparent by light microscopy, confirmed by electron microscopy (Figs. 6D, 6E). The degenerative changes in the retina were present in the same region as the fundus lesions, typically in the superior temporal quadrant, which showed the highest expression of the transgene. No significant histologic abnormality was present in the retinas of the other four lines that expressed lower levels of the transgene.

**DISCUSSION**

More than 30 different types of proteins have been shown to be involved in the synaptic vesicle cycle, to enable the cyclical production of synaptic vesicles and release of neurotransmitters from them. HRG4 is presently unique among known synaptic proteins, in that it is highly enriched in, if not restricted to, photoreceptor ribbon synapses. Although its precise function is not yet known, its ultrastructural localization is consistent with an association with synaptic vesicles, indicating a possible role in synaptic vesicle function. The present results strongly support the importance of HRG4 in synaptic function, in that a transgenic model that expresses a
The presence of the disease mostly in the consistent with the expression of the transgene from the rho-site of expression of the mutated level of expression of the transgene, was consistent with the synapse in the transgenic mouse, which correlated with the retinal degeneration with prominent degeneration of the synapses. Student’s t-test: ***

Of interest in the transgenic retina was the suggestion of transsynaptic degeneration, which presumably occurred as a result of the photoreceptor synaptic degeneration. Evidence of vacuolation was present in the outer plexiform and the inner nuclear layers with significant reduction in the number of inner retinal nuclei and many surviving inner nuclear layer cells, showing evidence of apoptosis with pyknotic nuclei at the ultrastructural level. Transsynaptic degeneration is a well-known phenomenon, both antero- and retrograde, as seen in the deep cerebellar nuclei of Purkinje cell degeneration (pcd) mutant mice, in the lateral geniculate nucleus of enucleated rhesus monkey, and in the olfactory bulb of rabbit and rat after the removal of olfactory mucosa. Loss of the ganglion cell layer has been reported in diseases of the outer retinal layer, such as cone–rod dystrophy and rod–cone dystrophy, presumably by transsynaptic degeneration. The disease observed in the transgenic retina may be consistent with this. One of the postulated mechanisms for this phenomenon, which can be studied in the transgenic model, is the release of neuroactive material from the degenerating terminals that affects the postsynaptic neurons.

The retinal disease in the human patient and the transgenic model based on the same mutation show multiple features in common. The observed age dependence of the disease in the human was evident in the transgenic model when the ERG abnormality of transgenic mice 1 year of age or more was compared with that of transgenic mice less than a year of age, with the older transgenic mice showing a significant reduction in the b-wave. The histopathologic changes also showed a clear-cut age-dependent progression, with overt retinal degeneration not occurring before the mice were at least middle aged (i.e., 1 year of age or more). We have shown that HRG4 is expressed in both rod and cone photoreceptor synapses. Thus it could be predicted that disease with both rod-predominant (retinitis pigmentosa) and cone-predominant (macular pattern dystrophy) characteristics would be produced, as in the case of rds/peripherin, which is also expressed in both rod and cone photoreceptors. Indeed, the phenotype of the human disease cone–rod dystrophy, at least in the one patient found to date, is certainly consistent with the expression of HRG4 in both rod and cone photoreceptors. The transgenic model, however, is theoretically not suitable for comparison in this respect, because the transgene is driven by a rhodopsin promoter and is expressed only in rod photoreceptors, resulting in rod photoreceptor degeneration. It is interesting, however, that the absence of a b-wave gain in the ERG of the line 452 transgenic mice at the higher stimulus

**FIGURE 5.** Electroretinography of HRG4 transgenic line 452 mice. (A) The b-wave amplitude is shown as a function of the log of stimulus intensity. The age of the transgenic mice examined ranged from 9 to 24 months, and age-matched normal animals were used as controls. The old transgenic mice were 1 year or more, and the young transgenic mice were less than 1 year of age. The old transgenic mice show a significant decrease in b-wave amplitude, which measures photoreceptor synaptic transmission and bipolar cell activation. This effect is statistically significant at stimulus intensities above ~4.0 log units. (B) The ratio of the c-wave, generated by a flux in subretinal K+ as a result of photoreceptor hyperpolarization, to the b-wave in the transgenic mice and age-matched controls is shown. The ratio was significantly increased in the transgenic mice, because of a decrease in the b-wave in the presence of an intact c-wave, consistent with intact hyperpolarizing photoreceptors but a defect in photoreceptor synaptic transmission. Student’s t test: ***P < 0.001, **P < 0.01, *P < 0.05.

mutant HRG4 (identical with that found in a patient with late-onset cone–rod dystrophy) in the photoreceptor synapse shows a progressive decrease in the ERG b-wave (independent of the photoreceptor’s ability to generate a c-wave), leading to retinal degeneration with prominent degeneration of the synapses.

