10-30-1998

Congenital Stationary Night Blindness in the Dog: Common Mutation in the RPE65 Gene Indicates Founder Effect

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PMID: 9808841
http://www.molvis.org/molvis/v4/p13

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Abstract

Purpose: To clone and characterize the canine RPE65 cDNA from normal dog, examine for mutations, and establish if the mutation identified in Swedish briard dogs with retinal dystrophy is present in dogs of the same breed that originated from the United States and other countries, and are affected with congenital stationary night blindness.

Methods: Fifteen briard dogs were studied, of which 10 were affected with *csnb*, and five were clinically normal. In addition, we tested samples from four Swedish dogs, and samples from a briard affected with progressive retinal atrophy. RPE65 cDNA was cloned a from retinal cDNA library by PCR, and from canine retina by RT-PCR. ERG and morphology were used to characterize *csnb*.

Results: The normal RPE65 cDNA spans 1724 nucleotides (GenBank accession number AF084537), and includes 1602 nucleotides of coding sequence; the deduced amino acid sequence shares 98%, 97%, and 93% identity with homologous human, bovine, and rat sequences, respectively. A homozygous four nucleotide (AAGA) deletion, representing nucleotides 487-490 of wildtype RPE65 sequence, was found only in *csnb* and retinal dystrophy affected dogs; heterozygous animals had normal and mutant alleles. The mutation produces a frameshift, causing a deduced mistranslation with a premature stop codon. The mutation causes retinal dysfunction and RPE accumulation of lipid vacuoles.

Conclusions: Identification of the same mutation in *csnb* and retinal dystrophy confirms the molecular identity of the two disorders. A common mutation in dogs derived from different countries suggests a founder effect causing the propagation of a common mutant allele in the population at risk.

Disciplines

Comparative and Laboratory Animal Medicine | Genetic Phenomena | Medicine and Health Sciences | Veterinary Medicine

Comments

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Molecular Vision **1998; 4:23** <http://www.molvis.org/molvis/v4/p13>
Received 5 October 1998 | Accepted 29 October 1998 | Published 30 October 1998

Congenital stationary night blindness in the dog: common mutation in the RPE65 gene indicates founder effect

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Purpose: To clone and characterize the canine RPE65 cDNA from normal dog, examine for mutations, and establish if the mutation identified in Swedish briard dogs with retinal dystrophy is present in dogs of the same breed that originated from the United States and other countries, and are affected with congenital stationary night blindness.
Methods: Fifteen briard dogs were studied, of which 10 were affected with *csnb*, and five were clinically normal. In addition, we tested samples from four Swedish dogs, and samples from a briard affected with progressive retinal atrophy. RPE65 cDNA was cloned a from retinal cDNA library by PCR, and from canine retina by RT-PCR. ERG and morphology were used to characterize *csnb*.
Results: The normal RPE65 cDNA spans 1724 nucleotides (GenBank accession number AF084537), and includes 1602 nucleotides of coding sequence; the deduced amino acid sequence shares 98%, 97%, and 93% identity with homologous human, bovine, and rat sequences, respectively. A homozygous four nucleotide (AAGA) deletion, representing nucleotides 487-490 of wildtype RPE65 sequence, was found only in *csnb* and retinal dystrophy affected dogs; heterozygous animals had normal and mutant alleles. The mutation produces a frameshift, causing a deduced mistranslation with a premature stop codon. The mutation causes retinal dysfunction and RPE accumulation of lipid vacuoles.
Conclusions: Identification of the same mutation in *csnb* and retinal dystrophy confirms the molecular identity of the two disorders. A common mutation in dogs derived from different countries suggests a founder effect causing the propagation of a common mutant allele in the population at risk.

The briard dog is affected with a recessively inherited retinal disorder characterized by congenital night blindness with various degrees of visual impairment under photopic illumination. Vision in affected dogs ranges from normal day vision to profound day blindness [1]. The disease was initially described in Swedish dogs as a stationary disorder analogous to human congenital stationary night blindness (CSNB [2]). More recently, the disease has been described as having a progressive component, and has been termed hereditary retinal dystrophy [3,4]. Along with the visual impairment, affected dogs have an abnormal electroretinogram (ERG); in general, the recorded responses are normal in waveform, but show a marked diminution of response amplitudes, similar to a "Riggs type" ERG in man. The ERG recorded under DC conditions shows complete absence of the a-, b-, and c-waves, with the latter waveform being replaced by a very slow negative potential which develops when the stimulus intensity is greater than 3 log units above the normal b-wave threshold. The authors interpret the abnormalities in the a- and b-waves as representing a delay in rod phototransduction [5]. A similar disease is also recognized in other countries, including France, Canada, and the United States. In the US, the disease is termed congenital stationary night blindness, and *csnb* has been designated as the gene symbol for the disease locus. Apart from the above studies in Swedish briard dogs, no other systematic investigation of the disease has been reported, nor has there been definitive proof that *csnb* and retinal dystrophy represent the same disorder.

During a presentation on the mutation spectrum of the RPE65 gene in childhood onset retinal dystrophies at the 1998 ARVO meeting, Andreas Gal and associates stated that a 4-nucleotide (AAGA) deletion in the RPE65 gene was responsible for hereditary retinal dystrophy in the Swedish briard dog [6]. This presentation prompted us to clone and characterize the canine RPE65 cDNA from normal dog, examine for mutations in the coding sequence of this gene, and establish if the mutation described in Swedish briards is present in dogs of the same breed that originated from the United States and other countries. Our results indicate that the same four nucleotide deletion in the RPE65 gene is the mutation causing *csnb* in the briard dog.

