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Genetics

Isolation of a candidate gene for choroideremia

(retina/retinal dystrophy/X chromosome)

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ABSTRACT **Choroideremia is an X chromosome-linked retinal dystrophy of unknown pathogenesis. We have isolated cDNAs from a human retinal library with a genomic probe located at the X chromosomal breakpoint in a female with choroideremia and an X;13 translocation. This cDNA spans the breakpoint in the X;13 translocation female and is deleted in males who have choroideremia as part of a complex phenotype including mental retardation and deafness. However, this cDNA detects no alterations in the DNA of 34 males with isolated choroideremia. Nonetheless, the cDNA does detect reduced or absent levels of mRNA in three-quarters of male patients with an apparently intact gene. These data support the hypothesis that this cDNA represents the gene in which mutations cause choroideremia.**

Choroideremia (*CHM* locus; McKusick no. 303100), a rare, X chromosome-linked retinal dystrophy, produces visual field constriction and night blindness in adolescence and results in blindness by the fourth to fifth decade of life (1, 2). The molecular and cellular pathogenesis is unknown (3, 4).

The identification of human disease genes based on precise chromosomal localization has been successful for a number of human disease genes (5–10) and has provided important insights into the biochemical basis of the associated diseases and the normal biological function of these genes. A similar approach to choroideremia was feasible because the locus for choroideremia has been mapped to Xq21.1–Xq21.2 by tight linkage to polymorphic DNA markers (11–16), by the study of individuals with choroideremia who have large deletions of this region (17–23), and by the characterization of two women with choroideremia and *de novo* X;autosomal translocations (24–26). Recently, Cremers *et al.* (27) isolated a candidate cDNA for the choroideremia gene that was disrupted in males with the disease by large [>40-kilobase (kb)] genomic deletions and in a female with the disease by an X;13 translocation. Because another gene in this region could have been disrupted by these events, however, additional evidence is required to demonstrate a role of this candidate gene in causing choroideremia.

By chromosome walking, from nearby probes, we have isolated genomic sequences from the translocation breakpoint in a female with choroideremia and an X;13 translocation (26) and used them to identify a candidate choroideremia cDNA.¶ This gene is very similar but not identical to that reported by Cremers *et al.* (27). Expression studies in 34 unrelated probands with isolated choroideremia indicate that, while the genomic structure of this region is unaltered in all these patients, mRNA levels for this gene in 25 of these patients are markedly reduced or absent compared to normal controls. These data provide additional evidence that this cDNA is an appropriate candidate for being the choroideremia gene.

METHODS

Cell Lines. Cell lines used were lymphoblast and fibroblast cell lines from the t(X;13) patient and hybrid CIII-1, a mouse/human somatic cell hybrid that contains the derivative chromosome 13 (24); GM01416, a lymphoblastoid cell line with the 48,XXXX karyotype (Human Genetic Mutant Cell Repository, Coriell Institute for Medical Research, Camden, NJ); and HRY79, a retinoblastoma cell line (American Type Culture Collection). Lymphoblastoid cell lines from choroideremia patients and from normal male and female individuals were established in our laboratory. Primary retinal pigmented epithelium cell cultures were established from human eyes obtained from the National Disease Research Interchange, Philadelphia.

Southern Blot Analysis. High molecular weight DNA isolation, Southern analysis, and probe preparation were by standard methods (28–30). When necessary, probes were preannealed with an excess of human placental DNA (31).

Library Screening. Chromosome walking was accomplished in three libraries, a cosmid library (Stratagene) and two genomic phage libraries [one from the American Type Culture Collection and one previously described (32)]. A human retinal cDNA library (in λgt10) was obtained from J. Nathans (Johns Hopkins University, Baltimore) and used for cDNA isolation. Prehybridization and hybridization conditions have been described (32). cDNA clone inserts were sized by PCR according to Clontech. Miniprep phage and cosmid DNA was prepared by standard protocols (33, 34). Restriction fragments from phage and cosmid clones were subcloned into pGEM-3Z or pBluescript and transformed into DH5α or XL1-Blue, respectively.

