

# Cloning and Characterization of a Human $\beta,\beta$ -Carotene-15, 15'-Dioxygenase That Is Highly Expressed in the Retinal Pigment Epithelium

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**Retinoids play a critical role in vision, as well as in development and cellular differentiation.  $\beta,\beta$ -Carotene-15,15'-dioxygenase (Bcdo), the enzyme that catalyzes the oxidative cleavage of  $\beta,\beta$ -carotene into two retinal molecules, plays an important role in retinoid synthesis. We report here the first cloning of a mammalian *Bcdo*. Human *BCDO* encodes a protein of 547 amino acid residues that demonstrates 68% identity with chicken Bcdo. It is expressed highly in the retinal pigment epithelium (RPE) and also in kidney, intestine, liver, brain, stomach, and testis. The gene spans approximately 20 kb, is composed of 11 exons and 10 introns, and maps to chromosome 16q21–q23. A mouse orthologue was also identified, and its predicted amino acid sequence is 83% identical with human *BCDO*. Biochemical analysis of baculovirus expressed human *BCDO* demonstrates the predicted  $\beta,\beta$ -carotene-15,15'-dioxygenase activity. The expression pattern of *BCDO* suggests that it may provide a local supplement to the retinoids available to photoreceptors, as well as a supplement to the retinoid pools utilized elsewhere in the body. In addition, the finding that many of the enzymes involved in retinoid metabolism are mutated in retinal degenerations suggests that *BCDO* may also be a candidate gene for retinal degenerative disease.** © 2001 Academic Press

## INTRODUCTION

Vertebrate phototransduction, the process by which light energy is converted into a neurochemical signal, is carried out by photoreceptor cells within the retina (Koutalos and Yau, 1996; Lagnado and Baylor, 1992; Palczewski *et al.*, 2000; Polans *et al.*, 1996; Pugh *et al.*,

1999; Rattner *et al.*, 1999) and is supported by the underlying cells of the retinal pigment epithelium (RPE) (Bok, 1993). It is initiated when a visual pigment, rhodopsin in rods or one of the color pigments in cones, absorbs a photon of light. These pigments consist of an opsin apoprotein joined in a Schiff base linkage to the chromophore 11-*cis*-retinal. Upon photoisomerization of the 11-*cis*-retinylidene group to an all-*trans*-retinylidene group, the pigment undergoes a sequence of conformational changes. These changes eventually result in the activation of the retinal G-protein transducin, and amplification of the light signal. A hydrolysis step then leads to the release of free all-*trans*-retinal.

Regeneration of fresh rhodopsin from opsin requires a constant supply of 11-*cis*-retinal. The major mechanism for supplying photoreceptors with fresh chromophore, which is known as the visual cycle (or retinoid cycle), involves recycling from the released all-*trans*-retinal (Crouch *et al.*, 1996). Upon release from the binding pocket of rhodopsin, the potentially reactive all-*trans*-retinal is transported to the cytoplasm by a mechanism that is thought to involve a photoreceptor-specific ATP-binding cassette transporter (ABCR) (Ahn and Molday, 2000; Haeseleer *et al.*, 1998; Rattner *et al.*, 2000; Sun and Nathans, 1997, 2000; Weng *et al.*, 1999). It is then reduced to all-*trans*-retinol (Haeseleer *et al.*, 1998; Rattner *et al.*, 2000) and subsequently translocated to the RPE. Within the RPE, a putative retinol isomerase catalyzes the conversion of all-*trans*-retinol into 11-*cis*-retinol (Bernstein *et al.*, 1987; Deigner *et al.*, 1989; Winston and Rando, 1998). It has been suggested that the esterified form of all-*trans*-retinol serves as the immediate substrate for the putative isomerohydrolase (Canada *et al.*, 1990; Ruiz *et al.*, 1999; Trehan *et al.*, 1990), but this issue is controversial (McBee *et al.*, 2000; Stecher *et al.*, 1999). RPE65, a protein of unknown function that is prefer-

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entially expressed in the RPE (Hamel *et al.*, 1993; Nicoletti *et al.*, 1995), appears to be required for isomerization. RPE65 null mice accumulate all-*trans*-retinyl esters in their RPE cells and are deficient in 11-*cis*-retinal (Redmond *et al.*, 1998; Van Hooser *et al.*, 2000). Finally, 11-*cis*-retinol generated by the isomerase reaction is oxidized to 11-*cis*-retinal by 11-*cis*-RDH (Simon *et al.*, 1995) and other oxidizing systems (Driesen *et al.*, 2000; Jang *et al.*, 2000) and transported back to the photoreceptor cells.