METHODS

Animals: Briard dogs affected with *csnb* and related and unrelated phenotypically normal dogs have been examined to characterize the disease phenotype, and examine for mutations in the RPE65 gene. Overall, we have studied 15 briard dogs, of which 10 were affected with *csnb*, and five were clinically normal. These dogs came primarily from the US and Canada, of breeding stock that originated from the US and

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France. In addition, we tested samples from four Swedish dogs, both purebred briard or briard-beagle crosses, of which two were affected and two heterozygous for the reported four nucleotide deletion in the RPE65 gene. Lastly, we tested samples from four littermate briard dogs that we had examined previously. One individual from this last group of four dogs was clinically affected with a retinal degenerative disorder. This dog, at 6 years of age, showed evidence of night blindness, hesitant behavior in bright light, and ophthalmoscopically visible retinal thinning and vascular attenuation characteristic of mid-stage progressive retinal atrophy (PRA [7]). ERG testing confirmed the retinal disorder, and indicated that only cone mediated responses were recordable (Aguirre, unpublished data).

All dogs studied were subjected to a comprehensive clinical ophthalmic examination, including indirect ophthalmoscopy and slit lamp biomicroscopy. In addition, a selected number of dogs underwent ERG testing as previously described [8]. Briefly, the ERG was recorded from the halothane anesthetized dog using a stimulus protocol that, by differentially eliciting rod and cone components of the ERG, allowed their separate evaluation [7-10]. Signal averaging of very low amplitude responses also was conducted to examine the waveform of these responses.

The eyes from two dogs, 4.3 and 10.7 months of age, were removed following euthanasia by barbiturate overdose, and processed for microscopic examination using methods we have previously described for embedding either in plastic (4.3 months, both eyes) [10] or in the synthetic wax diethylene glycol distearate (DGD; 10.7 months, one eye) [11]; the tissues were sectioned at 1 µm and stained with azure II/methylene blue. The retina of the fellow eye of the 10.7-month-old dog was isolated under sterile conditions, and kept frozen at -70 °C until used for these studies. All procedures involving animals were undertaken in strict compliance with the guidelines of the US Public Health Service's policy on the Humane Care and Use of Laboratory Animals, and the ARVO Resolution on the Use of Animals in Ophthalmic and Vision Research.

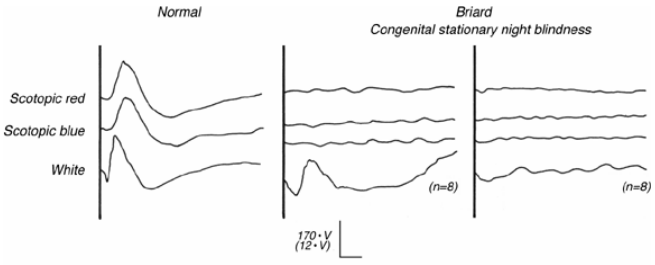

Figure 1. Electroretinographic responses in *csnb*. Representative dark adapted ERG responses recorded from a normal dog (left) and two briard dogs with *csnb* (center and right). The normal dog responds to scotopically balanced red and blue light stimuli with responses which are similar in waveform and amplitude. The response to a single high intensity (4.0 log-foot Lamberts) white light stimulus is biphasic, with a prominent a-wave, and an overall shorter latency b-wave response. In contrast, dogs affected with *csnb* have minute responses which are barely discernible over the baseline noise. When signal averaged over eight responses (n = 8), a distinct ERG response is evident, and the waveform is more characteristic of the normal response in the younger (4 months, center column) than in the older (2 years, right column) animal. This difference is not a characteristic finding with aging. (n = 8) is the number of responses averaged; vertical calibration is 170 µV for single responses, and 12 µV for averaged responses; horizontal calibration = 50 ms.

Genomic DNA and RNA samples: Genomic DNA was isolated using standard techniques [12] from either blood samples collected in citrate anticoagulant tubes, or from splenic samples from deceased dogs. Retina from the enucleated fellow eye of the 10.7-month-old affected dog was utilized for RNA extraction; total RNA was isolated from retina using the guanidinium-phenol procedure previously described [13].

Screening of a canine retinal cDNA library for RPE65 clone by polymerase chain reaction (PCR): The canine retinal cDNA library was custom made (Stratagene Cloning Systems, La Jolla, CA) from poly-A+ RNA isolated from retinas of homozygous normal miniature poodles. RPE65 cDNA sequence was retrieved from the cDNA library by polymerase chain reaction (PCR) based screening of the library. A forward primer (RPE65-1; 5'-CAA TGC CCT TGT TAA TGT CTA CCC AG-3') and a reverse primer (RPE65-3; 5'-CCT GCT TAA TTG TCT CCA AGG TCT C-3') were designed from the consensus region of human (GenBank accession number U18991), bovine (GenBank accession numbers L11356 and X66277) and rat (GenBank accession number AF035673) RPE65 cDNA sequences. The gene specific forward primer, RPE65-1, was used in combination with a vector specific reverse primer (pBK-V; 5'-CCG CTC TAG AAG TAC TCT CGA GTT-3') to amplify the 3'-region of the canine homologue of RPE65 cDNA. Similarly, the gene specific reverse primer, RPE65-3, was used in combination with the vector specific forward primer (pBK-III; 5'-GGT CGA CAC TAG TGG ATC CAA AG-3') to amplify the 5'-region of the canine RPE65 cDNA that would have an overlapping region with the amplified 3' cDNA fragment. PCR was done for 30 cycles (94 °C for 1 min, 60 °C for 1 min, 72 °C for 2 min with a final extension at 72 °C for 10 min) using 0.4 µM of each primer pair in a volume of 50 µl containing 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 2 mM $MgCl_2$, and 0.2 mM each dATP, dCTP, dGTP and dTTP.