DNA Sequencing. DNA sequencing was performed by the Sanger dideoxy chain-termination procedure (35) with T7 DNA polymerase (Pharmacia) on double-stranded DNA in pBluescript SK. PCR-amplified genomic DNA was sequenced on the Applied Biosystems automated sequencer with *Taq* DNA polymerase in a cycle sequencing protocol (Applied Biosystems) with dideoxynucleotide fluorescent terminators.

Nucleic acid sequence analysis was performed with either an Intelligenetics or a Genetics Computer Group software package.

RNA Isolation and Analysis. RNA was isolated from lymphoblasts, fibroblasts, HRY79, and primary cultures of retinal pigmented epithelium by the guanidinium isothiocyanate method (36). Northern analysis was performed by standard

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Abbreviation: RT-PCR, reverse transcription–PCR.

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¶The sequence reported in this paper has been deposited in the GenBank data base (accession no. M83773).

methods in formaldehyde/agarose gels (1.2%) in Mops buffer (37). Analysis of the 5′ end by primer extension was performed according to a modification of the protocol described by Inoue and Cech (38).

Amplification of RNA by Reverse Transcription–PCR (RT–PCR). PCR amplification of mRNA was performed as follows: first-strand cDNA was synthesized from total RNA by reverse transcription from a 3′ primer complementary to bases 842–858 of the CHR sequence (see *Results*) (5′-GGTGCCTTTCATGCATG-3′) and then amplified between this 3′ primer and a 5′ primer corresponding to bases 624–640 of the CHR sequence (5′-GAGAATAATCTCTGAGC-3′) (see Fig. 5). As a control, RT–PCR amplification was carried out simultaneously with primers for β-actin on the same RNA sample.

RNAs were first treated with RNase-free DNase I (Stratagene), according to the manufacturer's conditions. After a 30-min incubation at 37°C, RNAs were phenol extracted, chloroform extracted, precipitated, and washed with 70% ethanol. Reverse transcription used 300 ng of the unlabeled 3′ CHR primer and 30 ng of unlabeled 3′ β-actin primer as in the primer extension experiments described above. After 30 min at 45°C, the reaction products were precipitated and washed well with 70% ethanol. PCR was performed with reagents from Perkin–Elmer/Cetus. Nucleotides were used at a concentration of 200 μM, and the Mg^{2+} concentration was 1.5 mM. Three hundred nanograms of the CHR 5′ primer and 30 ng of the β-actin 5′ primer were added, along with 300 ng more of the CHR 3′ primer. Fifteen cycles of amplification were carried out (1 min at 94°C, 1 min at 47°C, and 3 min at 72°C). The PCR products were separated by electrophoresis on a 3% NuSieve/1% agarose gel, transferred to Zetabind in 10× standard saline citrate, and hybridized sequentially with a 1-kb *Eco*RI subfragment of CHR (pCHor1.0) and β-actin as probes. The filters were stripped and exposed between hybridizations to ensure the complete removal of signal. The CHR RT–PCR product is 234 base pairs (bp) and the β-actin RT–PCR product is 250 bp. Exposure of the CHR hybridization was for 24 hr at room temperature, and exposure of the β-actin hybridization was for 1.5 hr at room temperature. Densitometric readings were performed with a Molecular Dynamics densitometer (Sunnyvale, CA).

Amplification of DNA. The primers (5′-ATATAGGATCCATCTCCTGCCTTCTTTAATAG-3′ and 5′-ATATAGGATCCTGGCTGTCTCTGATGTCATTG-3′) contain positions 180–201 and the complement of positions 374–395 of the sequence shown in Fig. 3 with synthetic *Bam*HI recognition and spacer sequences (underlined). Genomic DNA from a normal female and a male were amplified with 30 cycles of 94°C for 1 min, 47°C for 1 min, and 72°C for 3 min; 10% of this product was then reamplified with the same primers and program. The fragments were gel purified, digested with *Bam*HI, and cloned into pBluescript SK for sequencing.

