Identification of a nonsense mutation in the rod photoreceptor cGMP phosphodiesterase \( \beta \)-subunit gene of the rd mouse

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ABSTRACT Retinal degeneration in the mouse mutant, rd, was previously shown to be a disorder of cyclic nucleotide metabolism involving a deficiency in the activity of the rod photoreceptor cGMP phosphodiesterase (PDE). We have characterized the normal and rd PDE \( \beta \)-subunit gene, and their respective transcripts, by PCR and direct sequence analysis. We show that the gene consists of at least 22 exons ranging in size from 48 base pairs to several hundred base pairs, covering >25 kilobases. Within a 67-base-pair exon of the rd PDE \( \beta \)-subunit gene, we identified a nonsense ochre mutation (a \( C \rightarrow A \) transition in codon 347) that truncates the normal gene product, eliminating more than one-half of the peptide chain, including the putative catalytic domain. The consequences of the truncation are consistent with the observed phenotypes in rd mice heterozygous and homozygous for the disorder. The nonsense mutation was also found in another related and in six unrelated strains displaying the rd phenotype, indicating that the rd allele arose from a single genetic event. The results strongly argue for the nonsense mutation being responsible for retinal degeneration in the rd mouse.

An autosomal recessively inherited retinal degeneration in the mouse, locus designation rd, was first identified in wild mice caught in Switzerland, England, and France and later was found in several inbred laboratory strains (reviewed in ref. 1). Mice homozygous for the mutation display a complete loss of rod photoreceptor cells by postnatal day 20 (2), yet cones survive much longer, and other retinal cells remain intact (3). The detection of high levels of cGMP, followed by photoreceptor degeneration, and absence of phosphodiesterase (PDE) activity suggested a defect in the enzyme itself (4). Using immunological methods it was shown that at least one of the two large subunits of PDE is present at low levels in the homozygous mutant mouse and that a normal subunit complex, \( \alpha \beta_{2} \gamma \) (5, 6), which is essential for regulation of cGMP levels in the phototransduction cascade, is not formed (7). Mice homozygous for the mutation have histologically normal rod photoreceptors but show a reduction in retinal cGMP levels, altered PDE kinetics, abnormalities of cGMP binding, and abnormal electroretinograms (8–11).

The rd gene was localized to mouse chromosome 5 (1) and the three subunits of rod PDE are encoded by genes located on chromosomes 18 (\( \alpha \)), 11 (\( \gamma \)), and 5 (\( \beta \)), excluding the \( \alpha \)- and \( \gamma \)-subunit genes as candidates for the rd locus (12, 13). The \( \beta \)-subunit gene was fine-mapped by interspecific backcross at or near the rd locus between \( A/p \) and \( Gus \) (14). The rd gene was also shown to be closely linked to the xenotropic provirus \( XmV-28 \) (15). The predominant transcript of the normal \( \beta \)-subunit gene is 3.3 kilobases (kb), and the rd \( \beta \)-subunit mRNA was reported to differ in size by up to 300 base pairs (bp) (16). Additionally, \( \beta \)-subunit mRNA levels were found to be significantly reduced prior to the onset of photoreceptor degeneration (16, 17). Partial and complete cDNA sequences of the normal PDE \( \beta \)-subunit have been described (17, 18), and functional domains based largely on sequence similarity have been proposed (19, 20). Neither the gene organization nor a precise molecular defect in the rd \( \beta \)-subunit is known. In this article we establish the PDE \( \beta \)-subunit gene structure and identify a nonsense codon in the rd \( \beta \)-subunit gene that truncates the normal gene product, eliminating more than one-half of the peptide, including the presumed catalytic domain.*

METHODS

Isolation of Genomic DNA. Genomic DNA from C57BL/6J, C57BL/6J rd le, and six other homozygous rd strains was purchased from The Jackson Laboratory. Genomic DNA from C57BL/6J (+/+) at the rd and le loci, C57BL/6J rd/+ le/+ , and C57BL/6J rd/rd le/le was isolated using a Fastack poly(A) mRNA isolation kit (Invitrogen, San Diego). Following oligo(dT) selection of RNA, the supernatant was diluted to 0.3 M NaCl and DNA was precipitated with isopropyl alcohol, washed with 70% ethanol, dissolved in Tris-HCl/EDTA, pH 8.0, and used directly for PCR.