Reverse transcription (RT)-PCR from canine retina : A 540 nucleotide long 5'-region of canine RPE65 cDNA was successfully amplified by PCR from the retinal cDNA library, but the 3'-region of the cDNA did not yield PCR product containing the remainder of the coding sequence. To clone the 3' end, a new consensus primer (RPE65-8; 5'-TGC TTG CTC AAC TCA GTG CTT TCT G-3') was designed, as described above, from the 3'-untranslated region (UTR) of mammalian RPE65. A 1400 bp DNA fragment was amplified by RT-PCR using the RPE65-1 and RPE65-8 primer pair from total RNA isolated from retina using RNA PCR kit (Perkin Elmer, Foster City, CA). The identity of the amplified DNA fragment was confirmed to be RPE65 by direct sequencing of the PCR product, and comparison with previously published homologous sequences using the BLAST service. PCR conditions were as above.

PCR using genomic DNA templates: To identify the presence of a mutation in the canine RPE65 gene and examine for

cosegregation of the mutation and the disease, PCR was undertaken for 30 cycles (94 °C for 30 s, 60 °C for 1 min, 72 °C for 1 min with a final extension at 72 °C for 5 min) using primers RPE65-1 and RPE65-3 selected from a single exon (putative exon 5) spanning the location of the mutation. The sizes of the DNA fragments amplified from homozygous normal and *csnb*-affected dogs were 109 bp and 105 bp, respectively. The amplified DNA fragments were electrophoresed in a 6% nondenaturing polyacrylamide gel using TBE buffer (0.089 M Tris-borate and 0.002 M EDTA, pH 8).

DNA sequencing: PCR amplified DNA fragments were used directly for sequencing after purification of the samples. Sequencing was accomplished by Taq cycle sequencing using BigDye™ fluorescent terminators (PE-Applied Biosystems, Foster City, CA) in an ABI 377 DNA sequencer (PE-Applied Biosystems) at the core sequencing facility of Cornell University. Sequence manipulation and comparison were undertaken using programs *Seqed* (ABI Applied Biosystems, Inc.) and *Gene Jockey II*(Biosoft, Cambridge, UK), and the Genbank BLAST service [14].

RESULTS

Clinical and morphologic characterization of csnb*:* We have examined 10 briard dogs affected with *csnb* that originated from stock in the US, Canada and France; in addition, we examined five clinically non-affected briards that were related to the affected dogs in this study. The obligate heterozygous animals had normal ophthalmic examination; prior studies have shown that visual function and the ERG of heterozygous animals is normal (Aguirre and Acland, unpublished data). In contrast, the affected dogs had a severe impairment of visual function that primarily affected night vision, but, in some cases, day vision was affected to various degrees. When young, some of the animals had distinct nystagmus which disappeared with aging, but could be induced with excitement; no other abnormalities were recognized on clinical ophthalmic exam. The one older affected dog that was available for examination at 4 years of age also showed no abnormalities on ophthalmologic examination.

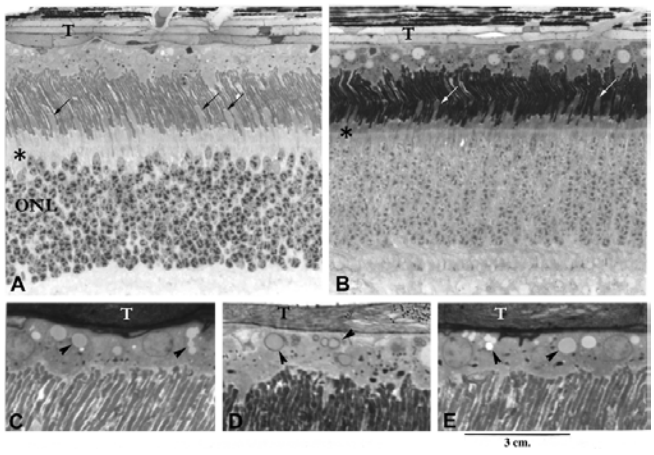

Figure 2. Retinal morphology in *csnb*. Histologic sections of plastic embedded retina from a 4.3-month-old dog affected with congenital stationary night blindness. Sections are taken from the tapetal zone (T) in the superior quadrant, near the posterior pole (A) or the mid-periphery (B). The retina is of normal thickness, and there is a normal number of photoreceptor cells and nuclei in the outer nuclear layer (ONL). The rod outer segments appear slightly irregular, particularly in the posterior pole (A), but are structurally better preserved in the mid-periphery (B). The variable shortening of rod inner segments results in outer segments of differing lengths. Cone inner segments appear elongated and distinct (A, B-oblique arrows). Cytoplasmic inclusions are present in the RPE (C-E, arrowheads). These occur as single small inclusions, or form aggregates or larger inclusions that can be homogeneous or vacuolated. * external limiting membrane. (A and B x500; C-D x1250).

Electroretinography of affected dogs showed that the rod and cone mediated responses were severely depressed in amplitude in comparison to those recorded from normals (Figure 1). In general, the responses appeared diminutive or non-recordable under most recording conditions, especially when the retina was stimulated with weak illumination. Higher intensity flickering light stimuli that elicited cone-mediated responses often resulted in low amplitude signals (data not shown). Signal averaging showed the presence of small amplitude responses that often had a normal waveform, similar to a “Riggs type” ERG in man [2].