The striking disease observed in the photoreceptor ribbon synapse in the transgenic mouse, which correlated with the level of expression of the transgene, was consistent with the site of expression of the mutated MRG4. The synaptic degeneration was observed in the rod photoreceptor spherules, consistent with the expression of the transgene from the rhodopsin promoter. The presence of the disease mostly in the superior temporal quadrant also correlated with the highest expression of the transgene in this region of the retina. The observed pathologic changes in the synapse, including vacuolation, swollen, watery dendritic processes containing flocculent material, and accumulation of osmiophilic granules or 10-15-nm neurofilaments matched those previously described in other examples of neuronal degeneration. The same types of changes were seen in the nerve terminals in the lateral geniculate nucleus after enucleation in rhesus monkeys and in the olfactory bulb of rabbit and rat after removal of the olfactory mucosa. These changes led to an electron-dense transformation of the neuron called “dark degeneration” that was clearly evident in some of the HRG4 transgenic photoreceptor ribbon terminals, which became filled with osmiophilic material.
intensities seemed to suggest the possibility of a cone system defect in addition to rod dysfunction. This may actually reflect a leakiness of the rhodopsin promoter, resulting in expression of the transgene in both rods and cones or a secondary effect of the rod degeneration on cone function. Regardless, a significant reduction in the ERG b-wave, consistent with a defect in photoreceptor synaptic neurotransmission, was seen in both the human patient and the transgenic model. Given all the above similarities to the human disease, the transgenic mouse model may be useful for analysis of the pathophysiological mechanism of the disease and approaches to treatment for this form of retinal degeneration. Screening of this gene in additional retinopathies is ongoing.

Our transgenic model was designed to achieve a dominant negative effect of a mutant MRG4, as observed in a patient with cone–rod dystrophy who had the heterozygous mutation, by the expression of a truncated MRG4 protein containing only the proximal proline-rich domain. Similar transgenic models based on the expression of a truncated gene product to achieve a dominant negative effect have been described.33–34 Because we have shown that the MRG4 transgene construct is capable of expressing the truncated N-terminal protein and that this protein is expressed in the line 452 transgenic mouse retina, the pathogenic mechanism must involve an interference of the function of the normal MRG4 gene product by the truncated protein—i.e., a dominant negative effect. Such interference may be by competition for the putative target protein, as we had predicted from the likely protein-interacting function of the proline-rich N-terminal region,12 or by a direct inhibition of normal MRG4 by the truncated protein.

The pathogenic mechanism is unlikely to involve haploinsufficiency, because the full complement of the normal MRG4 gene is expressed in the transgenic mouse, yet the degeneration occurs. An overexpression of the transgene in the retina was also ruled out as the cause of the retinal degeneration. Although the transgene message level was 172% of the endogenous MRG4 message, the level of the transgene protein was quite low at 8% of the total MRG4 protein. The low level of the abnormal truncated protein may explain the slow progress of the disease. It is possible that most of the expressed abnormal protein is being degraded in the photoreceptors. The transgenic model should be useful for testing hypotheses regarding the pathophysiological mechanism involving the mutant MRG4 and for studying the mechanism of the subsequent retinal degeneration.

The precise function of HRG4 is not known yet. Its importance in synaptic neurotransmission and its capacity for pathogenicity, however, have been amply supported by the presence of an HRG4 mutation in a patient with cone–rod dystrophy and by the phenotype produced in our transgenic model expressing the identical mutation. Its precise function will begin to be elucidated by the identification of its target protein by such strategies as the yeast two-hybrid system.35,36 HRG4 represents the first example of a synaptic protein that is

![FIGURE 6. Light microscopy of HRG4 transgenic line 452 mouse retinas. A range of retinal disease is seen in transgenic mice of different ages with fundus and ERG abnormalities. Compared with the intact retina of a normal 12-month-old mouse (A), a 9-month-old transgenic retina reveals a disorganized, loosely packed outer nuclear layer with some migrating photoreceptor nuclei (B; arrows). (C) A 24-month-old transgenic retina showing clear evidence of photoreceptor degeneration, with the outer nuclear layer consisting of only 4 to 5 rows of nuclei instead of the usual 10 to 12. There was also a reduction in the number of inner nuclear layer cells and appearance of vacuolation of the inner retina. (E) At higher magnification, vacuolation is evident in the outer plexiform layer, the site of the photoreceptor ribbon synapses. Numerous inner nuclear layer cells show evidence of apoptosis with pyknotic nuclei. (D) These features are not seen in the normal mouse retina of this age. INL, inner nuclear layer; OPL, outer plexiform layer; ONL, outer nuclear layer; IS, inner segment; OS, outer segment; RPE, retinal pigment epithelium.](http:// iovs.arvojournals.org)
highly enriched in, if not specific to, the photoreceptor ribbon synapse and which is pathogenic when mutated.

References


