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Isolation of a candidate cDNA for the gene causing retinal degeneration in the *rd* mouse

(recombinant DNA/subtractive and differential cloning/chromosome mapping)

CATHY BOWES*†, MICHAEL DANCIGER‡, CHRISTINE A. KOZAK§, AND DEBORA B. FARBER*¶

*Jules Stein Eye Institute and †Department of Pathology, University of California, Los Angeles, School of Medicine, Los Angeles, CA 90024; ‡Loyola Marymount University, Los Angeles, CA 90045; and §National Institute of Allergy and Infectious Diseases, Bethesda, MD 20892

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ABSTRACT **The inherited retinal degeneration of the *rd* mouse results in the exclusive loss of one cell type, the photoreceptors. We took advantage of this visual-cell loss to devise a strategy for the isolation of photoreceptor-specific cDNAs based on the use of subtractive and differential hybridizations. The resulting pool of photoreceptor-specific cDNAs was screened for a candidate cDNA for the *rd* gene, and a putative *rd* cDNA that maps to mouse chromosome 5, the chromosome to which the *rd* gene has been assigned, was identified. On Northern blots the candidate *rd* cDNA hybridizes a 3.3-kilobase RNA species from 9- to 11-day-old developing normal retina and, much more faintly, a 3.6-kb RNA species from age-matched *rd* retina. The 0.3-kilobase difference in the size of the mRNAs hybridized suggests that a structural alteration in the gene corresponding to the candidate *rd* cDNA has occurred in the *rd* mouse. This was further supported by the detection of polymorphisms between *rd/rd* and +/+ mouse genomic DNA after digestion with restriction endonucleases and probing with the candidate *rd* cDNA. Expression of mRNAs hybridized by the candidate *rd* cDNA is detected in normal and diseased retinas at postnatal day 1 but the signal intensity is considerably lower in the *rd* retina. To our knowledge, this is the earliest molecular defect reported in the *rd* retina that is observed prior to any phenotypic signs of photoreceptor degeneration.**

The primary lesions affecting most inherited retinal diseases are unknown; however, several studies of animal models for the human disease retinitis pigmentosa have revealed that biochemical defects related to cyclic nucleotide metabolism are associated with some of these disorders (1–3). The *rd* mouse, the most thoroughly investigated animal model, is affected with an autosomal-recessive retinal degeneration characterized by an early onset and a rapid progression. The *rd* mutation has been localized to mouse chromosome 5 (4) and its expression is restricted to the photoreceptor cell layer (5, 6). Elevated levels of cGMP present in the *rd* retina by postnatal day 6, 2 days before pathological signs are observed (1), lead to the degeneration of the visual cells (7) and are the result of deficient cGMP phosphodiesterase activity (8). Photoreceptor maturation in the *rd* retina appears to parallel development in the normal retina until postnatal day 8, when ultrastructural changes become evident (9–12). By 20 postnatal days the *rd* photoreceptor layer consists of a single row of cone nuclei (13) and by day 30 the *rd* retina is virtually devoid of rod photoreceptors, whereas the small cone population (3% of the total visual cells) has been reduced to between one-third and one-half of the original number (13, 14). The inner cell layers of the retina remain intact and apposed to the pigment epithelium. We took advantage of this phenotypic expression of the disease to devise a strategy for the isolation of a candidate cDNA for the *rd* gene.

