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4 June 2001; accepted 1 August 2001

Triallelic Inheritance in Bardet-Biedl Syndrome, a Mendelian Recessive Disorder

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Bardet-Biedl syndrome (BBS) is a genetically heterogeneous disorder characterized by multiple clinical features that include pigmentary retinal dystrophy, polydactyly, obesity, developmental delay, and renal defects. BBS is considered an autosomal recessive disorder, and recent positional cloning efforts have identified two *BBS* genes (*BBS2* and *BBS6*). We screened our cohort of 163 BBS families for mutations in both *BBS2* and *BBS6* and report the presence of three mutant alleles in affected individuals in four pedigrees. In addition, we detected unaffected individuals in two pedigrees who carry two *BBS2* mutations but not a *BBS6* mutation. We therefore propose that BBS may not be a single-gene recessive disease but a complex trait requiring three mutant alleles to manifest the phenotype. This triallelic model of disease transmission may be important in the study of both Mendelian and multifactorial disorders.

Locus heterogeneity in Mendelian disorders is the phenomenon whereby mutations in different genes result in a similar or identical clinical phenotype. In most reported instances, mutations at a single locus suffice to cause disease, although rare cases have been reported where mutations at two loci are necessary for pathogenesis (*1*) or exacerbate the severity of the phenotype (*2*, *3*). BBS is a typical example of a rare, genetically heterogeneous disorder. BBS patients manifest a complex and variable phenotype that includes pigmentary retinal dystrophy, polydactyly, central obesity, hypogonadism, learning difficulties, and renal dysplasia; additional features such as asthma and diabetes mellitus may also be present (*4*, *5*). The segregation of the disorder in families and population isolates led to the hypothesis that the syndrome is inherited in an autosomal recessive manner. On the basis of this model, six *BBS* loci have been identified: *BBS1* on 11q13 (*6*), *BBS2* on 16q21 (*7*), *BBS3* on 3p12 (*8*), *BBS4* on 15q22.2-q23 (*9*), *BBS5* on 2q31 (*10*), and *BBS6* on 20p12 (*11*), with evidence for at least one more locus (*12*). Two *BBS* genes have been cloned recently: *BBS6* (*11*, *13*), which encodes a putative chaperonin (*14*), and *BBS2*, which encodes a protein of unknown function (*15*).

During our analysis of *BBS6* in 163 patients, we identified eight pedigrees with mutations. A high frequency of these (seven of eight) harbored only a single mutant *BBS6* allele (*12*). Furthermore, we described one consanguineous pedigree in which both the affected and unaffected sibs carried a heterozygous A242S mutation in *BBS6*, but the affected sib also exhibited homozygosity by descent (HBD) across the *BBS2* locus. This observation suggests either that BBS arises through multiallelic inheritance or that the A242S allele is a rare polymorphism (*12*).

The recent identification of *BBS2* enabled us to test the former hypothesis by screening the same cohort of BBS patients (*16*) for coding sequence alterations in *BBS2*, irrespective of haplotype-inferred chromosomal assignment for any given pedigree or previously obtained mutational data for *BBS6*. Upon deducing the intron-exon structure of *BBS2* (*17*), we designed suitable primers and amplified and sequenced each of the 17 exons of this gene in our entire patient cohort (*18*).

We identified numerous sequence alterations. In 19 unrelated patients (Table 1), the alterations fulfilled our minimal criteria for mutations, in that they caused a nonconservative amino acid change and that they were not found in a minimum of 192 control chromosomes from ethnically matched samples (*19*). Initial segregation analyses confirmed that *BBS2* mutations were likely to be pathogenic. We identified six pedigrees (AR171, PB005, PB020, PB026, PB058, and K059) in which two independent mutations segregated with the disease (Table 1) (*20*). However, despite complete DNA sequence coverage of the *BBS2* open reading frame (ORF) and exon-intron boundaries, we identified only a single mutant allele in eight pedigrees. We considered four possible explanations for this result: (i) the single alleles found may not be pathogenic but rare polymorphisms, (ii) by chance alone, we were detecting rare *BBS2* carriers, (iii) the second mutation might reside in the regulatory regions or introns of *BBS2* or might be undetectable by sequenc-

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ing because of deletions of the whole or part of the gene, or (iv) additional mutations lie in another gene, and it is the combination of mutant alleles at two or more loci that causes the BBS phenotype.