Light microscopic examination of the retinas showed pathologic changes limited to the retinal pigment epithelium (RPE) and photoreceptor layers. These abnormalities were most distinct in plastic embedded sections of the retina of the younger dog (4.3 months), but were also evident in the eye from the older affected dog that was embedded in DGD. The photoreceptor outer segments appeared normal, particularly in the periphery, but showed slight disorganization in the posterior pole and equator (Figure 2A). Additionally, there was an uneven shortening of the rod inner segments that caused the rod outer segments to have a variable length, even when structurally normal (Figure 2B). The shortening of the rod inner segments resulted in increased prominence of the cones in the photoreceptor layer. The most remarkable abnormalities, however, were present in the RPE, and consisted of the accumulation of cytoplasmic inclusions of variable size. These inclusions were single to multiple, and were vacuolated or appeared homogeneous (Figure 2C-E). The RPE inclusions appeared to coalesce and were much larger in the 10.7-month-old dog. The RPE appeared somewhat reactive in that the cells were slightly hypertrophied, and their apical surfaces were irregular. At the two ages examined, there were no other pathologic changes in the retina, and no evidence of photoreceptor degeneration or cell death as indicated by the presence of an outer nuclear layer of normal thickness.

Characterization of the canine RPE65 cDNA: Overlapping fragments of normal canine RPE65 cDNA were amplified from the retinal cDNA library by PCR, and from retinal RNA by RT-PCR. The characterized region of normal canine RPE65 cDNA spans 1724 nucleotides (GenBank accession number AF084537), and includes 1602 nucleotides of coding sequence predicted to encode a protein of 533 amino acids (61 kDa), 27 nucleotides of 5'-*UTR* and 94 nucleotides of 3'-UTR (Figure 3). Over the coding region, the canine RPE65 gene shares 88-89% nucleotide sequence identity with homologous human and bovine sequences, and 83% identity with rat sequence. The deduced amino acid sequence shares 98%, 97%,

and 93% identity with homologous human, bovine, and rat sequences, respectively.

csnb *results from the same mutation in the RPE65 gene causing retinal dystrophy in Swedish briard dogs:* Once we characterized the normal canine RPE65 cDNA, the cDNA was amplified from *csnb*-affected retinal RNA and compared with the normal. We observed that the four nucleotide (AAGA) deletion reported to cause retinal dystrophy in Swedish briards [6] is present in *csnb*-affected briards in USA and Canada. The deleted nucleotides (AAGA) represent nucleotides 487-490 of wildtype canine RPE65 sequence, and correspond to nucleotides 340-343 of human exon 5 (Genbank accession number U20479). The mutation produces a frameshift, causing a deduced mistranslation of (now) nucleotides 487 through 645, with a stop at (now) codon 205 (nucleotides 643-645 of mutant sequence). No other disease causing mutations were identified in the sequence obtained from the affected dogs.

To identify the mutation from genomic DNA in suspected dogs, a region of putative exon 5 encompassing the site of the mutation was amplified. As shown in Figure 4, PCR using genomic DNA from *csnb*-affected and normal briard dogs resulted in amplification of DNA fragments 105 bp and 109 bp long, respectively. As expected, PCR product from an obligate heterozygote dog contained both the alleles. Also, the presence of two distinct heteroduplex bands with slower mobility in the polyacrylamide gel is a typical observation associated with PCR products of heterozygous samples containing two alleles resulting from a short insertion or deletion. To determine if the mutation identified in *csnb*-affected dogs is the same as the one described for Swedish dogs with retinal dystrophy, two affected and two heterozygous dogs were analyzed, and the results were identical to the observation made for *csnb* alleles in US and Canadian briard dogs (Figure 4). Sequencing of the amplified DNA fragments from the normal and *csnb*-affected briard revealed deletion of four nucleotides (AAGA).

Cosegregation of the RPE65 mutation in csnb*-affected briard pedigree:* Once the four nucleotide deletion in the RPE65 gene was identified in *csnb*affected dogs, we examined a briard pedigree informative for *csnb* to determine if the mutation cosegregated with the disease. In the animals available for molecular testing, we found complete cosegregation of the mutation with the disease; affected dogs were homozygous for the four nucleotide deletion while obligate carriers were heterozygous for the normal and mu-

(A)