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MATERIALS AND METHODS

cDNA Library Preparation. Care of mice used for all experiments was in accordance with institutional guidelines. Total RNA was isolated as reported (15) from quick-frozen adult (at least 31 postnatal days) C57BL/6J *rd/rd* and from 9- to 11-day-old heterozygous, morphologically normal, C57BL/6J *rd*/+ mouse retinas by using the guanidine thiocyanate/CsCl procedure (16). Total RNA and not poly(A)-selected RNA was used to decrease the amount of RNA needed to perform these studies. Double-stranded cDNAs with cohesive *Eco*RI ends were synthesized from 50 μg of total adult *rd/rd* retina RNA by using a cDNA synthesis kit (Pharmacia) with one modification: 5 μg of RNase A (type XII-A; Sigma) was added with the Klenow fragment of DNA polymerase I after second-strand synthesis to help degrade any residual RNA. The cDNAs were then ligated into the *Eco*RI site of plasmid vector pGEM7zf (Promega Biotec) and used to transform DH5α competent cells (BRL). The resulting library was estimated to contain at least 10^6 recombinants. Plasmid DNA was isolated from the *rd/rd* library by alkali lysis and ultracentrifugation in a CsCl/ethidium bromide gradient (17). After digestion with *Apa* I (New England Biolabs), the plasmid DNA was end-labeled with the biotinylated nucleotide, 5-[*N*-(*N*-biotinyl-ε-aminocaproyl)-3-aminoallyl]uridine 5′-triphosphate (Bio-11-dUTP; Sigma) in a scaled up version of the reaction described by Rigas *et al.* (18), with the addition of dCTP in a 1:1 ratio with Bio-11-dUTP. A phagemid library of 9- to 11-day-old normal *rd*/+ retinal cDNAs was constructed in the same manner as the plasmid library described above using the vector λZAP II (Stratagene). The cDNA/phagemid ligation mixture was packaged with Gigapack packaging extracts and transfected into *Escherichia coli* XL-1 Blue cells (Stratagene). The titer was estimated to be 5×10^6 plaque-forming units.

Probe Preparation. A single-stranded [^{32}P]cDNA probe was prepared from 50–60 μg of total RNA isolated from 9- to 11-day-old *rd*/+ retinas by using excess oligo(dT) (Collaborative Research) as a primer. The procedure was as follows: total RNA was denatured at 65°C for 7 min and then was incubated in 50 mM Tris·HCl, pH 8.3/75 mM KCl/50 mM dithiothreitol/15 mM $MgCl_2$/0.5 mM spermidine/1 mM dATP/1 mM dGTP/1 mM dTTP/33 μM dCTP/5 μg of $(dT)_{12-18}$/120 units of RNasin (Promega Biotec)/200 μCi of [α-^{32}P]dCTP (1 Ci = 37 GBq) with 1000 units of Maloney murine leukemia virus reverse transcriptase (BRL) for 1 hr at 37°C. After removal of the RNA by heating in 0.1 M NaOH (19), unincorporated nucleotides were separated using Sephadex G-50 spin columns (Worthington). Typically 1–2 μg of cDNA was synthesized. Biotinylated driver adult *rd/rd* cDNA (104 μg) was mixed with 1.65 μg of 9- to 11-day-old

¶To whom reprint requests should be addressed at: Jules Stein Eye Institute, University of California, Los Angeles, School of Medicine, 800 Westwood Plaza, Los Angeles, CA 90024-1771.

rd/+ first-strand retinal [^{32}P]cDNA, denatured, and hybridized using the phenol emulsion reassociation technique (20) as described by Travis and Sutcliffe (19). After 50 hr of hybridization and removal of phenol by $CHCl_3$ extraction and ethanol-precipitation, the single-stranded photoreceptor-enriched [^{32}P]cDNA was separated from the biotin-DNA·[^{32}P]-DNA duplexes by passage over a streptavidin-agarose column (BRL) as described by Rigas *et al.* (18). Most of the subtracted photoreceptor-enriched probe came off the column immediately (in the first 3 ml) after loading the DNA.

cDNA Library Screening. Approximately 100,000 plaque-forming units of the 9- to 11-day-old *rd*/+ retina cDNA library in the phagemid vector λZAP II were plated, and duplicate "plaque lifts" were prepared by the method of Benton and Davis (21) using nylon Hybond N filters (Amersham). One set of plaque lifts was hybridized with the radiolabeled, subtracted, photoreceptor-enriched, 9- to 11-day-old *rd*/+ cDNA probe (5×10^6 cpm) and the other set was hybridized with single-stranded [^{32}P]cDNAs synthesized from adult *rd*/*rd* retinal RNAs as described (15), for at least 72 hr. Washing after hybridization was performed in 0.2× SSC (1× SSC = 0.15 M NaCl/0.015 M sodium citrate, pH 7.0) plus 0.1% $NaDodSO_4$ at 65°C.