The first hypothesis is unlikely because changes caused by several of the "singleton" mutant alleles are predicted to be severe (Table 1). For instance, conceptual translation of the single mutation in pedigree AR124 causes a frameshift affecting a valine at position 158 and then results in premature termination of translation at position 200 (V158fsX200; Fig. 1 and Table 1). Likewise, the mutation found in pedigree AR050 was an arginine to STOP (R275X), also causing premature termination (Table 1). Furthermore, this R275X mutation was also found in the homozygous state and segregated with the disorder in pedigree PB005 (*20*). None of these alterations were found in 192 ethnically matched control chromosomes.

The possibility of incidental detection of BBS carriers is even less likely. The frequency of BBS is 1:125,000 to 1:150,000 in the European and North American populations (*4*). Furthermore, the contribution of *BBS2* to all BBS has been estimated at about 10 to 20% (*12*, *15*, *21*), which at Hardy-Weinberg equilibrium leads to an expected carrier frequency of 1:400 to 1:700 for *BBS2*. Therefore, the association of five mutations with *BBS2* (in North American pedigrees AR050, AR124, AR237, AR238, and AR596; Table 1) in a sample of 163 patient DNAs is significant ($\chi^2 = 52.9$; $P < 0.0001$).

We explored the possibility that the second mutation was not located in the ORF or was a large deletion by genotyping microsatellites around *BBS2* and examining whether the segregation pattern of the parental chromosomes in any given pedigree with a single mutation was consistent with linkage to *BBS2* (*22*). Six (of eight) pedigrees provided sufficient information for this investigation. Only one pedigree, AR029, was consistent with linkage to *BBS2*.

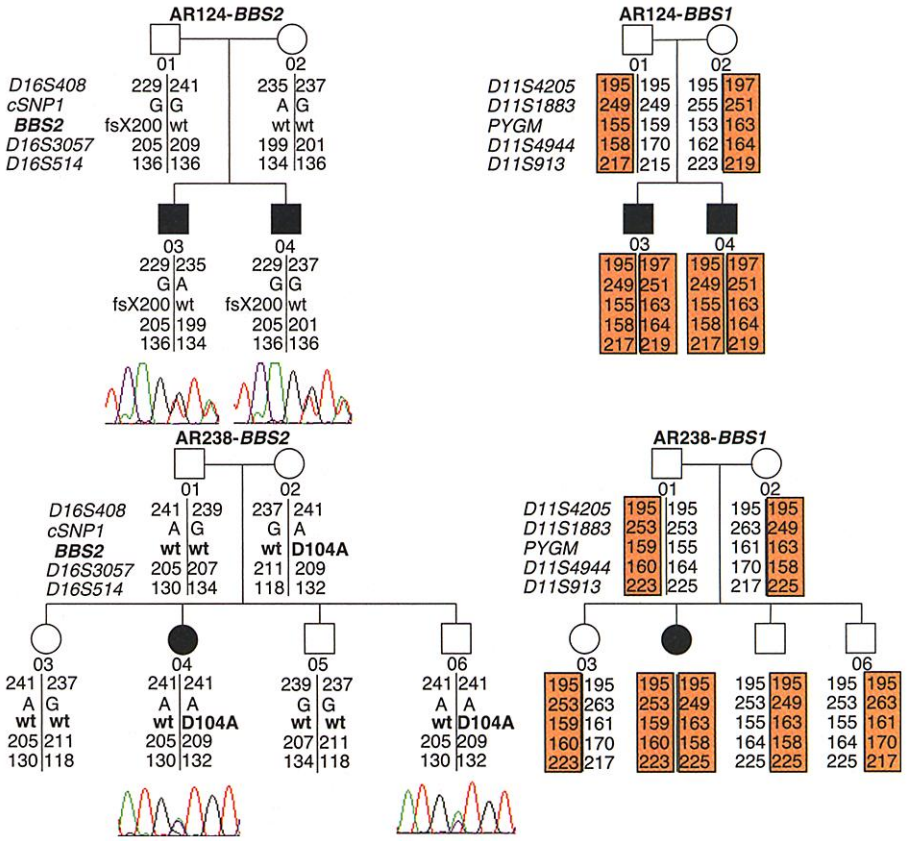

Fig. 1. Pedigrees with one *BBS2* mutant allele that have been excluded genetically from the *BBS2* region. In AR124, the two affected siblings carry different maternal chromosomes. In AR238, the pedigree is excluded from *BBS2* because the single affected sib AR238-04 has an identical haplotype to that of unaffected sib AR238-06. The genotypes across the *BBS1* critical interval (*31*) of both pedigrees are also shown, where the haplotypes are consistent with linkage.