A second complementary mechanism for providing retinoids involves their generation from dietary retinoids and retinoid precursors. Since animals cannot synthesize vitamin A (retinol) *de novo*, vitamin A and its derivatives are derived from the oxidative cleavage of C<sub>40</sub> carotenoids. In one pathway, the enzyme  $\beta,\beta$ -carotene-15,15'-dioxygenase (EC 1.13.11.21;  $\beta,\beta$ -carotene dioxygenase; *Bcdo*) catalyzes the conversion of  $\beta,\beta$ -carotene into two molecules of all-*trans*-retinal. However, the biochemistry and molecular biology of this pathway and its biological importance in vertebrates are not well understood, partly due to difficulties in purifying and studying the enzyme(s) *in vitro*. In fact, it was only recently that cDNAs for vertebrate (chicken) and invertebrate (*Drosophila*) *Bcdo* were cloned (von Lintig and Vogt, 2000a; Wyss *et al.*, 2000). Of particular interest to the studies presented here is the recent finding that mutations in *Drosophila Bcdo* are responsible for the *ninaB* photoreceptor degeneration (von Lintig *et al.*, 2000b).

In the process of characterizing clones from a human RPE cDNA library that are preferentially expressed in the RPE, we identified a clone that shows homology with *RPE65* and also with chicken *Bcdo* and other retinoid dioxygenases. In this paper we present the initial molecular characterization of the murine and human cDNAs and the human *BCDO* gene, explore the expression pattern of the human gene, and demonstrate that human *BCDO* does indeed encode a protein with *Bcdo* activity, thus providing the first evidence for a cloned mammalian *Bcdo*. We also suggest that *Bcdo* in the RPE may play an important role in a local secondary pathway for the synthesis of all-*trans*-retinal, particularly under bright-light conditions where the normal visual cycle may be stressed.

## MATERIALS AND METHODS

**Cloning of the human *BCDO* gene.** A human RPE cDNA library in Uni-ZAP XR (Stratagene) was *in vivo* excised, and 2000 random clones were grown on LB/ampicillin plates. Using a subtracted bovine RPE cDNA library as template (Chang *et al.*, 1997, 1999), polymerase chain reaction (PCR) products were generated with primers (W8, 5'-AGGAATTCGGCAGCAGANN-3' and W9, 5'-CGGGCCCCCCTCGAGTT-3') and labeled with [ $\alpha$ -<sup>32</sup>P]dCTP by random prime labeling. The labeled products were used as probes to screen the above-mentioned human RPE cDNA library, and selected clones were sequenced by standard methods (Thermo Sequenase Cycle Sequencing Kit, Amersham, Piscataway Pharmacia Biotech). *BCDO* 5'-RACE (Marathon-ready cDNA, Clontech) was conducted according to the manufacturer's instructions using the following

primers: adaptor primer 1 (5'-CCATCCTAATACGACTCACTAT-AGGGC-3'); *BCDO* antisense primer 1 (W121, 5'-CGGGCACAAA-CAGGGGTTC-3'); adaptor primer 2, 5'-ACTCACTATAGGGCTC-GAGCGC-3'); and *BCDO* antisense primer 2 (5'-ACCCG-TGGAAGCTCTAAGCCTTCA-3'). Resulting PCR products were subcloned into the pCR2.1 vector using a TA cloning kit (Invitrogen) according to the manufacturer's direction and sequenced.

To obtain the gene structure of *BCDO*, RPCI-11 Human Male BAC library high-density filters (Segment 1, Bacpac Resource) were screened using a <sup>32</sup>P-labeled PCR fragment generated with the primers W131 (5'-AAAGGAGGTTTCTTAGAAGGACTG-3') and W133 (5'-CAGCCATTGTCTCTACTGATCC-3'). Four clones containing the entire human *BCDO* gene were isolated, and intron/exon boundaries were determined by sequencing the BAC clones directly or PCR products generated using the BAC clones as templates.

A mouse orthologue was also identified by searching the EST database (National Center for Biotechnology Information) (Altschul *et al.*, 1990, 1997), and the EST clone was obtained and sequenced on both strands.