Oligonucleotide Primers. The following oligonucleotides were used for amplification or direct sequencing [nucleotide positions in parentheses refer to a sequence previously published (18)]: 118 (461–430), 119 (721–701), 129 (580–601), 120 (809–829), 122 (1147–1126), 128 (967–988), 134 (1085–1105), 135 (1264–1244), 127 (1583–1563), 114 (1865–1842), 115 (1485–1506), 91 (2578–2558), 133 (988–967), 136 (1105–1085), 124 (1359–1375), 141 (1463–1443), 127 (1583–1563), 126 (1622–1642), 116 (1726–1705), 112 (2075–2096), 111 (2137–2159), 87 (568–587), 88 (2242–2261), 110 (2488–2509), 132 (701–721). Primer 131 (5'-CCAGGCACAGCACGACAG-3') is located at position –26 to –4 of a full-length clone (MBP-71). Oligonucleotides 80, 81, 82, 83, 84, 89, 97, 98, 100, 101, 102, and 108 were described earlier (18). Intron primers 149 and 150 are depicted in Fig. 3.

PCR Analysis. One microgram of retinal poly(A) RNA isolated from 9- to 11-day-old C57BL/6J rd/rd mice was used as a template to synthesize cDNA in a volume of 25 \( \mu \)l using a reverse transcription kit (Promega). Priming of the cDNA was performed with primer pairs as described herein and an anchoring primer (21). Thirty-one PCR cycles were performed in a Precision GTC-1 thermal cycler with an initial denaturation of 4 min at 94°C and a final extension of 10 min at 72°C. For a second round of amplification (rd cDNA only), PCR products from amplified cDNA were diluted 1:100 with water and 1 \( \mu \)l was used for reamplification. For amplification of genomic DNA, 31 cycles were performed at 92°C/1 min, 55°C/2 min, and 72°C/3.5 min. Pilot reactions were performed in 25–50 \( \mu \)l of a solution containing 200 \( \mu \)M dNTP, 0.5–1 unit of Taq polymerase, 50 mM KCl, 10 mM Tris (pH 8.3)

Abbreviations: PDE, phosphodiesterase; RFLP, restriction fragment length polymorphism.

*The sequence reported in this paper has been deposited in the GenBank data base (accession no. M75166).
genomic DNA was amplified with primers 149 and 150 specific for introns f and g (Fig. 3). Dde I digestion was carried out directly in a 10-μl volume by adding 8 μl of PCR product, 1 μl of Dde I 10× buffer, and 8–10 units of Dde I (Promega).

Direct Sequencing. For direct sequencing the PCR reaction was scaled up to 400 μl and the products were purified from agarose gels using glass beads (GeneClean, Bio101, La Jolla, CA). Sequencing reactions were carried out with a Sequenase kit (United States Biochemical) according to the double-stranded protocol except that the primer (100 ng) and template (200–400 ng) were mixed in a final volume of 8 μl, boiled 3 min, immediately chilled to 0°C, and used directly in the sequencing reactions.

RESULTS

Amplification of Normal and rd PDE β-Subunit cDNA. To characterize the PDE β-subunit gene without cloning, we adopted a strategy in which normal and rd mouse DNA fragments are amplified directly from cDNA and genomic templates with appropriate primers. The amplified material can then be directly sequenced avoiding artifacts of PCR cloning (22). Primers were designed according to our previously published mouse PDE β-subunit cDNA sequence (18). Using cDNA templates reverse transcribed from poly(A) mRNA of 9- to 11-day-old rd and adult normal retina, we amplified overlapping segments of the protein coding region with three sets of sequence specific primers, 80/83, 89/81, and 82/102. In addition, using an anchoring primer hybridizing to the poly(A) tail, mcs23 (21), and sense primer 100 located near the C terminus, the 3′ untranslated region was amplified. No size differences were observed in any of the amplified products, as judged by their mobilities on agarose gels (Fig. 1A) in contrast to an apparent 0.3-kb difference reported from RNA blot analysis (16). Given a sensitivity of ±25 bp on the gel system used, this result would be consistent only with a much smaller alteration or a point mutation downstream from the 5′-most primer (89, starting at nucleotide position 21 in ref. 18) or an alteration in the 5′ untranslated region not covered by the amplification primers.