Table 1. Mutational, genetic, and phenotypic analysis of pedigrees with mutations in *BBS2*. The different aspects of the BBS phenotype in each pedigree are indicated as present (+), normal (Nml), or not recorded (NR). The primary features of BBS were examined: retinitis pigmentosa (RP), obesity (Ob), polydactyly (PD), developmental delay (Dev), and renal (Ren) and gonadal malformations (Gon). HBD, homozygous by descent; the "fs" annotation for some mutations indicates a frameshift; IVS, intervening sequence. *, **, ***, and **** indicate recurrent mutations.

Pedigree	Allele 1	Allele 2	Allele 3	RP	Ob	PD	Dev	Ren	Gon
AR171†	D104A (*BBS2*)*	R634P (*BBS2*)		+	+	+	Nml	Nml	NR
PB005†	R275X (*BBS2*)**	R275X (*BBS2*)**		+	+	+	+	+	NR
PB020†	Y24X (*BBS2*)***	Y24X (*BBS2*)***		+	+	+	NR	+	NR
PB026†	D170fsX171 (*BBS2*)	D170fsX171 (*BBS2*)	(HBD for *BBS1*)	+	+	+	+	+	NR
PB058†	C210fsX246 (*BBS2*)	C210fsX246 (*BBS2*)		+	+	+	+	+	NR
K059†	R315W (*BBS2*)	R315W (*BBS2*)	(HBD for *BBS4*)	+	+	+	+	+	+
AR029†	Y24X (*BBS2*)*	*BBS2*-linkage		+	+	+	+	+	+
AR724‡	Q59X (*BBS2*)****	Unknown		+	+	+	+	+	+
PB045‡	IVS1-1G->C	Unknown		+	+	+	+	+	+
AR050‡	R275X (*BBS2*)**	*BBS1*	*BBS1*	+	+	+	Nml	+	+
AR124‡	V158fsX200 (*BBS2*)	*BBS1*	*BBS1*	+	+	+	+	+	Nml
AR238‡	D104A (*BBS2*)*	*BBS1*	*BBS1*	+	+	+	Nml	Nml	Nml
AR596‡	IVS4+1G->C	*BBS3*	*BBS3*	+	+	+	+	Nml	+
AR237‡§	N70S (*BBS2*)	Y37C (*BBS6*)	Y37C (*BBS6*)	+	+	+	+	+	+
AR579§	L168fsX170 (*BBS2*)	R216X (*BBS2*)	C499S (*BBS6*)	+	+	+	+	+	+
NFB14§	Y24X (*BBS2*)*	Y24X (*BBS2*)*	A242S (*BBS6*)	+	+	+	+	+	+
AR241‖	IVS1+1G->C/R315Q	R315Q (*BBS2*)	Unmapped	+	+	+	Nml	NR	+
AR259§‖	Y24X (*BBS2*)***	Q59X (*BBS2*)****	Q147X (*BBS6*)	+	+	+	Nml	+	NR
PB043‖	T560I (*BBS2*)	T560I (*BBS2*)	(HBD for *BBS4*)	Nml	+	+	+	Nml	NR

Pedigrees have been divided into the following: †two *BBS2* mutations segregating with the disease, ‡one *BBS2* mutation but excluded genetically from the *BBS2* locus, §three *BBS* mutations, and ‖two *BBS2* mutations found in unaffected individuals.

The remaining five pedigrees could be excluded genetically from *BBS2* either because the affected individuals shared only one parental chromosome at the locus (e.g., AR124; Fig. 1) or because the affected and unaffected sibs had inherited the same parental chromosomes and both were carriers for the single mutation found. Despite the dense microsatellite spacing, there remained a remote chance that a recombination between any marker and the locus might have led to a deceptive exclusion; we eliminated this possibility by genotyping all individuals with a highly informative single nucleotide polymorphism in exon 3 of *BBS2* (Fig. 1). When we used polymorphic microsatellites from all *BBS* loci to monitor the segregation of the parental chromosomes, we observed that three out of five pedigrees could be excluded from all *BBS* loci except for *BBS1*, the major *BBS* locus, suggesting that these three pedigrees may harbor *BBS1* mutations. One pedigree was consistent with mapping only to *BBS3*, and one pedigree, AR237, could be assigned to *BBS6* (Fig. 2A).