RESULTS

Chromosome Walking. Clone pCH4 was previously isolated by chromosome jumping from DXS165 (26) and was shown to lie within 45 kb of the X;13 breakpoint in a female with choroideremia. We screened phage and cosmid libraries with pCH4 and isolated cosmid clone c18 and phage clones λCHR10–14 (Fig. 1). A subclone of λCHR10, pC10A, was found to map proximal to the t(X;13) breakpoint and identified altered *Bam*HI and *Kpn* I fragments in the X;13 cell line (data not shown). The probe pC10A was used subsequently in a chromosome walk to isolate the phage clone λCHR15. Four contiguous *Pst* I fragments in λCHR15 were mapped with respect to the translocation breakpoint; these results are shown in Fig. 2. Probe Pst3 detects no restriction fragments in the hybrid CIII-1 and therefore lies proximal to the breakpoint; Pst4 and Pst1, which do detect fragments in CIII-1, are distal; and fragment Pst2 spans the X;13 breakpoint (data not shown).

Pst3 identified cross-hybridizing sequences in dog, sheep, and rabbit at reduced stringency (40% formamide, 42°C) (data not shown). We used probe Pst3 to screen the retinal cDNA library and identified three clones that mapped to the Xq21.2 region. The insert of the largest cDNA clone, λCHR (assigned DXS703 in the Genome Data Base), measured 2.8 kb; the insert was subcloned as two *Eco*RI fragments into pBluescript (SK−) (pCHR1.0 and pCHR1.8) and sequenced (Fig. 3).

The sequence of λCHR insert predicts a 1095-nucleotide open reading frame, which encodes a protein of 365 amino acids (estimated M_r of 42,000). The first ATG is in a good consensus sequence for a valid translational start (39, 40). Neither the 5′ nor the 3′ end of the gene is present, as evidenced by primer extension analysis (described below) and the absence of a polyadenylylation signal or a poly(A) tail.

The λCHR cDNA sequence reported here is, for the most part, identical to that described in the report of Cremers *et al.* (27), with two notable exceptions.

The first discrepancy is located at the 5′ end of the cDNA sequence. We found no homology between the first 287 nucleotides of our sequence and the analogous nucleotides at the 5′ end of the sequence reported by Cremers *et al.* (27). To explore this discrepancy, we generated antisense primers complementary to nucleotides 91–111 (well within the region of the first 287 nucleotides of our sequence, the region that diverged from Cremers' sequence) and carried out primer extension analysis with total RNA from retinoblastoma cells as a template. Primer extension analysis using a labeled primer spanning nucleotides 91–111 from the 5′ end of CHR showed a major product, which predicted a start of transcription ≈90 bases 5′ to the 5′ end of CHR (data not shown). There was also an additional smaller fragment of 110 bp, suggesting that either there is an additional start at or near the 5′ end of CHR or that an RNA that is alternatively spliced 5′ to the primer results in a smaller product. In contrast, Cremers *et al.* (27) found that the 5′ end of their cDNA sequence was not fully protected in an S1 analysis and thus is unlikely to reflect the predominant mRNA species.

The second discrepancy is a single base change at position 295 in our sequence (Fig. 3)—we found a guanine at base 295, whereas Cremers *et al.* (27) report a thymine at the analogous

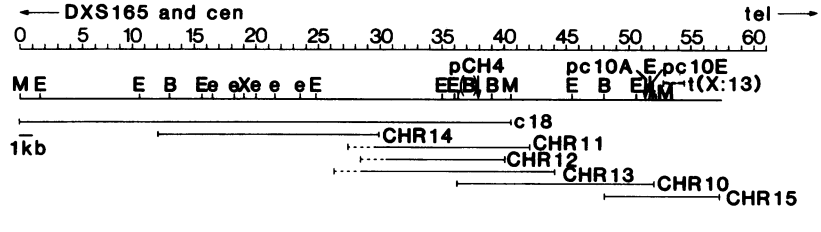

FIG. 1. Chromosome walking experiments around pCH4. The relative order of *Eco*RI sites in lowercase letters in λCHR14 and cosmid c18 were not determined. The *Bam*HI site in parentheses is present in C18 but not in phage clones λCHR10–13. B, *Bam*HI; E, *Eco*RI; M, *Mbo* I; X, *Xho* I; cen, centromere; tel, telomere.