Analytical Procedures. *In vivo* excision and rescue of double-stranded recombinant pBluescript SK(−) plasmids out of the λZAP II phagemids was performed as described by the manufacturer (Stratagene). Plasmid DNA was isolated on Qiagen-pack 100 columns (Qiagen, Studio City, CA). cDNA inserts generated by restriction enzyme digestion were obtained after fractionation on 0.7–1.5% agarose gels by electroelution in an Elutrap (Schleicher & Schuell). cDNA fragments recovered in this manner or whole plasmids were radiolabeled with [α-^{32}P]dCTP by random priming (22) and blots containing RNAs from normal and affected mouse retinas as well as from other tissues, used to probe blots as described by Bowes and Farber (15). RNA blots were prepared at least three times with independent RNA samples and reprobed with a cDNA clone (p1B15) of rat cyclophilin.‖ This probe recognizes a ubiquitously expressed mRNA (24, 25) and was used as a control for gel loading and RNA integrity, as reported (19). The size of the RNAs hybridized by the candidate cDNA clone that we isolated, zr.408 clone, was estimated from an RNA ladder (BRL).

Chromosome Mapping and Southern Blot Analysis. Hamster–mouse somatic cell hybrids were derived from the fusion of E36 Chinese hamster cells with peritoneal or spleen cells from BALB/c or NFS.Akv-2 mice (26). The chromosome content of most hybrids was determined by trypsin–Giemsa banding followed by staining with Hoechst 33258; hybrids were also typed for specific marker loci on all of the 20 mouse chromosomes (26–28). Chinese hamster–mouse hybrid and parental DNAs were prepared as described (29) and digested with *Hin*dIII.

Adult *rd*/*rd*, *rd*/+, and +/+ mouse brains were dissected, immediately frozen in liquid nitrogen, and pulverized. Genomic DNA was isolated by the method of Fodor and Doty (30) and aliquots were digested with various restriction enzymes. Seventeen endonucleases were tested for their capability to produce restriction fragment length polymorphisms between *rd*/*rd* and +/+ DNA.

All DNAs were electrophoresed in 1.0, 1.2, or 1.5% agarose gels with 6 μg of DNA per lane and transferred to nylon membranes (31). The blots were prehybridized, hybridized, and washed as described (32).

‖Cyclophilin was obtained from G. H. Travis (Research Institute of Scripps Clinic) and cloned as described by Danielson *et al.* (23)

RESULTS

Cloning Strategy. Our approach for the isolation of a candidate cDNA for the *rd* gene was based on the assumption that the *rd* gene codes for an mRNA that would be expressed differently in the developing photoreceptor cells of affected *rd*/*rd* mouse retinas (9- to 11-day-old C57BL/6J *rd*/*rd*) compared to that of morphologically normal heterozygous *rd*/+ mouse retinas (9- to 11-day-old C57BL/6J *rd*/+). At 9–11 postnatal days, the *rd*/*rd* and *rd*/+ littermates provide the maximum amount of age-matched retinal photoreceptor tissue that can be obtained before full degeneration of the *rd*/*rd* visual cells has occurred (9–14). We used subtractive cloning for the isolation of a pool of photoreceptor-specific cDNAs since the abundance and expression of the *rd* mRNA (whether it would be absent or manifested as an increased or decreased amount of mRNA) was unknown. Subtractive cloning allows isolation of cDNAs representing mRNAs with much lower abundances than differential cloning (as low as 0.001% versus 0.05%) (19, 33, 34).

The first step in the isolation of photoreceptor-specific clones was the preparation of a radiolabeled probe consisting of photoreceptor-enriched cDNAs, as outlined in Fig. 1. An excess of plasmid DNA derived from an adult, photoreceptorless, *rd*/*rd* retinal library was biotinylated, denatured, and hybridized with radiolabeled single-stranded cDNAs of 9- to 11-day-old normal *rd*/+ mouse retina in a phenol emulsion to enhance the hybridization kinetics (20). After hybridization, the biotinylated driver DNA and the non-photoreceptor-specific *rd*/+ cDNAs hybridized to it were separated from the unhybridized *rd*/+ cDNAs on a streptavidin-Sepharose column. These subtracted cDNAs enriched for photoreceptor transcripts, in parallel with radiolabeled single-stranded cDNAs of adult *rd*/*rd* retina, were used independently to probe a phagemid library of 9- to 11-day-old normal *rd*/+ retina. Since any clone able to hybridize the adult *rd*/*rd* mouse cDNAs had to be non-photoreceptor-specific, the differential screening with adult *rd*/*rd* cDNAs was used to eliminate any of these clones that may have hybridized among the pool of cDNAs selected with the subtracted probe. Of approximately 100,000 clones tested, 588 clones gave a hybridization signal detectable in autoradiograms with the subtracted *rd*/+ retina probe and not with the adult *rd*/*rd* cDNAs. These putative photoreceptor-specific clones were rescreened by differential hybridization with three probes: radiolabeled single-stranded cDNAs from adult *rd*/*rd*, 9- to 11-day-old *rd*/+, and 9- to 11-day-old *rd*/*rd* mouse retinas.