**Northern blot hybridization.** Total RNA from human RPE and neural retina was extracted from human donor eyes (Maryland Eye Bank) using Trizol reagent (Life Technologies); all other total RNA used was purchased from Clontech. Northern blots with 10  $\mu$ g of total RNA from RPE, retina, liver, brain, and testis were prepared and hybridized by standard methods using either a PCR-generated PCR probe (primers W78, 5'-CCAGGACTTCAAGGAGAACTCCAG-3', and W121) or a human  $\beta$ -actin probe. A human Multiple Tissue Northern blot (MTN) (Clontech), containing 2  $\mu$ g poly(A)<sup>+</sup> RNA from a variety of tissues, was also hybridized with the same probes as previously described (Chang *et al.*, 1999).

**Reverse transcription-PCR (RT-PCR) analysis.** One microgram of total RNA was reverse transcribed with an oligo(dT) primer using SuperScript II reverse transcriptase (Life Technologies) according to the manufacturer's protocol. Aliquots of first-strand cDNA were PCR amplified using primers for human *BCDO* (W78 and W121) and for human glyceraldehyde phosphate dehydrogenase (*GAPD*) (W226 5'-GGGGAGCCAAAAGGGTCAT-3' and W227 5'-GCCCCAGCGTCAAAGGTGGA-3') and then analyzed by agarose gel electrophoresis.

**Chromosomal localization of the human *BCDO* gene.** Radiation hybrid mapping of the human *BCDO* gene was carried out using the Stanford G3 panel and primers W131 and W133, following the supplier's instructions (Research Genetics). A Chinese hamster-human somatic cell hybrid panel was also analyzed with the same set of human *BCDO* primers as used for the G3 panel, according to the supplier's instructions (Coriell).

**Primer extension.** Primer W180 (5'-TTCCCTCCTTCACATCTCTG-3') was labeled with [ $\gamma$ -<sup>32</sup>P]ATP by T4 polynucleotide kinase. Ten micrograms of total human RPE RNA was denatured with 5 pmol of labeled primer at 80°C for 90 s and annealed at 50°C for 20 min. Primer extension was carried out with ThermoScript reverse transcriptase (Life Technologies) at 45°C for 15 min and then at 65°C for 60 min, and the resulting products were incubated with RNase A1/H at 37°C for 15 min, extracted with phenol/chloroform, ethanol precipitated, and separated on a 6% sequencing gel. The sequence ladder was created using the same primer (W180) with one of the human *BCDO* clones as template.

**Generation of anti-*BCDO* polyclonal antibody.** A rabbit polyclonal antibody against *BCDO* was generated by standard methods. The peptide RLTSVPTLRRFVAVPLHVDK, corresponding to human *BCDO* codons 348–367, was synthesized, purified to greater than 90% by HPLC, and then conjugated to keyhole limpet hemocyanin (KLH) as carrier. Two rabbits were immunized with the KLH peptide. Hyperimmune serum was processed over an immunosorbent to capture antibodies specific for the peptide. The affinity-purified antibody was then titered by ELISA methods.

**Immunoblot analysis of *Bcdo* protein expression.** Bovine tissues were sonicated in buffer (50 mM sodium phosphate, pH 7.8, 300 mM NaCl, 1  $\mu$ g/ml leupeptin, 1  $\mu$ g/ml aprotinin, 0.1 mM PMSF) and

centrifuged at 14,000g for 30 min at 4°C to separate soluble and insoluble fractions. The protein concentration of the supernatant and pellet extracts was determined by the Bradford method (Bio-Rad Laboratories). Ten micrograms of protein from each set of extracts was electrophoresed on 4–15% gradient Bio-Rad Ready gels by standard methods. After electrophoresis, the gel was soaked in transfer buffer (25 mM Tris-HCl, 192 mM glycine, 20% methanol) and semi-dry transferred to a nitrocellulose membrane following the manufacturer's instructions (Bio-Rad Laboratories). The nitrocellulose membrane was fixed in methanol destain solution (25% methanol, 10% acetic acid) for 10 min at room temperature. After being blocked with 5% dry milk in 1× TBST (50 mM Tris-HCl, pH 7.4, 200 mM NaCl, 0.1% Tween 20) for 1 h at room temperature, the membrane was incubated overnight at 4°C with the rabbit polyclonal antibody against BCDO (1:8000) in 5% dry milk, washed four times with 1× TBST, and incubated with horseradish peroxidase-conjugated anti-rabbit antibody (1:2000) in 5% dry milk in 1× TBST for 1 h at room temperature. After washing, antibody binding was visualized using ECL plus reagent (Amersham Pharmacia Biotech).