These data suggest that mutations at more than one locus may be present in BBS patients. We therefore integrated and expanded all sequence data from the *BBS2* and *BBS6* mutation screens that yielded a complete scan of the ORFs of the two genes, and we constructed detailed haplotypes across both genomic regions. Of the 19 pedigrees with one or two *BBS2* mutations, we identified four pedigrees with a third potential mutation (AR237, AR579, NFB14, and PB043). AR237 was reported in our initial discovery of *BBS6*, because it harbored a homozygous $Tyr^{37} \rightarrow Cys$ (Y37C) mutation in *BBS6* (*11*). We then found a heterozygous $Asn^{70} \rightarrow Ser$ (N70S) alteration in *BBS2* in this family (Fig. 2A and Table 1). In AR259, we found two heterozygous *BBS2* nonsense mutations [$Tyr^{24} \rightarrow X$ (Y24X) and $Gln^{59} \rightarrow X$ (Q59X)] and one *BBS6* nonsense mutation [$Gln^{147} \rightarrow X$ (Q147X)]. Similar findings were seen in pedigrees AR579 and NFB14 (Table 1 and Fig. 2, B and C).

The probability that we had detected three mutant alleles by chance is minuscule, given the rarity of this disorder and the observation that *BBS6* contributes only ~4% to BBS (*11*, *12*). That the third allele is not a mutation is possible but is not likely. First, nonsense mutations are unlikely polymorphisms. Second, the $Ala^{242} \rightarrow Ser$ (A242S) allele identified in NFB14 has been observed on two other occasions, once in the Old Order Amish, associated with McKusick-Kaufman syndrome (*14*), and subsequently in a BBS patient, but not in 384 control chromosomes (Table 2). The N70S (*BBS2*) and $Cys^{499} \rightarrow Ser$ (C499S) (*BBS6*) alleles are more challenging to interpret in the absence of a functional assay for either gene, but neither has been found in 384 control chromosomes. More importantly, however, the cumulative data indicate that, among 19 pedigrees