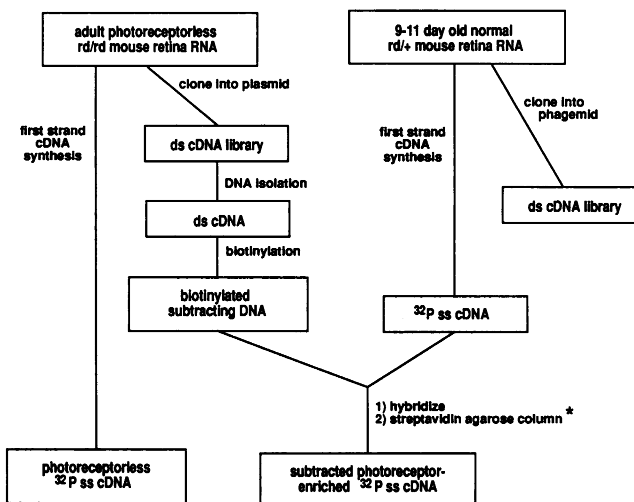

FIG. 1. Preparation of probes used to select photoreceptor-specific cDNAs from 9- to 11-day-old *rd*/+ cDNA library. ss, Single stranded; ds, double stranded. * Column retains biotinylated hybrids.

Four hundred plaques (67.5%) hybridized only with the cDNAs of the transcripts from developing *rd*/+ or *rd*/*rd* retina after rescreening and not with the photoreceptorless adult *rd*/*rd* retina cDNAs, strongly suggesting that these clones were, in fact, photoreceptor-specific.

Candidate cDNAs for the *rd* gene were selected from the photoreceptor-specific cDNAs by searching for clones that were hybridized differently by the 9- to 11-day-old *rd*/+ and the 9- to 11-day-old *rd*/*rd* retinal probes in autoradiograms of duplicate lifts hybridized independently with the two probes. Three clones gave a weaker signal when hybridized with the affected *rd*/*rd* probe than with the *rd*/+ probe.

Analysis of the Putative *rd* cDNAs. The three clones containing inserts selected as candidate *rd* cDNAs were released from their flanking phage in the phagemid vector sequences by *in vivo* excision. The rescued plasmid DNAs were then isolated, radiolabeled, and used to probe Northern blots of developing and adult normal and diseased mouse retinal RNA. Analysis of the blots showed that one of the three clones (zr.408) continued to hybridize to the developing *rd*/*rd* retinal RNA more faintly than to the age-matched normal RNA (Fig. 2*A*), whereas the other photoreceptor-specific clones hybridized with the same intensity in the 9- to 11-day-old normal and the diseased RNA samples (for example, Fig. 2*C*). The difference in the pattern of hybridization of *rd* cDNA with diseased and normal mRNA was confirmed by reprobing with a cDNA (p1B15) for the ubiquitously expressed rat cyclophilin (Fig. 2 *B* and *D*). The zr.408 clone contains an insert of 3.45 kilobases (kb) with one internal *Eco*RI site. Both isolated *Eco*RI fragments (1.65 and 1.8 kb) hybridized with the 1.65-kb fragment (Fig. 2*A*).