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 1                                                          cgaccgtctgtcctgccctgggagaca
       Met Ser Ile Gln Val Glu His Pro Ala Gly Gly Tyr Lys Lys Leu Phe Glu Thr Val Glu    20
 28    ATG TCC ATC CAA GTG GAG CAT CCC GCC GGC GGT TAC AAG AAG CTG TTT GAA ACC GTG GAA
       Glu Leu Ser Ser Pro Leu Thr Ala His Val Thr Gly Arg Ile Pro Leu Trp Leu Thr Gly    40
 88    GAG CTG TCG TCG CCG CTC ACC GCC CAC GTG ACA GGC AGG ATC CCG CTC TGG CTC ACG GGC
       Ser Leu Leu Arg Cys Gly Pro Gly Leu Phe Glu Val Gly Ser Glu Pro Phe Tyr His Leu    60
148    AGT CTC CTC CGA TGC GGA CCG GGG CTC TTC GAG GTT GGA TCT GAA CCA TTT TAC CAC CTG
       Phe Asp Gly Gln Ala Leu Leu His Lys Phe Asp Phe Lys Glu Gly His Val Thr Tyr His    80
208    TTT GAC GGA CAA GCC CTT CTG CAC AAG TTC GAC TTT AAA GAA GGA CAC GTC ACC TAT CAC
       Arg Arg Phe Ile Arg Thr Asp Ala Tyr Val Arg Ala Met Thr Glu Lys Arg Ile Val Ile   100
268    AGA AGG TTC ATC CGC ACC GAT GCT TAC GTC CGG GCA ATG ACC GAG AAA AGG ATC GTC ATA
       Thr Glu Phe Gly Thr Cys Ala Phe Pro Asp Pro Cys Lys Asn Ile Phe Ser Arg Phe Phe   120
328    ACG GAA TTT GGC ACC TGT GCG TTC CCA GAT CCC TGC AAG AAT ATA TTT TCC AGG TTT TTT
       Ser Tyr Phe Arg Gly Val Glu Val Thr Asp Asn Ala Leu Val Asn Val Tyr Pro Val Gly   140
388    TCT TAC TTC CGA GGA GTG GAG GTC ACT GAC AAT GCC CTT GTT AAC GTC TAC CCA GTA GGG
       Glu Asp Tyr Tyr Ala Cys Thr Glu Thr Asn Phe Ile Thr Lys Ile Asn Pro Glu Thr Leu   160
448    GAA GAT TAC TAC GCC TGC ACG GAG ACC AAC TTC ATT ACA AAG ATT AAT CCT GAG ACC CTG
       Glu Thr Ile Lys Gln Val Asp Leu Cys Asn Tyr Val Ser Val Asn Gly Ala Thr Ala His   180
508    GAG ACA ATT AAG CAG GTT GAT CTC TGC AAC TAC GTC TCT GTC AAT GGA GCC ACC GCT CAC
       Pro His Ile Glu Asn Asp Gly Thr Val Tyr Asn Ile Gly Asn Cys Phe Gly Lys Asn Phe   200
568    CCC CAC ATT GAA AAT GAT GGG ACT GTT TAC AAC ATT GGT AAT TGC TTT GGG AAA AAT TTT
       Ser Ile Ala Tyr Asn Ile Val Lys Ile Pro Pro Leu Gln Ala Asp Lys Glu Asp Pro Ile   220
628    TCG ATT GCC TAC AAT ATT GTA AAG ATC CCT CCA CTC CAA GCA GAC AAG GAA GAT CCA ATA
       Ser Lys Ser Glu Val Val Val Gln Phe Pro Cys Ser Asp Arg Phe Lys Pro Ser Tyr Val   240
688    AGC AAG TCC GAG GTC GTC GTA CAA TTC CCC TGC AGC GAC CGA TTC AAG CCA TCG TAC GTC
       His Ser Phe Gly Leu Thr Pro Asn Tyr Ile Val Phe Val Glu Thr Pro Val Lys Ile Asn   260
748    CAT AGT TTT GGT TTG ACT CCC AAC TAT ATT GTT TTT GTG GAG ACG CCA GTC AAA ATT AAC
       Leu Leu Lys Phe Leu Ser Ser Trp Ser Leu Trp Gly Ala Asn Tyr Met Asp Cys Phe Glu   280
808    CTG CTC AAG TTC CTT TCT TCG TGG AGT CTT TGG GGA GCC AAC TAC ATG GAT TGT TTT GAG
       Ser Asn Glu Thr Met Gly Val Trp Leu His Ile Ala Asp Lys Lys Arg Lys Lys Tyr Leu   300
868    TCC AAT GAA ACC ATG GGG GTT TGG CTT CAC ATC GCT GAC AAA AAA AGA AAA AAG TAT CTC
       Asn Asn Lys Tyr Arg Thr Ser Ser Phe Asn Leu Phe His His Ile Asn Thr Tyr Glu Asp   320
928    AAT AAT AAG TAC AGG ACC TCT TCC TTT AAT CTC TTC CAT CAT ATC AAT ACT TAC GAA GAC
       Asn Glu Phe Leu Ile Val Asp Leu Cys Cys Trp Lys Gly Phe Glu Phe Val Tyr Asn Tyr   340
988    AAT GAG TTT CTG ATT GTG GAT CTC TGC TGC TGG AAA GGA TTT GAA TTC GTC TAC AAT TAC
       Leu Tyr Leu Ala Asn Leu Arg Glu Asn Trp Glu Glu Val Lys Lys Asn Ala Arg Lys Ala   360
1048   TTG TAT TTA GCC AAT TTA CGT GAG AAC TGG GAA GAG GTG AAA AAA AAT GCC AGA AAG GCT
       Pro Gln Pro Glu Val Arg Arg Ser Val Leu Pro Leu Asn Ile Asp Lys Ala Asp Thr Gly   380
1108   CCG CAG CCT GAA GTT AGG AGA TCC GTG CTT CCT TTG AAT ATC GAC AAG GCC GAC ACA GGC
       Lys Asn Leu Val Thr Leu Pro Asn Thr Thr Ala Thr Ala Thr Leu Arg Ser Asp Glu Thr   400
1168   AAG AAC CTA GTC ACC CTT CCC AAC ACG ACG GCC ACT GCA ACT CTG CGC AGC GAC GAG ACC
       Ile Trp Leu Glu Pro Glu Val Leu Phe Ser Gly Pro Arg Gln Ala Phe Glu Phe Pro Gln   420
1228   ATC TGG CTG GAA CCT GAG GTT CTC TTC TCA GGG CCT CGT CAA GCC TTT GAG TTT CCT CAA
       Ile Asn Tyr Gln Lys Tyr Gly Gly Lys Pro Tyr Thr Tyr Ala Tyr Gly Leu Gly Leu Asn   440
1288   ATC AAC TAT CAG AAG TAT GGC GGG AAG CCT TAC ACG TAC GCG TAT GGA CTT GGC TTG AAT
       His Phe Val Pro Asp Arg Leu Cys Lys Leu Asn Val Lys Thr Lys Glu Thr Trp Val Trp   460
1348   CAC TTC GTT CCG GAC AGG CTC TGC AAG CTG AAC GTC AAG ACT AAA GAA ACG TGG GTA TGG
       Gln Glu Pro Asp Ser Tyr Pro Ser Glu Pro Ile Phe Val Ser His Pro Asp Ala Leu Glu   480
1408   CAA GAG CCC GAC TCA TAC CCA TCA GAA CCC ATC TTT GTT TCT CAC CCA GAT GCC TTG GAA
       Glu Asp Asp Gly Val Val Leu Ser Val Val Val Ser Pro Gly Ala Gly Gln Lys Pro Ala   500
1468   GAA GAT GAT GGT GTA GTT CTG AGT GTG GTG GTG AGC CCT GGG GCA GGA CAA AAG CCT GCT
       Tyr Leu Leu Ile Leu Asn Ala Lys Asp Leu Ser Glu Val Ala Arg Ala Glu Val Glu Ile   520
1528   TAT CTT CTG ATT CTG AAT GCC AAG GAT TTG AGT GAA GTT GCC AGG GCT GAA GTG GAG ATT
       Asn Ile Pro Val Thr Phe His Gly Leu Phe Lys Lys Ser  *                            533
1588   AAC ATC CCT GTC ACC TTT CAT GGA CTG TTC AAA AAA TCC TAA gtacattctagcaaattatattt
1653   ctattgacaaagtcaagaaaaagtgaggtctgcaatcaaattctgttcaattttagcctgctgtattacagg
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(B)