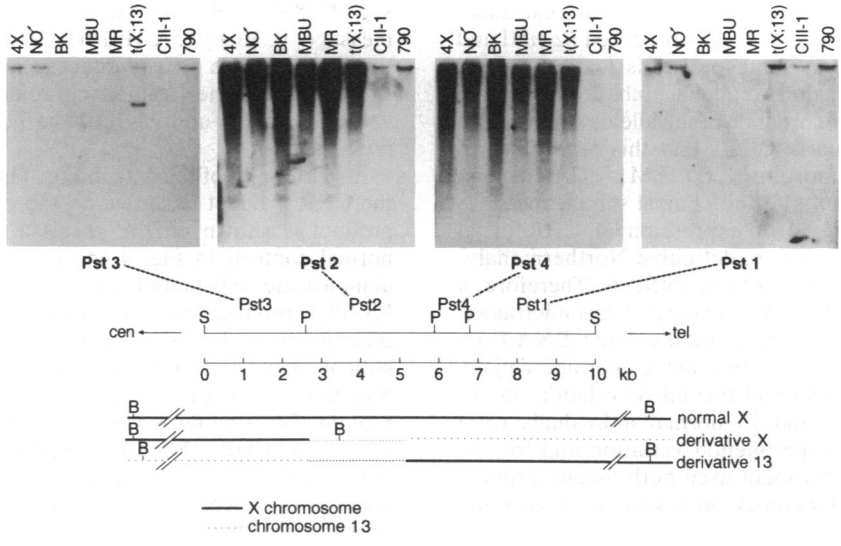

FIG. 2. Autoradiographs of Southern blot filters containing genomic DNA digested with *Bam*HI and hybridized with four contiguous *Pst* I fragments from λCHR15. From left to right in each filter are DNA samples from the 48,XXXX cell line (lane 1); a normal 46,XY male (lane 2); probands BK, MBU, and MR, who all have large deletions of the choroideremia locus (lanes 3–5); the female proband with choroideremia and t(X;13) (lane 6); hybrid from the t(X;13), which retains the derivative 13 (lane 7); and a somatic cell hybrid containing a single human X chromosome in a Chinese hamster background (lane 8).

position. This difference (GAA encoding glutamic acid versus TAA, a stop, respectively) is significant because it extends an opening reading frame 147 nucleotides further 5′ through the region of divergence between our sequence and that reported by Cremers *et al.* (27) and augments the predicted protein by 49 amino acids. To confirm the GAA rather than TAA codon at this position (G vs. T at position 295), DNA samples from a male and a female were amplified by PCR with primers that span the region in which the base discrepancy occurs. Two independent samples of PCR-amplified products from each individual were cloned and sequenced. All four sequences showed GAA (glutamatic acid) rather than TAA at this position.

Proband Analysis. Southern blot analysis of 34 probands with isolated choroideremia was performed with DNA samples digested by the enzymes *Taq* I, *Hin*dIII, and *Bam*HI and

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   1  CGAATTCCGATTAAAGACATCTGCTCTGGAACACGGAACCTGTTCCACTCGTCCTTCTCGAAATGCAAGAATCCTGGTAATATTTTTAAACTCTGCATATCGACTAACATTAGATTTGAT  120

 121  TAGAAGATCAATTAGTAATCCTCGAGAATACAGCAGCTTTGATACTAAATCAATATTAAATCTCCTGCCTTCTTTAATAGCAAACAACTTACTATGGTAGAAAAGCGAATGCTAATGAAA  240
                                                                                                    MetValGluLysArgMetLeuMetLys

 241  TTTCTTACATTTTGTATGGAATATGAGAAATATCCTGATGAATATAAAGGATATGAAGAGATCACATTTTATGAATATTTAAAGACTCAAAAATTAACCCCCAACCTCCAATATATTGTC  360
      PheLeuThrPheCysMetGluTyrGluLysTyrProAspGluTyrLysGlyTyrGluGluIleThrPheTyrGluTyrLeuLysThrGlnLysLeuThrProAsnLeuGlnTyrIleVal