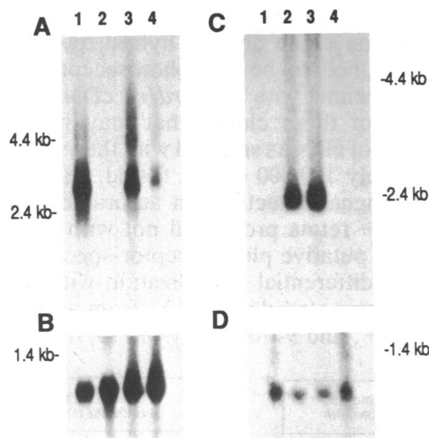

FIG. 2. Characterization of mouse RNAs hybridized by the putative *rd* cDNA clone (*A*), another photoreceptor-specific cDNA (*C*), and a cDNA for rat cyclophilin (*B* and *D*). (*A*) Northern blot of total retinal RNA hybridized to the candidate *rd* cDNA, zr.408 (1.65-kb *Eco*RI fragment of clone zr.408). Lanes: 1, 7.5 μg of adult *rd*/+ RNA (>31-days-old); 2, 15 μg of adult *rd*/*rd* RNA (>31-days-old); 3, 15 μg of 11-day-old *rd*/+ RNA; 4, 15 μg of 11-day-old *rd*/*rd* RNA. Washed in 0.3× SSC plus 0.1% NaDodSO4 at 57°C. (*B*) Same Northern blot as in *A* but hybridized to p1B15, a cDNA for the ubiquitously expressed rat cyclophilin mRNA, used as a control for RNA integrity and reproducible sample loading. Washed in 0.3× SSC plus 0.1% NaDodSO4 at 60°C. (*C*) Northern blot of total mouse RNA (15 μg per lane) isolated from adult *rd*/+ spleen (lane 1), 9- to 11-day-old *rd*/+ retina (lane 2), 9- to 11-day-old *rd*/*rd* retina (lane 3), and adult *rd*/*rd* retina (lane 4) and probed with mouse clone zr.494 (plasmid plus 2.0-kb insert), selected as photoreceptor-specific by the combined subtractive and differential plaque screenings. Washed to same stringency used in *A*. (*D*) Same Northern blot as in *C* probed as in *B* with a cDNA for rat cyclophilin. Washed in 0.1× SSC plus 0.1% NaDodSO4 at 60°C. Autoradiograms were exposed 22 hr in *A*, 40 hr in *B*, 48 hr in *C*, and 20 hr in *D*. RNA size standards are in kb.

To confirm that clone zr.408 is photoreceptor-specific, RNA from several mouse tissues was isolated and immobilized. Dot-blot analysis of these RNAs showed that the candidate *rd* cDNA hybridizes in a retina-specific manner (Fig. 3*A*). All brain, kidney, liver, lung, and spleen RNAs gave negative results. The integrity of the immobilized RNA was demonstrated by hybridization with the cyclophilin cDNA (Fig. 3*B*).

Developmental Analysis of mRNA. Expression of the putative *rd* clone was detectable by dot-blot analysis on postnatal day 1 in both normal and diseased retinas (Fig. 4) and it increased steadily during development but was always lower in the *rd*/*rd* retina. A sharp decrease in levels of mRNA hybridized by the candidate *rd* cDNA in 14-day-old *rd*/*rd* retina coincided with the 50% loss of visual cells that had occurred by this age (36) (Fig. 4*B*). At 31 postnatal days, the levels of zr.408 mRNA were still increasing in the normal retina but, as expected, no signal above background was detectable in the diseased retina (Fig. 4*B*).

Chromosome Assignment. DNA from a panel of 14 hamster–mouse somatic cell hybrids was analyzed for sequences homologous to the putative *rd* cDNA. Digestion of the Chinese hamster and mouse parental DNAs and hybridization with ^{32}P-labeled probe showed two main bands of 9.4 kb and 2.9 kb for the hamster DNAs and three main bands of 6.2 kb, 5.8 kb, and 1.9 kb for the mouse parental DNAs (Fig. 5, lanes 1 and 2). Typical results of hybrid DNAs either positive or negative for the putative *rd* sequences are shown in Fig. 5, lanes 3–8. Two of 14 hybrids tested contained all scoreable mouse-specific fragments (Fig. 5, lanes 3 and 8), whereas the remaining 12 hybrids had none. Statistical analysis of all the data, compiled in Table 1, shows that there are no discor*dancies* for the presence of the candidate *rd* gene on mouse chromosome 5, whereas there are at least two discordancies for its presence on any other chromosome.