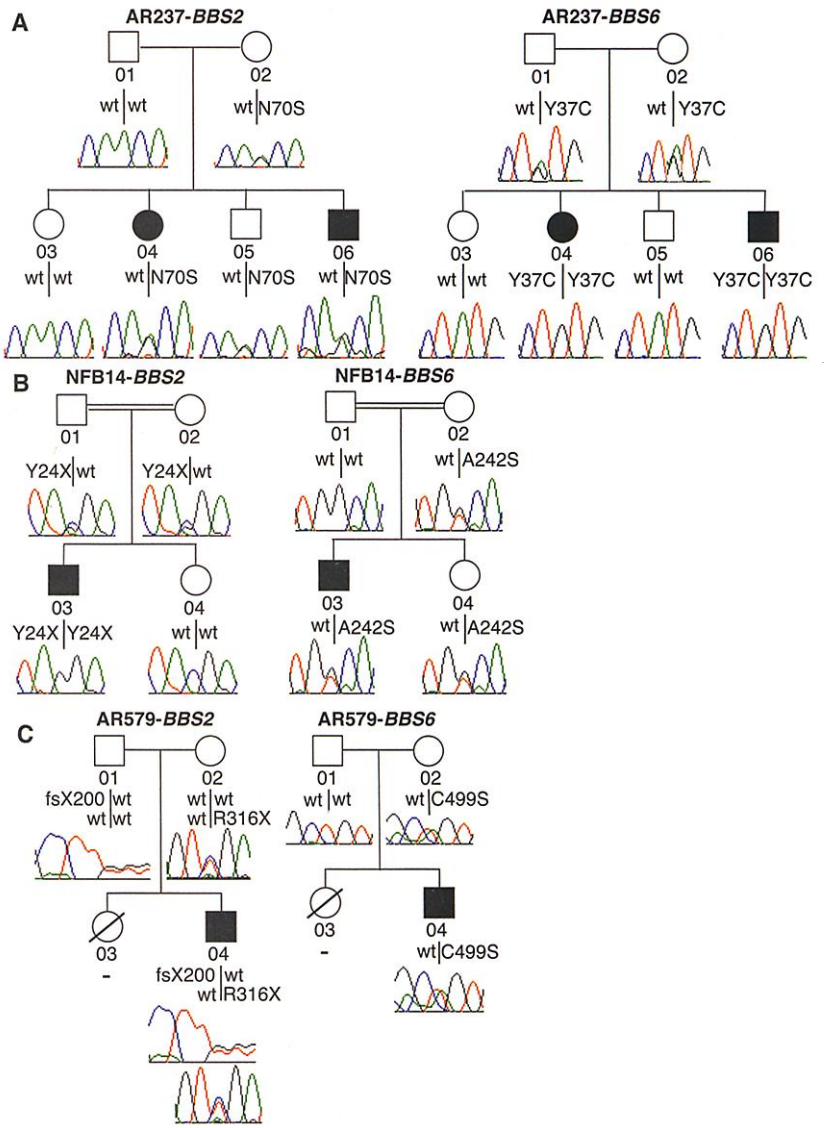

Fig. 2. Pedigrees carrying three *BBS* mutations. (**A**) Pedigree AR237 has two *BBS6* mutations and one *BBS2* mutation; (**B** and **C**) the converse is the case for pedigrees NFB14 and AR579.

with *BBS2* mutations, sequence or genetic evidence for involvement of another locus occurs in at least nine families (47.3%). The same is also true for *BBS6*: Of the eight outbred pedigrees where mutations have been identified (*12*), three harbor *BBS2* mutations (37.5%). The combination of the genetic data described earlier and the mutational data for the only two genes known to be mutated in BBS suggest that multiple alleles may act in concert to cause pathogenesis. We therefore propose a model of triallelic inheritance, in which three mutant alleles segregate with the disorder.

In the absence of either complete gene identification for all *BBS* loci or an animal model, it is not clear whether two mutations at a single locus may sometimes suffice to cause BBS. The recessive segregation pattern in several *BBS2* pedigrees initially suggests that this may be the case and that "biallelic" inheritance may occur for some instances. However, the *BBS6* homozygous Y37C mutation in pedigree AR237 segregates perfectly with the disease, yet this pedigree harbors an additional *BBS2* mutation (Fig. 2A). In fact, in three additional pedigrees, three disease alleles are identified in affected individuals: two *BBS2* alleles with one *BBS6* allele (Figs. 2, B and C, and 3). Consistent with our triallelic hypothesis, individuals with two mutant alleles at *BBS2* who are either wild type for *BBS6* (AR259-05; Fig. 3) or map genetically to another locus (*BBS4* in PB043; Table 1) are unaffected. The absence of these alterations in 384 control chromosomes and the nature of the *BBS2* mutations in AR259-05, in particular (Y24X and Q59X), suggest that these are not polymorphisms (Table 2).

Inheritance of multiple alleles has been documented previously. Paired single mutations in *ROM1* and *RDS*, for example, are the cause of some cases of retinitis pigmentosa (*1*). Subse-

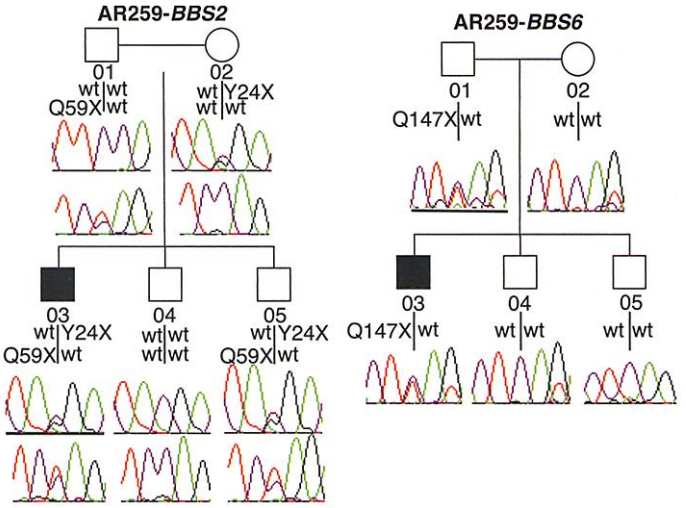

Fig. 3. Pedigree AR259 carrying three nonsense *BBS* mutations. Individual AR259-05 carries two nonsense *BBS2* mutations but no *BBS6* mutations and is phenotypically normal.

Table 2. Summary of the number of the third BBS mutations found in our cohort of 163 patients, and 384 control chromosomes. The exon affected by splice junction mutations is indicated as "x."