Identification of a Nonsense Codon in rd mRNA. To identify any defects that may exist in the PDE β-subunit gene, we first sequenced the overlapping amplified segments of the rd and normal β-subunit cDNA. All but the 5′ untranslated region and the first 58 nucleotides of the coding region were determined. The sequence obtained from the PDE β-subunit cDNA of normal mouse was identical to our previously published cDNA sequence (18). The rd β-subunit fragments revealed nine differences in comparison with normal cDNA. Three of the differences (positions 2582 (G → C), 2612 (C → T), and 2657 (C → A)) occur in the 3′ untranslated region, and five are silent changes within the coding region (nucleotide positions 86 (TTG → CTG), 331 (ACG → ACA), 826 (TAT → TAC), 1156 (GTC → GTT), 1834 (CAA → CAG)). The only significant difference (a C → A transversion at position 1048) produces a nonsense mutation converting codon 347, tyrosine (TAC), to a stop codon (TAA), thereby creating a new Dde I restriction site (see Fig. 3). Digestion of amplified products (not shown) of normal and rd cDNA produces the expected Dde I restriction fragment length polymorphism (RFLP). The mutation at codon 347 would lead to premature chain termination during translation, predicting a truncated β-subunit of about 40 kDa.

β-Subunit Gene Structure. To establish the β-subunit gene structure and to confirm the presence of the nonsense codon in the rd β-subunit gene, we amplified and sequenced 0.4- to 3.5-kb overlapping segments of normal and rd β-subunit genomic DNA using exon-specific primers. Apart from two segments near the 5′ end (between 118/87 and 132/120), containing large introns, the amplification results show that no major insertions or deletions are present in the rd gene (Fig. 1B). Introns a (located between nucleotide positions 465 and 560) and c (positions 715–801) are estimated to be at least 4.8 and 4.1 kb in length based on DNA blot analysis using cDNA probes that span the location of the introns. Direct sequencing of the amplified material with PDE β-subunit-

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**Fig. 1.** Agarose gel electrophoresis of PCR products. (A) First strand β-subunit cDNA. First strand cDNA produced from normal (left lane of each bracketed pair) and rd (right lane) retinal poly(A) mRNA amplified with primers covering the majority of the coding region and the entire 3′ untranslated region (21) are shown. Purified fragments from amplifications of normal retina cDNA and reamplification of rd retina cDNA were electrophoresed in 1% agarose gels. (B) PDE β-subunit gene. Purified PCR products amplified from genomic DNA of normal and rd mouse with exon-specific primers are shown. Lane M contains DNA size markers (1-kb ladder, BRL). Primer pairs used for amplification are below the lanes of each pair of products.
specific primers allowed the determination of the gene structure in the normal and mutant genomes (Fig. 2). The fragment amplified with primers 131/118, which does not contain an intron, allowed completion of the rd coding sequence up to and including 3 nucleotides upstream of ATG (position 5 in ref. 18).

The β-subunit gene consists of at least 22 exons varying in length from 46 bp to several hundred base pairs and 21 introns varying in size from 70 bp to several kilobases. Introns up to 350 bp in length have been completely sequenced (d, g, m, n, p, q, Fig. 2) and at least 300 bp of the other introns have been determined. The positions of 8 of these introns (j to r, Fig. 2) are identical with the positions of corresponding introns thus far determined in the bovine α-subunit gene (25). The exact conservation of the lengths of exons covering most of the catalytic domain in the bovine α- and mouse β-subunits is consistent with a common ancestral PDE gene.

Overall, 51 nucleotide differences, including one single base-pair insertion, nine single base-pair deletions, and one 27-bp deletion, were found in introns of the rd β-subunit gene in comparison with the normal gene. Only nine differences reside within the exons of the gene, matching the differences found in the rd cDNA. No differences in the sequences of the splice junctions for introns b and d–u (Fig. 2) were found in the rd β-subunit gene compared to the normal gene. Since normal and rd cDNA sequences are identical from 3 nucleotides upstream of ATG to intron d, the splice junctions of introns a and c are unaltered. Not a single discrepancy was identified in the sequence determined from amplified cDNA fragments compared to amplified genomic fragments emphasizing the reliability and accuracy of direct sequencing of PCR products.

Identification and Distribution of the Nonsense Mutation in Mus musculus Strains. Direct sequencing of the rd/rd genomic fragments revealed that the nonsense mutation at codon 347 of the rd cDNA is present in exon 7, of only 67 bp, that is flanked by introns f and g (Fig. 3). As expected, the heterozygous (rd/+) DNA, possessing a normal and a mutant allele, shows a TAC and a TAA codon (Fig. 4), effectively ruling out the possibility of a PCR artifact in the normal and homozygous rd sequences. To determine the presence of the nonsense mutation in strains carrying the rd allele, we amplified with primers 149/150 a 298-bp genomic fragment that contains two Dde I sites in the rd β-subunit allele and none in the normal allele (Fig. 3). One of the sites is created by the C → A transversion, and the other site is created by the insertion of an adenine 117 bp downstream of the start of intron g. Digestion of the PCR amplified products with Dde I from normal, heterozygous, and rd DNA revealed the predicted RFLP pattern (not shown).