Southern Blot Analysis of Genomic DNA. *rd*/*rd*, *rd*/+, and +/+ mouse genomic DNAs were digested in separate experiments with a total of 17 restriction enzymes. Four of these enzymes (*Alu* I, *Bal* I, *Msp* I, and *Rsa* I) revealed polymorphic fragments either present in *rd*/*rd* and *rd*/+ and

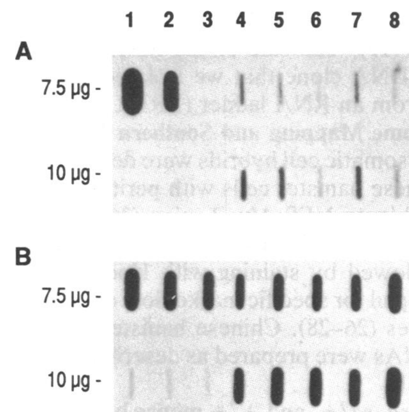

FIG. 3. Tissue specificity of candidate *rd* cDNA. Hybridization of candidate *rd* cDNA (*A*) and a cDNA for cyclophilin (*B*) to mouse RNAs isolated from various tissues. Total RNA was denatured in formamide, immobilized on a nylon membrane by using a "slot blotter," and hybridized in *A* to the candidate *rd* cDNA, zr.408 (as in Fig. 2*A*). Columns: 1, 11-day-old *rd*/+ retina; 2, 10-day-old *rd*/*rd* retina; 3, 4-day-old *rd*/+ liver; 4, 41-day-old *rd*/+ lung; 5, 41-day-old *rd*/+ kidney; 6, 41-day-old *rd*/+ spleen; 7, 41-day-old *rd*/+ brain; 8, 11-day-old *rd*/*rd* brain. Washed as in Fig. 2*A*. There is no RNA loaded in the 10-μg sample slots in columns 1, 2, or 3. (*B*) Same RNA dot blot as in *A* hybridized to rat cyclophilin cDNA, p1B15 as a control for RNA loading. Washed as in Fig. 2*B*. Autoradiograms were exposed 24 hr in *A* and 20 hr in *B*. The amount of RNA loaded per slot is indicated to the left of each row.

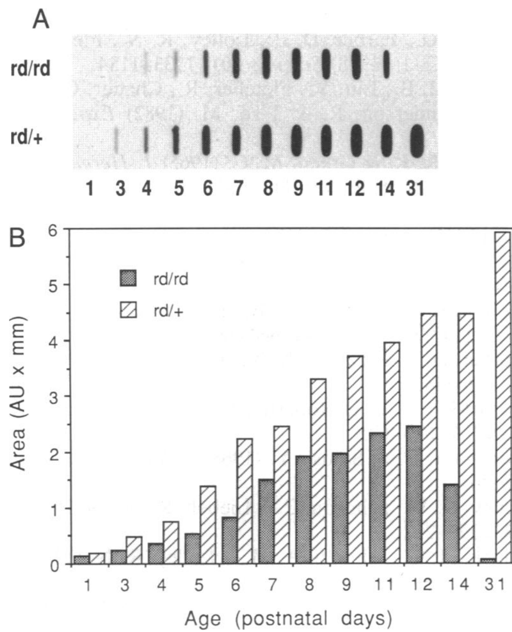

FIG. 4. Developmental expression of mRNA coding for the candidate *rd* clone in *rd*/+ and *rd*/*rd* mouse retina. (*A*) Total RNA (12.5 μg per slot) was denatured, applied to a nylon membrane, and hybridized with a 1.65-kb *Eco*RI fragment of the zr.408 clone as in Fig. 3. Washing after hybridization was as in Fig. 2*A*. Age is indicated in postnatal days below autoradiogram. Autoradiogram was exposed for 16 hr at 22°C. Reproducible sample loading and RNA integrity was confirmed by reprobing the RNA slot blot with the photoreceptor-specific clone zr.494 (as in Fig. 2*C*) and with the cDNA for the ubiquitously expressed cyclophilin mRNA (as in Figs. 2 *B* and *D* and 3*B*) (data not shown). (*B*) Densitometric histograms derived from laser densitometry analysis of autoradiogram in *A* (35). Each value has been normalized with the values obtained by hybridization with the cDNA for cyclophilin. Area = AU (absorption units at 633 nm) × mm.