 361  ATGCATTCAATTGCAATGACATCAGAGACAGCCAGCAGCACCATAGATGGTCTCAAAGCTACCAAAAACTTTCTTCACTGTCTTGGGCGGTATGGCAACACTCCATTTTTGTTTCCTTTA  480
      MetHisSerIleAlaMetThrSerGluThrAlaSerSerThrIleAspGlyLeuLysAlaThrLysAsnPheLeuHisCysLeuGlyArgTyrGlyAsnThrProPheLeuPheProLeu

 481  TATGGCCAAGGAGAACTCCCCCAGTGTTTCTGCAGGATGTGTGCTGTGTTTGGTGGAATTTATTGTCTTCGCCATTCAGTACAGTGCCTTGTAGTGGACAAAGAATCCAGAAAATGTAAA  600
      TyrGlyGlnGlyGluLeuProGlnCysPheCysArgMetCysAlaValPheGlyGlyIleTyrCysLeuArgHisSerValGlnCysLeuValValAspLysGluSerArgLysCysLys

 601  GCAATTATAGATCAGTTTGGTCAGAGAATAATCTCTGAGCATTTCCTCGTGGAGGACAGTTACTTTCCTGAGAACATGTGCTCACGTGTGCAATACAGGCAGATCTCCAGGGCAGTGCTG  720
      AlaIleIleAspGlnPheGlyGlnArgIleIleSerGluHisPheLeuValGluAspSerTyrPheProGluAsnMetCysSerArgValGlnTyrArgGlnIleSerArgAlaValLeu

 721  ATTACAGATAGATCTGTCCTAAAAACAGATTCAGATCAACAGATTTCCATTTTGACAGTGCCAGCAGAGGAACCAGGAACTTTTGCTGTTCGGGTCATTGAGTTATGTTCTTCAACGATG  840
      IleThrAspArgSerValLeuLysThrAspSerAspGlnGlnIleSerIleLeuThrValProAlaGluGluProGlyThrPheAlaValArgValIleGluLeuCysSerSerThrMet

 841  ACATGCATGAAAGGCACCTATTTGGTTCATTTGACTTGCACATCTTCTAAAACAGCAAGAGAAGATTTAGAATCAGTTGTGCAGAAATTGTTTGTTCCATATACTGAAATGGAGATAGAA  960
      ThrCysMetLysGlyThrTyrLeuValHisLeuThrCysThrSerSerLysThrAlaArgGluAspLeuGluSerValValGlnLysLeuPheValProTyrThrGluMetGluIleGlu

 961  AATGAACAAGTAGAAAAGCCAAGAATTCTGTGGGCTCTTTACTTCAATATGAGAGATTCGTCAGACATCAGCAGGAGCTGTTATAATGATTTACCATCCAACGTTTATGTCTGCTCTGGC  1080
      AsnGluGlnValGluLysProArgIleLeuTrpAlaLeuTyrPheAsnMetArgAspSerSerAspIleSerArgSerCysTyrAsnAspLeuProSerAsnValTyrValCysSerGly

1081  CCAGATTGTGGTTTAGGAAATGATAATGCAGTCAAACAGGCTGAAACACTTTTCCAGGAAATCTGCCCCAATGAAGATTTCTGTCCCCCTCCACCAAATCCTGAAGACATTATCCTTGAT  1200
      ProAspCysGlyLeuGlyAsnAspAsnAlaValLysGlnAlaGluThrLeuPheGlnGluIleCysProAsnGluAspPheCysProProProProAsnProGluAspIleIleLeuAsp

1201  GGAGACAGTTTACAGCCAGAGCGTGCAGAATCCAGTGCCATACCAGAGGCTAACTCGGAGACTTTCAAGGAAAGCACAAACCTTGGAAACCTAGAGGAGTCCTCTGAATAATGGATATAC  1320
      GlyAspSerLeuGlnProGluArgAlaGluSerSerAlaIleProGluAlaAsnSerGluThrPheLysGluSerThrAsnLeuGlyAsnLeuGluGluSerSerGluEnd

1321  ACCAAACTGGATACCCAACTTTGGAAATTCTGACTGGTCTCAGAGTCTACTTGATAGAAGGACTCTTTGAGAAATGTTAGAAAGCAGCAGCAATTATAAGGCAAAATAGGTAATAGAAAT  1440