To address the possible heterogeneity of defects in other strains carrying the rd allele, we used the primer pair 128/136 to amplify a 2.8-kb fragment spanning the region of the rd mutation. As shown in Fig. 4, we analyzed a strain closely related to C57BL/6J (CBA/J) and six strains shown to contain a genetically identical defect found in stocks thought to be of independent origin (ST/Bj, WB/ReJ-W, PL/J, SWR/J, SJL/J, BDP/J). The nonsense mutation was also found in the retinal degeneration strains FVB/N and Bub/J completing the analysis of presently available rd strains. Direct sequence analysis of the amplified products showed that all of the strains contain the nonsense mutation (Fig. 4), indicating that a single genetic event produced the rd allele. In seven normal strains analyzed (SM/J, AKR/J, DBA/2J, 129/Rr; I/LnJ, LP/J, NZB/Blnd), codon 347 in the β-subunit gene was identified as the tyrosine codon TAC characteristic for normal PDE (not shown). All of the strains analyzed in this study produced the expected Dde I RFLP. The RFLP pattern thus provides a tool for verification of the presence or absence of the rd allele in genetic analyses.

**DISCUSSION**

The gene encoding the PDE β-subunit covers at least 25 kb exhibiting a remarkable structural complexity. It is split into

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**Fig. 2.** cDNA and gene structure of the PDE β-subunit (A) cDNA structure. ATG and TAA denote the beginning and end of the coding sequence (hatched box). Functional domains (19, 23, 24) of the PDE β-subunit are indicated above the box. The asterisk near ATG denotes the nonsense codon present in the rd β-subunit gene. (B) Gene structure. Exons (filled rectangles) are numbered from 1 to 22, and introns (horizontal lines) are lettered from a to u. The lengths of exons were derived from direct sequencing of amplified genomic DNA and comparison to the cDNA sequence. The lengths of introns longer than 150 bp were estimated according to the size of the amplified material. Introns b and d–u are inserted immediately after nucleotide positions (18) 628, 859, 935, 999, 1066, 1114, 1264, 1408, 1474, 1621, 1729, 1840, 1927, 2028, 2136, 2200, 2274, 2359, and 2516. A poly(A) signal is presumed to be at the 3' end of exon 22 based on previous analysis (18). Exon 7 contains the nonsense mutation, indicated by an asterisk, of the rd β-subunit gene. Below the gene structure, an enlarged view of exons 6–9 is shown. Introns f–h are not drawn to scale. The positions of primers (122, 128, 149, 150) used for amplification (sense primers, above the line; antisense primers, below the line) are indicated. Primer 149 (Fig. 3) was used to sequence the region of the nonsense mutation in PCR products amplified with the primer pair 122/128 (Fig. 4).
Fig. 3. Sequence of exon 7 of the rd PDE β-subunit. Intron sequences are in lowercase letters and exon sequences are in uppercase letters. Primer sequences used for DNA sequencing and PCR amplification are in boldface type. Numbers indicate nucleotide positions and amino acid residue positions, respectively. Codon 347 containing the nonsense mutation in the rd β-subunit cDNA is boxed. Dde I sites are present. Two additional Dde I sites are present in the rd gene within the amplified product (see text).

at least 22 exons, separated by 21 introns (Fig. 2). The complexity is further enhanced by our recent finding that the β-subunit gene undergoes alternative splicing (18) at two acceptor sites that are only 10 bp apart. Determination of the gene structure allows assignment of the alternative splice sites to the 5′ end of exon 21. The sequences of exon 21 and the 3′ end of intron t (Fig. 2) are identical in the normal and rdβ-subunit gene. The rd gene also produces an alternatively spliced mRNA that was identified by PCR (unpublished results). The alternative splicing event is thus unrelated to the rd mutation. A related cyclic nucleotide PDE gene, dunci, in Drosophila melanogaster, has also been shown to be very complex. The dunci locus extends over >100 kb and utilizes alternative splicing, producing no less than six different transcripts, and is predicted to utilize multiple promoters (26). Within a large upstream intron, at least two other genes are known to reside (27). It is possible that such complexity is a general feature of PDE genes.

Two distinct characteristics have been described that result from the rd mutation.