absent from +/+ or present in +/+ and *rd*/+ and absent in *rd*/*rd* mouse DNAs. Fig. 6 shows an *Msp* I digest of DNA from the three mouse genetic types. At least 10 hybridizing fragments are present on the blot. Six of these fragments are present in all the +/+, *rd*/+, and *rd*/*rd* lanes (11.0, 7.7, 6.4, 5.6, 4.5, and 3.8 kb). Three other fragments are seen only in the *rd*/+ and +/+ lanes (13.0, 5.2, and 2.6 kb) and one of the fragments (2.4 kb) is observed only in the *rd*/+ and *rd*/*rd* lanes. These restriction fragment length polymorphisms reflect differences in the mutant *rd* and wild-type DNAs at sites homologous to the putative *rd* cDNA. Furthermore, the

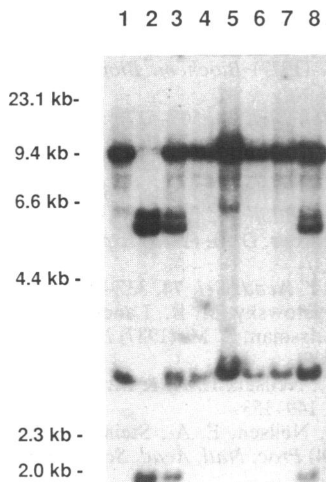

FIG. 5. Chromosome mapping of the candidate *rd* cDNA, zr.408, using somatic cell hybrids. Southern blot of hamster–mouse hybrid DNAs (6 μg per lane) digested with *Hin*dIII and hybridized with the candidate *rd* cDNA (1.65-kb insert fragment of clone zr.408). Lanes: 1, Chinese hamster parental DNA; 2, mouse parental DNA, NFS.-Akv-2; 3–8, representative somatic cell hybrid DNAs (lanes 3 and 8 contain somatic cell hybrid DNAs with mouse chromosome 5). Size markers (λ DNA digested with *Hin*dIII) are indicated in kb on the left. Autoradiogram was exposed 12 days.

Table 1. Correlation between candidate *rd* cDNA-homologous sequences and a specific mouse chromosome in 14 somatic cell hybrids

Mouse chromosome in hybrid	Number of hybrids*				% discordant
	+/+	−/−	+/−	−/+	
1	1	6	1	4	41.7
2	1	3	1	9	71.4
3	1	6	1	2	30.0
4	1	6	1	5	46.2
5	2	10	0	0	0.0
6	0	5	2	7	64.3
7	1	1	1	11	85.7
8	0	6	2	5	53.8
9	1	8	1	4	35.7
10	0	12	2	0	14.3
11	0	7	2	0	22.2
12	0	2	2	6	80.0
13	1	2	1	9	76.9
14	0	9	2	2	30.8
15	1	0	1	5	85.7
16	1	7	1	3	33.3
17	1	6	1	6	50.0
18	1	5	1	5	50.0
19	0	5	2	6	61.5
20	1	8	1	3	30.8

Symbols indicate the presence (+/) or absence (−/) of mouse sequences homologous to the candidate *rd* cDNA as related to the presence (/+) or absence (/−) of a particular mouse chromosome in the somatic cell hybrids analyzed.

presence of all the +/+ and *rd*/*rd* polymorphisms in the *rd*/+ DNA confirms the differences between the *rd* and wild-type DNAs and provides further evidence for identity between restriction fragment length polymorphisms hybridized by the candidate cDNA and the genetic trait.