**Expression of human BCDO in insect cells.** DNA containing the entire human BCDO coding region was amplified by PCR from a pBluescript BCDO cDNA containing plasmid using primers FH350 (5'-GCAATGGATATAATATTGGCAGG-3') and FH351 (5'-CCATCAGGTCAGAGGAGCC-3') and 25 cycles at 94°C for 30 s, 60°C for 30 s, and 68°C for 2 min. The PCR product was cloned into the pCRII-TOPO vector (Invitrogen) and sequenced using the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems). The coding region of human BCDO was then transferred as a BamHI-XhoI fragment into the BamHI-XhoI sites of pFASTBac (Life Technologies). A BCDO recombinant baculovirus was obtained by transposition in DH10BAC bacteria and amplified after transfection into Sf9 cells. The expression of recombinant proteins was tested 3 days postinfection.

**Assay for BCDO activity.** Sf9 cells containing either the BCDO recombinant baculovirus or the empty bacmid were solubilized with 0.1% Triton X-100 in 20 mM BTP (1,3-bis[tris(hydroxymethyl)methylamino]propane) (pH 7.4) in a 1:9 ratio (cell pellet to buffer) at 0°C for 30 min. The solubilization mixture was then centrifuged at 98,600g, and the resulting supernatant was used to assay for BCDO activity. The unused supernatant was aliquoted and flash-frozen with liquid nitrogen without affecting the enzymatic activity. The assay mixture (100  $\mu$ l) contained BTP (final concentration, 45–70 mM, pH 7.4), NaCl (33–65 mM), Triton X-100 (0.095%), FeSO<sub>4</sub> (10  $\mu$ M), ascorbic acid (10 mM), the solubilized enzyme (33–132  $\mu$ g protein), and 2  $\mu$ l of  $\beta,\beta$ -carotene (40  $\mu$ M) in *N,N*-dimethylformamide, which was added last to initiate the reaction. The reaction was incubated at 37°C for various times and terminated with 300  $\mu$ l of methanol and 200  $\mu$ l of 0.4 M NH<sub>2</sub>OH (pH 6.5, freshly prepared). The mixture was then placed on a mixer at room temperature for 30 min and extracted with 500  $\mu$ l hexane. After mixing and separating phases, 400  $\mu$ l of the upper phase was removed and dried using a Speedvac. The extraction was repeated once. The residue was dissolved in 120  $\mu$ l hexane. One hundred microliters of the hexane solution was analyzed by normal-phase HPLC (Alltech, silica 5  $\mu$ m, 2.1 × 250 mm) with 4% ethyl acetate/96% hexane at a flow rate of 0.5 ml/min using an HP1100 with a diode-array detector and HP Chemstation A.06.03 software. The (*syn*) all-*trans*-retinal oxime fraction (the major product) was collected and quantified for the  $\beta,\beta$ -carotene-15,15'-dioxygenase activity.

## RESULTS

### Cloning of a Human cDNA with Homology to Bcdo

To identify human cDNAs representing genes that are preferentially expressed in the RPE, a nonsubtracted human RPE cDNA library was screened with a PCR probe generated from a previously described subtracted bovine RPE cDNA library (Chang *et al.*, 1997,

1999). Among the multiple novel positive clones identified, the sequence of one showed 37% identity with human RPE65 at the deduced amino acid level. Based on the findings described below, we called this clone BCDO.

The open reading frame of the human BCDO cDNA encodes a predicted protein of 547 amino acids with a calculated molecular mass of 62 kDa. The predicted amino acid sequence shows 67% identity and 80% similarity with a recently published chicken Bcdo sequence (Wyss *et al.*, 2000) (Fig. 1). Homology between human and chicken Bcdo is distributed fairly equally throughout the entire protein, but human BCDO has an extra 20 amino acid residues at its carboxyl terminus. Recent analysis of GenBank entries identified both human and murine Bcdo EST clones (GenBank Accession Nos. AK001592 and AW107279, respectively). The open reading frame of the full-length murine EST clone, which we obtained and sequenced, encodes a protein of 566 amino acids that has 83 and 66% identity with the human and chicken clones, respectively (Fig. 1). Most of the sequence variation between the murine and the human clones is located at the carboxyl terminus. Overall, chicken Bcdo, murine Bcdo, and human BCDO appear to be orthologues of one another. A *Drosophila*  $\beta,\beta$ -carotene dioxygenase was also recently cloned (von Lintig and Vogt, 2000a). The amino acid homology between human BCDO and *Drosophila* Bcdo is 24%, while the homology between chicken Bcdo and *Drosophila* Bcdo is 31%.