Third *BBS* mutation	BBS chromosomes	Control chromosomes
Q59X*	1	0
Splice acceptor x2*	1	0
R275X*	3	0
V158fsX200*	1	0
Splice donor x4*	1	0
D104A*	2	0
N70S*	1	0
C499S†	1	0
A242S†	3	0
Q147X†	1	0

**BBS2* mutation. †*BBS6* mutation.

quent functional studies confirmed these genetic observations by modeling the mutations in vitro and showing defective assembly of the ROM/RDS complex (*23*). Dominant Hirschsprung disease also provides a similar, potentially digenic example, where mutations in both *RET* and *GDNF* were found in patients (*24*, *25*). However, given that knockout mice for either gene display the Hirschsprung phenotype (*26*–*29*), it is likely that the combination of mutations modulates the severity of the phenotype. Likewise, in recessive junctional epidermolysis bullosa (JEB), mutations in both *COL17A1* and *LAMB3* lead to a phenotype with features of both severe JEB (Herlitz form, associated with *LAMB3* mutations) and generalized atrophic benign epidermolysis bullosa (caused by *COL17A* mutations) (*30*). In contrast, we have no evidence that the different *BBS* mutations modulate the phenotype (Table 1), although in principle, the phenotype/genotype relationship may be the combinatorial result of both the type of mutation at each locus and the number of loci involved.

The frequency of multiallelic inheritance in recessive, genetically heterogeneous disorders is unclear. A model of a dominant loss-of-function mutation requisite to unmask recessive mutations may explain, however, the genetic heterogeneity seen in many instances in inbred populations, where both one dominant and two recessive mutations are required for pathogenesis. This model is similar to the inheritance patterns seen in some complex traits, such as type I diabetes (IDDM1), where a dominant HLA genotype is necessary but not sufficient to cause disease; a second, recessively inherited alteration must also be present (*31*). The presence of a common dominant "master locus" but variable "partners" may also explain the inconsistency in replicating genetic linkage data from discrete populations in various complex traits such as asthma, diabetes, and schizophrenia, because each population isolate will be enriched for a limited number of the recessive mutations that, by themselves, will not satisfy all genetic criteria for pathogenicity. A modification of the theoretical model of disease transmission in these disorders may provide important insights.

To our knowledge, there is only one other clearly documented mechanistic example with a three-allele requirement to manifest a Mendelian trait, exemplified by the segmental duplication associated with Charcot-Marie-Tooth neuropathy type 1A (CMT1A). That model, however, represents a dominant trait requiring three copies (alleles) of a normal gene to manifest disease (*32*). Our observations on BBS described herein are distinct from the CMT1A mechanism and different from a digenic model in that a combination of three distinct alleles from two (or more) loci appears necessary and sufficient for trait manifestation. As such, triallelic inheritance may represent a transmission model that bridges classic Mendelian disorders with complex traits.

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16. The diagnosis of BBS was based on established criteria in which three of six cardinal features must be present (*4*). In several cases, the diagnosis was ascertained by local physicians and verified through extensive examination of medical records by one or more of the coauthors.
17. The published cDNA sequence for *BBS2* (GenBank accession number AF342736) was screened through the assembled human genomic sequence with the BLAST algorithm (www.ncbi.nlm.nih.gov/genome/seq/HsBlast.html). Assembly scaffold NT_024816.3 exhibited >99.5% identity to the cDNA sequence. The sequence was downloaded and aligned with the *BBS2* cDNA with programs from the GCG software package (*11*). The sequence flanking all coding exons was identified, and primers were designed to amplify both exons and intronic splice junctions with the Primer v3 program (www.genome.wi.mit.edu/cgi-bin/primer/primer3.cgi).
18. Amplified polymerase chain reaction products from patients, relatives, and control samples were sequenced (*11*). *BBS2* exon amplification primers are available at www.imgen.bcm.tmc.edu/molgen/lupski/index.htm.
19. Sequences were aligned with the wild-type cDNA and genomic sequences, and alterations were confirmed on both strands and evaluated by the Sequencher sequence alignment program (GeneCodes, Ann Arbor, MI).
20. Examples of Mendelian recessive segregation of mutations are shown for pedigrees AR171 and PB005 and are available as supplemental Web material on *Science* Online at www.sciencemag.org/cgi/content/full/293/5538/2256/DC1.
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34. We thank the families and the referring physicians for their cooperation and A. Beaudet, H. Bellen, and J. Belmont for constructive comments. Supported by grant EY12666 from the National Eye Institute (NIH) (N.K.), the March of Dimes (N.K. and J.R.L.), the Foundation Fighting Blindness, USA (R.A.L. and J.R.L.), the Canadian Institutes of Health Research (W.S.D.), the National Kidney Research Fund (B.E.H.), the Birth Defects Foundation, and the Wellcome Trust (P.L.B.).

18 June 2001; accepted 6 August 2001