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   1   Met Ser Ile Gln Val Glu His Pro Ala Gly Gly Tyr Lys Lys Leu Phe Glu Thr Val Glu    20
  21   Glu Leu Ser Ser Pro Leu Thr Ala His Val Thr Gly Arg Ile Pro Leu Trp Leu Thr Gly    40
  41   Ser Leu Leu Arg Cys Gly Pro Gly Leu Phe Glu Val Gly Ser Glu Pro Phe Tyr His Leu    60
  61   Phe Asp Gly Gln Ala Leu Leu His Lys Phe Asp Phe Lys Glu Gly His Val Thr Tyr His    80
  81   Arg Arg Phe Ile Arg Thr Asp Ala Tyr Val Arg Ala Met Thr Glu Lys Arg Ile Val Ile   100
 101   Thr Glu Phe Gly Thr Cys Ala Phe Pro Asp Pro Cys Lys Asn Ile Phe Ser Arg Phe Phe   120
 121   Ser Tyr Phe Arg Gly Val Glu Val Thr Asp Asn Ala Leu Val Asn Val Tyr Pro Val Gly   140
 141   Glu Asp Tyr Tyr Ala Cys Thr Glu Thr Asn Phe Ile Thr Leu Ile Leu Arg Pro Trp Arg   160
 161   Gln Leu Ser Arg Leu Ile Ser Ala Thr Thr Ser Leu Ser Met Glu Pro Pro Leu Thr Pro   180
 181   Thr Leu Lys Met Met Gly Leu Phe Thr Thr Leu Val Ile Ala Leu Gly Lys Ile Phe Arg   200
 201   Leu Pro Thr Ile Leu Stop                                                         205
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Figure 3. Canine RPE65, normal (wildtype) and *csnb*-mutant sequence. (A) Nucleotide (cDNA) and deduced amino acid sequences of canine RPE65 (wildtype allele). Nucleotides 487-490 (AAGA), shown in green, is the sequence deleted in the mutant allele. Nucleotides 417-442, and 523-499, shown in blue, are binding sites for primers RPE65-1 (forward) and RPE65-3 (reverse), respectively, used to amplify across the mutant site. The amino acids that differ from human sequence (codons 225, 262, 308, 322, 368, 395, 397, and 427) are shown in red. The "Start" (Met) and "Stop" (*) codons, nucleotides 28-30 and 1627-9, respectively, are shown in purple. The 5' and 3' UTRs (nucleotides 1-27 and 1630-1725, respectively) are shown with lower case letters. (B) Deduced amino acid sequence of canine mutant RPE65. Amino acids that differ from wildtype sequence are shown in red.

tant alleles. In this small pedigree, it was possible to readily differentiate the homozygous normal from heterozygous samples from phenotypically normal animals that were either genetically normal or carriers (Figure 5).

Progressive retinal atrophy (PRA) in the briard dogs is not associated with the RPE65 mutation causing csnb *and retinal dystrophy:* To determine if this mutation in the RPE65 gene is associated with PRA in the briard, we tested samples from four littermate dogs of this breed in which a diagnosis of PRA had been made in one of them on the basis of the characteristic visual, ophthalmoscopic and ERG abnormalities. We found no abnormality in the region of the RPE65 gene that harbors the mutation responsible for *csnb*, either in the PRA-affected dog or in its normal littermates (Figure 4, right panel).

DISCUSSION

The clinical, electrophysiologic and pathologic features of retinal dystrophy in the briard dog have been reported in a series of very detailed studies from Sweden [1-5]. The disease has a characteristic clinical phenotype, consisting of profound visual impairment present soon after the dog is sufficiently mature to test visual function (5-6 weeks of age), and a normal appearing fundus, at least for the first 3-4 years of age. Older dogs may show subtle retinal abnormalities indicative of a slowly progressive retinal degenerative process. The ERG responses, both rod and cone mediated, are also abnormal, and the DC ERG suggests a defect in the phototransduction process [5]. Surprisingly, the photoreceptor cells do not show extensive pathologic abnormalities, at least in the early stages of the disease, that would be expected for animals with such functional deficits. The RPE has shown a dramatic accumulation of lipoidal inclusions [3,15] that, until now, appeared to be an unexplained byproduct of the disease process (see below).