1441  CCAAAAGGGGATTTT  1455
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FIG. 3. Partial DNA sequence and predicted amino acid sequence of cDNA insert from λCHR. The star marks the location of a discrepancy (G vs. T) at which the guanine seen in λCHR and in PCR-amplified genomic DNA differs from the thymine seen in the published sequence of Cremers *et al.* (27). The sequence in λCHM that is 5′ to the overline differs completely from that of Cremers *et al.* (27).

probed with pCHR1.0. No obvious structural rearrangements of this region were seen except for altered *Bam*HI and *Hin*dIII fragments seen in some probands and in several normal control families; the Mendelian inheritance of the altered fragments and concordance of the alleles with the two enzymes in any individual suggests that this represents an insertion/deletion polymorphism (D.E.M., R.A.L., and R.L.N., unpublished results) of no clinical significance.

Because of the low level of expression of CHR in all tissues, especially lymphoblasts, definitive Northern analysis of these patients has proven to be difficult. Therefore, a more sensitive assay, an RT–PCR analysis, was undertaken. The RT–PCR products made from total cellular RNA from lymphoblasts from 34 patients with isolated choroideremia, 6 patients with choroideremia and mental retardation due to large deletions in Xq21, and 13 normal individuals were studied. To control for experimental variation and for the amount of RNA, this experiment used both β-actin primers and CHR primers simultaneously on a sample in the same tube (see *Methods*).

A representative sample of the Southern hybridizations of these RT–PCR experiments are shown in Fig. 4. As expected, the mentally retarded choroideremia patients who have large deletions in Xq21 (45-06, MBU, CH05-01, CH06-01, and 137-02) show no CHR RT–PCR product. Most significantly, however, several patients with choroideremia have no message (128-02, CH03-01, and 149-02) or reduced amounts of message (CH04-01, CH01-03, 95-02, and 115-02), even though they have grossly intact genes by Southern analysis. In addition, some isolated choroideremia patients (77-02, 94-03, CH07-03, and 73-01) have normal amounts of CHR message.

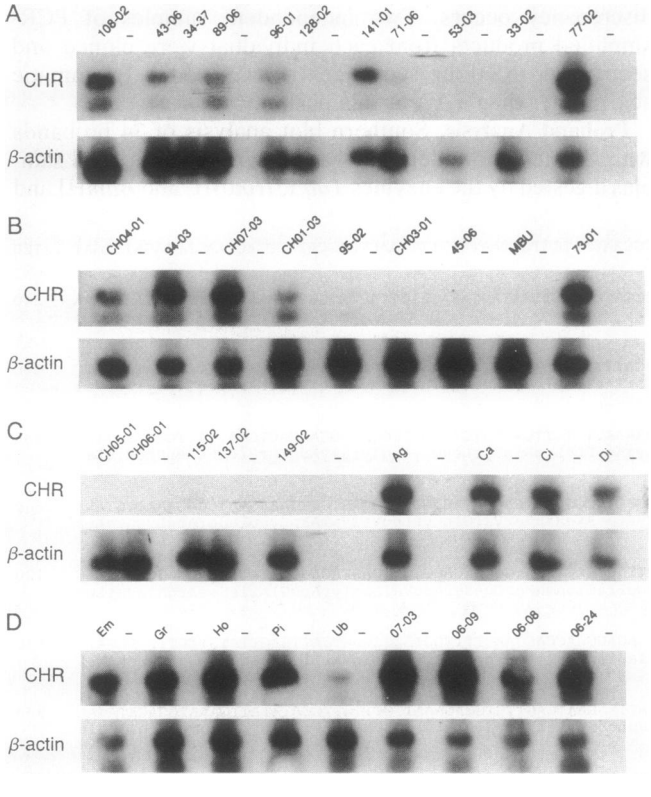

FIG. 4. Autoradiographs of Southern blots of cDNA synthesized by RT–PCR from total RNA of lymphoblastoid cells from normals and choroideremia probands and probed with pCH1.0, the 1-kb *Eco*RI subfragment of λCHR, or with a fragment of the β-actin cDNA. Lanes marked "—" correspond to a sample for which a mock RT–PCR was carried out with no reverse transcriptase present, as a control for PCR amplification of genomic DNA. (*A*) Probands with isolated choroideremia. (*B*) Probands with isolated choroideremia except for 45-06 and MBU, who have large deletions in Xq21 that include the region of the candidate choroideremia gene. (*C*) Three patients with large Xq21 deletions (CH05-01, CH06-01, and 137-02), two patients with isolated choroideremia without deletions (155-02 and 149-02), and four normal controls (Ag, Ca, Ci, and Di). (*D*) Normal individuals.