DISCUSSION

Although a considerable amount of biochemical information has accumulated through the years about the *rd* mouse disease, the only data regarding the gene that causes this retinal degeneration at the time we began this study was that it mapped to mouse chromosome 5 (4). With the experiments

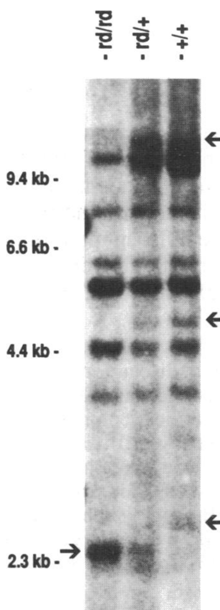

FIG. 6. Genomic Southern blot. Southern blot of adult mouse DNAs (6 μg per lane) digested with *Msp* I and hybridized with the candidate *rd* cDNA (1.65-kb *Eco*RI cDNA fragment of clone zr.408). Lanes: *rd*/*rd*, *rd*/*rd* mouse DNA; *rd*/+, *rd*/+ mouse DNA; +/+, +/+ mouse DNA. Arrows on the right point to the three polymorphisms common to *rd*/+ and +/+ but absent from *rd*/*rd* DNAs and the arrow on the left points to the polymorphism common to *rd*/+ and *rd*/*rd* but absent from +/+ DNAs. DNA size markers (λ DNA digested with *Hind*-III) are indicated in kb. Autoradiogram was exposed 9 days.

described above, we have come closer to finding the molecular genetic defect affecting the *rd* retina.

Our approach for the isolation of the putative *rd* cDNA involved the use of biotin–streptavidin subtraction cDNA cloning methods, which have the advantage of enhancing the detection of low-abundance cDNA clones. Conventional subtraction procedures are limited by the amount of retinal tissue required for the isolation of RNA and the conditions needed for hybridization and separation of DNA·RNA hybrids by hydroxyapatite chromatography (19, 37–39).

The photoreceptor-specific cDNA from normal retina that we have isolated (clone zr.408) has many characteristics that make it a likely candidate for the *rd* gene. When used as a probe to compare normal and *rd* mRNAs, consistently, with RNA samples extracted from different sets of retinas, the candidate *rd* cDNA hybridizes a normal mRNA of 3.3 kb whereas the *rd/rd* mRNA hybridized is 3.6 kb. This difference in mRNA size could be due to alternative splicing, polyadenylylation site differences, or the presence of a structural alteration in the *rd* gene. Polymorphisms between the mutant and wild-type DNAs revealed by genomic Southern blot analysis suggest that in fact there is a structural difference between the *rd/rd* and +/+ genes hybridized by the candidate *rd* cDNA. This has been further supported by the presence in the digested *rd*/+ DNA of all DNA polymorphic fragments from digested *rd/rd* or +/+ samples—results obtained with four endonucleases.

We also used the candidate cDNA and a panel of hamster–mouse somatic cell hybrids to assign the chromosomal location of the putative gene. The zr.408 clone mapped without discordancy to mouse chromosome 5, the same chromosomal assignment of the *rd* locus (Fig. 5 and Table 1).

Two pieces of evidence seem to indicate that our *rd* candidate cDNA hybridizes a single-copy gene: (*i*) after digestion of genomic DNAs from homozygous normal, heterozygous, and homozygous diseased mice with *Kpn* I and Southern analysis, only one fragment of 17.0 kb was hybridized by the candidate *rd* cDNA in all three samples (data not shown) and (*ii*) the assignment of the gene to a single chromosome.

Our data show that expression of the putative *rd* cDNA (clone zr.408) is abnormal in the *rd/rd* retina by postnatal day 1, several days prior to the first pathological signs identified by biochemical means [i.e., the accumulation of cGMP in the photoreceptors observed by postnatal day 6 (1)]. To the best of our knowledge, this is the earliest molecular lesion demonstrated in the *rd* mouse. The decrease in the amount of mRNA that we observed beginning at 14 postnatal days in the *rd/rd* retina has also been described for other photoreceptor-specific mRNAs in the degenerating *rd/rd* retina, including those coding for opsin, the α subunit of transducin, and the 48-kDa protein (35).

In summary, we have identified a cDNA clone that by several criteria would fit the classification of "strong candidate" for the *rd* gene. These include the differences in size and signal intensity observed on Northern blots between the normal and *rd/rd* mRNAs hybridized by the zr.408 clone, the different pattern of genomic DNA fragments hybridized by zr.408 after digesting the normal and diseased DNAs, and the chromosomal assignment of the gene corresponding to zr.408.

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