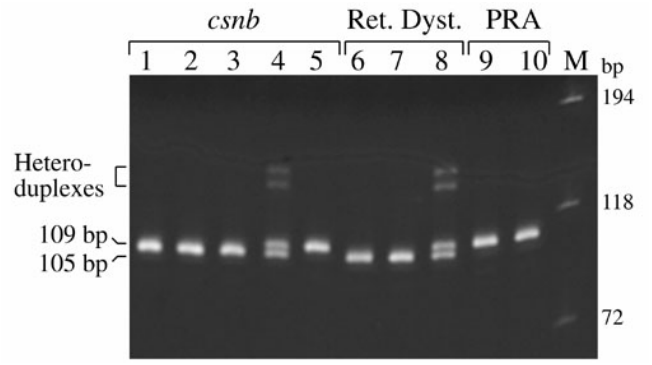

Figure 4. Amplification of canine RPE65 gene fragment from briard dogs. Amplification of putative exon 5 of RPE65 gene from briard dogs affected with *csnb*, retinal dystrophy (Ret. Dyst.) and progressive retinal atrophy (PRA). A small region of exon 5 was amplified by PCR from (a) *csnb*-affected (lanes 1-3), carrier (lane 4), and normal (lane 5) dogs of US and Canadian origin; (b) Swedish briard dogs affected with retinal dystrophy (lanes 6 and 7) or carrier for the disease (lane 8); and (c) PRA-affected (lane 9) and nonaffected littermate (lane 10) briard dogs of US origin. The PCR products were separated in 6% non denaturing polyacrylamide gel. The sizes of the amplified DNA fragments from the two alleles, and the relative location of the heteroduplexes formed between these two alleles in the gel are indicated. The marker lane (M) contains 100 bp DNA ladder.

Because of the clinical similarities in phenotype between retinal dystrophy and *csnb*, a disease identified in briard dogs from the United States and Canada, we examined a selected population of briards using methods which would evaluate the clinical, functional and morphologic characteristics of *csnb* in a manner that was analogous to the studies done on the Swedish dogs. With the limitation imposed by using slightly different methods, our results are totally compatible to those published by Narfström and associates in their studies [1,2,4]. At least on a clinical and morphologic basis, we can conclude that *csnb* and retinal dystrophy appear to represent the same disorder. Based on the four nucleotide deletion in the RPE65 gene that was reported to be causally associated with retinal dystrophy in briards [6], we cloned and characterized the canine RPE65 cDNA to determine if a mutation in this gene is present in *csnb*, and if it is the same as that causing retinal dystrophy in the Swedish dogs.

Previous reports have indicated that the RPE65 gene is exclusively expressed in the RPE [16,17]. However, we decided to characterize the cDNA from a retinal cDNA library on the premise that even a low level of expression of the gene in the tissue would be sufficient for amplification of the coding sequence, and characterization of the *UTR*. The characterized region of normal canine RPE65 cDNA spans 1724 nucleotides (GenBank accession number AF084537), and includes 1602 nucleotides of coding sequence predicted to encode a protein of 533 amino acids (61 kDa), 27 nucleotides of 5'-*UTR* and 94 nucleotides of 3'-UTR. Comparison of the sequence between the normal and *csnb*-affected dog indicated that in the affected there was a four nucleotide deletion (AAGA) in the putative exon 5 of the RPE65 gene that was the same as described by Gal and associates for dogs with retinal dystrophy. The deleted nucleotides (AAGA), represent nucleotides 487-490 of wildtype canine RPE65 sequence, and correspond to nucleotides 340-343 of human exon 5. The mutation produces a frameshift, causing a deduced mistranslation with a stop at (now) codon 205 (nucleotides 643-645 of the mutant sequence), and a presumably non-functional RPE65 gene product.

To establish if the observed mutation was causally associated with the disease, we amplified from genomic DNA a region of the putative exon 5 encompassing the site of the mutation. In a three generation pedigree informative for *csnb*, we could establish the cosegregation of the mutant allele with 100% concordance. These dogs were part of a larger sample of 15 briard dogs whose disease status was known, and derived from breeding stock that originated from the US and France. In all cases, affected dogs showed the homozygous four nucleotide deletion of the RPE65 gene, while obligate heterozygous dogs had the mutation in only one allele. Lastly, we tested four Swedish briard or briard-beagle crosses, two affected and two heterozygous for retinal dystrophy [1], and found the same mutation. Identification of the same mutation in briards with *csnb*and retinal dystrophy confirms the molecular identity of the two disorders. Furthermore, because some of the dogs tested in this study were apparently unre-

lated, the finding of a common mutation in dogs derived from different countries suggests a founder effect causing the propagation of a common mutant allele in the population at risk.

Progressive retinal atrophy also is present sporadically in the briard breed, and the clinical and functional abnormalities identified in the intermediate stages of the disease could be compatible with those present in older dogs affected with *csnb*. To exclude the four nucleotide deletion in the RPE65 gene from causal association with PRA, we tested samples from four littermate briard dogs, one PRA-affected and three non-affected. For the region of the RPE65 gene examined by PCR, we did not find the four nucleotide deletion that results in *csnb*. Thus, this mutation could be excluded as a cause of PRA in this dog breed.