A scatter plot of the data in Fig. 4 is shown in Fig. 5, where the CHR product is shown on the ordinate and the β-actin product is shown on the abscissa. Although the data for normal controls in Fig. 4 and the scatter plot in Fig. 5 do demonstrate substantial variation in the amount of CHR RT–PCR product, even when corrected by comparison with β-actin, these data show clearly that some choroideremia patients have no CHR message, whereas the amounts of β-actin message are similar to that of controls. All together, 8 of 34 (24%) patients with normal Southern blot patterns showed no CHR mRNA in a multiplex RT–PCR assay with β-actin as control. An additional 17 (50%) have reduced levels of the mRNA, whereas 9 (26%) had no mRNA for the choroideremia gene.

DISCUSSION

We have isolated and characterized cDNA sequences corresponding to a candidate gene for choroideremia and have demonstrated alteration of the expression of this gene in some patients with choroideremia who show no abnormality on Southern blot. That patients with isolated choroideremia have absent or reduced levels of message for this gene in the absence of any detectable structural rearrangements further strengthens the hypothesis that this is a good candidate for being the gene involved in choroideremia.

The function of the protein encoded by this candidate gene is unknown. A data base search (GenBank, August 1991) for nucleotide and amino acid sequence similarity revealed a short stretch of 35 amino acids that shows 80% similarity and 63% identity with the recently reported bovine smg p25A GDP-dissociation inhibitor (41) that regulates a ras-like guanine nucleotide-binding regulatory protein (G protein). This homology was recently described by Fodor *et al.* (42), who suggested that the choroideremia candidate gene may be involved in signal-transduction pathways involving G proteins. Cremers *et al.* (27) identified a PEST sequence (i.e., a region rich in proline, glutamic acid, serine and threonine),

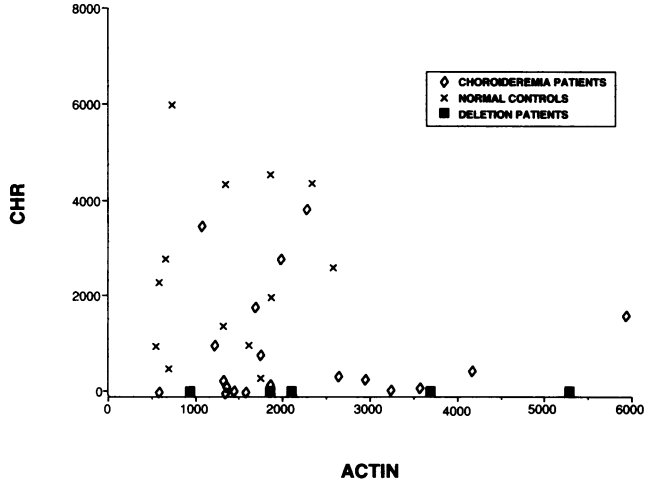

FIG. 5. Scatter plot of densitometric measurements of the RT–PCR experiments shown in Fig. 4. Each point represents an RT–PCR experiment from a single individual; the absolute density of the autoradiographic signal with the candidate choroideremia gene probe (on the ordinate) is plotted versus the absolute density of the signal obtained with the β-actin probe (on the abscissa).

described in many regulatory proteins with half-lives of 2 hr or less, at amino acids 301–351 (43, 44). These observations suggest that the choroideremia gene product may be a short-lived cytoplasmic protein involved in regulation of a signal-transduction pathway. Given that the genes responsible for other retinal dystrophies have been shown to encode proteins in the visual transduction pathway (45, 46), it is interesting that the choroideremia gene product appears to have some similarity to a protein that regulates G-protein activity; one may speculate that this gene product too may be involved in the visual-transduction pathway.

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