(i) A deficiency in PDE catalytic activity and subsequent accumulation of cGMP (4). The truncation produced by the nonsense codon in exon 7 would occur within the region encoding the second putative cGMP noncatalytic binding domain, eliminating the membrane binding domain and the domain presumed to be essential for enzymatic function of cyclic nucleotide PDEs (26, 36). The truncated peptide of ~40 kDa, however, may still be able to bind PDE-γ and/or cGMP, which may explain the reduction in retinal cGMP levels, altered PDE kinetics, abnormal electoretinograms, and abnormalities of cGMP binding observed in adult mice heterozygous for the rd locus (8–11). Deficiency of PDE catalytic activity but the presence of 88-kDa and 11-kDa immunoreactive components consisting of at least one of the two large subunits (7, 28) are consistent with premature chain termination during translation of the rd β-subunit mRNA. Since a truncated form of the β-subunit would be produced in the rd retina, the 88-kDa immunoreactive component described must be the α-subunit. The presence of a 230-residue domain that is conserved in cyclic nucleotide PDEs in the normal α- and β-subunits (18, 23) raised the possibility that two subunits may be independently active enzymes that can be activated by transducin-α/GTP and inhibited by the PDE γ-subunit. The presence of the α-subunit and most likely the γ-subunit in the rd retina but the absence of PDE activity strongly suggest that the α- and γ-subunits alone are unable to effectively regulate cGMP levels in photoreceptors. Most likely, the α- and β-subunits are required to form, together with the γ-subunit, a functional and stable PDE holoenzyme (7).

(ii) A significant reduction in the levels of rd β-subunit mRNA (16). Reductions of mRNA levels caused by the presence of a nonsense mutation have been shown for mutant transcripts of the β-globin and the dhfr genes (29, 30). Transcripts of Drosophila rhodopsin mutants (ninaE<sup>mdw</sup>) with an amber nonsense mutation at codon 251 are reduced to 10% of the normal level (31). Thus, the reduction in rd β-subunit mRNA levels based on the occurrence of a nonsense mutation is entirely consistent with similar effects in various other systems. Although the effects of nonsense mutations are well documented, a precise model to account for this feedback control has not been determined (32). We have also found that the level of α-subunit mRNA in 9- to 11-day-old rd retinas is similarly reduced (data not shown), suggesting that the genes encoding the two subunits may be coordinately regulated.

![DNA sequence of a 34-nucleotide window of exon 7 from several strains exhibiting retinal degeneration. The first two sets on the left are from homozygous C57BL/6J normal (+/+) and heterozygous (rd/+); all products were amplified with primers 122/128. The location of the mutated codon is bracketed. The arrow on the right identifies the location of the boundary of exon 7 and intron g (Fig. 3). The lane order is ACGT.](image-url)
Since the development of the mouse as an organism of genetic study, many inbred strains carrying heritable gene defects have been developed (33). The first retinal degeneration phenotype identified, termed rodless mouse, is now extinct, leaving the controversy over the relationship of this mutant to rd unresolved (1, 34). The lineage of the rd mutation is difficult to follow, since it appears to be a very old mutation present in many inbred strains. Keeler (34) suggested that this rodless mutation, which was identified in a Bagg albino colony, arose from the Bluhm albino stock in Berlin and was subsequently spread throughout the world mouse population. He also provided genetic evidence to support the suggestion that its rodless mutant and rd are genetically identical (34). We analyzed the strains C3H/HeJ and CBA/J, which originated from crosses of Bagg albino females to DBA males. The rd allele was placed into C57BL/6J through crosses with C57H designed to study the pigment gene, le, which is tightly linked to rd (35). Since rd and rodless originated from the Bagg albino stock and we have found that the C3H, CBA, and C57BL/6J strains all contain the nonsense mutation (Fig. 4), the identity of these two mutants is likely. Moreover, preliminary analysis of heterozygous rodless DNA isolated from archival retina sections indicates the presence of the Dde I RFLP (S.J.P., unpublished results). We have also shown that the nonsense mutation is present in strains that are not directly related to the Bagg albino stock, which is consistent with the known retinal degenerations of this type being due to a single genetic event.

Our results show that the rd β-subunit mRNA has no insertions, deletions, or alterations in splicing between translation start point and poly(A) tail. Of nine identified point mutations in the exons of the gene, one (codon 347) produces an ochre nonsense mutation. The mutation and its consequences are consistent with biochemical (defect in subunit assembly, inability of PDE to hydrolyze cGMP in rd/rd mice), altered kinetics in rd/+ and molecular biological phenomena (reduction in β-subunit mRNA levels) assigned to the rd phenotype. Moreover, three strains of related origin and eight unrelated strains with retinal degenerations carry the nonsense mutation, not found in any of the normal strains analyzed. These data strongly argue for the nonsense mutation being the defect responsible for the rd phenotype.

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