In their 1997 paper, Gu and associates described five different mutations in the RPE65 gene responsible for autosomal recessive childhood-onset severe retinal dystrophy [18]. Most patients had severe visual deficits present at birth or within the first decade of life. Ophthalmoscopic abnormalities varied from vascular attenuation and optic disc atrophy without bone spicules, to lesions typical of advanced retinitis pigmentosa in adults. In these patients, the disease progresses to severe visual impairment and blindness, and concomitant ophthalmoscopic abnormalities indicative of advanced retinal degeneration [18]. Similar abnormalities have been described in a second study of the disease [19]. More recently, mutations in this gene have been causally associated with autosomal recessive RP or Leber congenital amaurosis [20]. Although the profound visual deficit early in life is similar in the human and dog, the lack of ophthalmoscopically visible advanced retinal degeneration in adult dogs is not, and may indicate a difference in the temporal course of the photoreceptor disease. After all, most dogs affected with the different forms of PRA show evidence of advanced fundus pathology by 5 years [7], an age that would be comparable to a 35-year-old human.

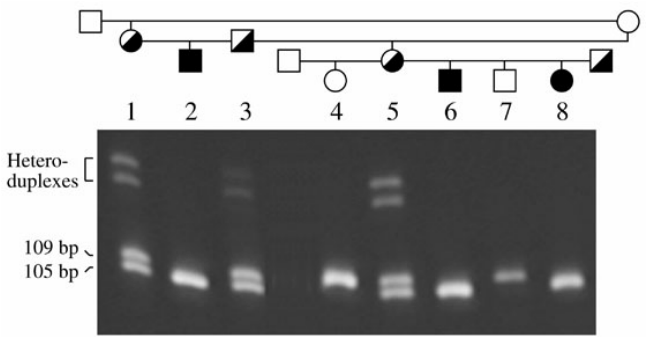

Figure 5. Cosegregation of RPE65 gene with *csnb* in briard dogs. Mendelian inheritance of the RPE65 mutation in a briard pedigree informative for *csnb*. Affected dogs are represented by black squares (males) and circles (females); obligate heterozygous and clinically non-affected dogs are represented by half filled and open symbols, respectively. The region of RPE65 exon 5 containing the mutation was amplified from genomic DNA of individual dogs, and the PCR products were separated in a 6% non denaturing polyacrylamide gel. Each lane in the illustration shows PCR product obtained from DNA samples of the dog corresponding to the lane. Some of the dogs of the pedigree from which the DNA samples were not available do not correspond to any marked lane of the gel. PCR products from the affected dogs (lanes 2, 6, and 8) contain only the smaller DNA fragment (105 bp) due to the presence of a four nucleotide deletion. Homozygous normal dogs (lanes 4 and 7) contain the normal allele, hence the larger DNA fragment (109 bp). As expected, PCR products from the obligate heterozygotes (lanes 1, 3, and 5) contain both of the DNA fragments, and the heteroduplexes formed between the complementary strands of the two alleles.

Mice with an RPE65 gene knockout were recently created [21]. Homozygous mutant mice show irregularities of the rod outer segments by 15 weeks of age, and these changes are associated with a 4.5 log unit increase in the dark adapted threshold, and a small amplitude ERG that is almost identical to that recorded under light adapted conditions. Even though the rod ERG is abolished, the results indicate that the cone ERG is normal (Redmond TM, Personal Communication, May, 1998). A recent commentary has suggested that the RPE65 gene product functions in retinoid metabolism in the RPE and retina [22]. Based on this putative function, the normal cone ERG function in the absence of rod mediated activity could be interpreted as supporting the hypothesis that rod and cones have different and independent pathways for visual pigment regeneration [23]. This difference, however, does not appear to exist in the dog since cone ERG function was compromised in all dogs with the mutation, and profound impairment of day vision was present in some of the affected animals. This issue merits further investigation as it may play a significant role in the evaluation of mice or dogs following RPE cell transplantation or vector-mediated gene therapy for the experimental treatment of the disease.

The salient pathologic abnormality in the retina of dogs with the four nucleotide deletion in the RPE65 gene, documented in this study or reported previously [3,15], is the accumulation of lipoidal inclusions of variable size within the pigment epithelium. Mice with the RPE65 gene knockout have no rhodopsin in the dark adapted retinal rods, but accumulate all-trans retinyl esters in the pigment epithelium (Redmond TM, Personal Communication, May, 1998). The accumulation of all-trans retinyl esters in the RPE suggests that the RPE65 protein functions in one of the metabolic steps involved in the conversion of all-trans retinyl esters to 11-cis retinol [22]. Although the precise function of the RPE65 protein in RPE retinoid metabolism is still to be determined, deficiency of the protein, either in naturally occurring cases or in transgenic knockout mice, results in the accumulation of all-trans retinyl esters in the RPE. Based on prior studies of vitamin A metabolism in the frog eye, these retinyl esters probably accumulate in oil droplets within the RPE which is the major storage depot for esterified vitamin A in the RPE [24]. These lipoidal inclusions are present in the RPE of affected dogs, and their number and size increases with age as reported here and in other studies [3,15].

ACKNOWLEDGEMENTS

Supported in part by the Briard Club of America, American Kennel Club-Canine Health Foundation, The Morris Animal Foundation/The Seeing Eye, Inc., The Foundation Fighting Blindness, NEI/NIH Grant EY 06855, and Swedish Medi-

cal Research Council grant 19X-09938. Three of the authors (GDA, GMA, KR) have a proprietary interest in a company that will perform the molecular test for *csnb*. The authors are grateful to the many owners and breeders who willingly participated in this study by making their dogs available, and to Julie Alling and Jill Czarnecki for excellent technical assistance.

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The typographical corrections below were made to the article on the date noted. These changes have been incorporated in the article and the details are documented here.

17 November 1998: In the caption of Figure 3A, the word "gray" was changed to "purple" and the color of the gray text was changed to purple.