The alignment of the BCDOs and human RPE65 shown in Fig. 1 reveals four areas of increased sequence conservation (labeled A–D). The homology of regions A and D is high and specific enough that database analysis using these regions with Blastp and an Expect (*E*) value of 1000 (Altschul *et al.*, 1990, 1997) yields significant matches only with dioxygenases and RPE65 homologues. Among the real and “hypothetical” dioxygenases that show homology to region A are proteins/genes from *Caenorhabditis elegans* (Accession Nos. CAB60367 and AAC67462), *Arabidopsis thaliana* (T10688), *Synechocystis* sp. (S76206 and S76169), and *Streptomyces coelicolor* (CAB56138). Since all these proteins presumably use iron cation as part of their oxidative mechanism, it is possible that region A or one of the other conserved sequences might be involved in iron cation binding.

### Genomic Structure of the Human BCDO Gene

To explore its possible relationship to human disease, the gene structure and chromosomal localization of human BCDO were determined. Four BAC clones containing the entire human BCDO gene were identified by screening a set of high-density BAC filters. The exon/intron boundaries of human BCDO were determined by sequence comparison of the genomic and cDNA clones. BCDO spans approximately 20 kb and consists of 11 exons and 10 introns (Table 1). The exons

H-BCDO	MDI-IFG--- RN---R--- -K----EQL -----	13
M-Bcdo	.E....--- Q.---K--- -E-----	13
C-Bcdo	.ET..N--- ..---K--- -E-----	13
D-Bcdo	.AAGV.KSFM .DFFAVKYDE QRNDPQA.R. DGNGRLYPNC SSDVWLRSC	50
H-RPE65	.S.-QVE--- HPAGGYK--- -.LFETV.E. S-----	23
<b>A</b>		
H-BCDO	----EPVRAK VTGKIPAWLQ <u>GTLLRNGPGM</u> HTVGESRYNH <u>WFDGLALLHS</u>	59
M-Bcdo	-----Q... ..S..... ..K.....	59
C-Bcdo	-----IK.E .Q.QL.T... .V..... .I.DTK.....	59
D-Bcdo	REIVD.IEGH HS.H..K.IC .S.....S WK..DMTFG. L..CS....R	100
H-RPE65	----S.LT.H ...R..L.T .S...C...L FE..SEPFY. L...Q...K	69
*		
H-BCDO	FTIRDGEVYY RSKYLRSPTY NTNIEANRIV VSEFGTMAYP DPCKNIFSKA	109
M-Bcdo	.S.....F. ....Q.... IA.....	109
C-Bcdo	.TFKN..... .C.....	109
D-Bcdo	.A..N.R.T. QNRFVDTE.L RK.RS.Q... .T...A.V. ...HS..DR-	149
H-RPE65	.DFKE.H.T. HRREFI.T.A. VRAMTEK... IT...C.F. ....RF	119
*		
H-BCDO	FSYLSHTIPD FTDNCLINIM KCGEDFYATS ETNYIRKINP QTLETLEKVD	159
M-Bcdo	.....E .....T .....D.....	159
C-Bcdo	.....E .....T.D.Y.... .F...D....	159
D-Bcdo	.AAIFRPDSG -...SM.S.Y PF.DQY.TFT ..PFMHR... C..A.EARIC	198
H-RPE65	...F-RGV-E V...A.V.VY PV...Y..CT ...F.T... E...IKQ..	167
H-BCDO	YRKYVAVNLA TSHPHYDEAG NVLNMGTSIV EKGKTKYVIF KIPATVPEGK	209
M-Bcdo	.....V. D..R.....	209
C-Bcdo	.S.....S... .I.....D..R...L...SS...KE	209
D-Bcdo	TTDF.G.VNH .....VLPS. T.Y.L..TMT RS.PA-.T.L SF.--HG.QM	245
H-RPE65	LCN..S..G. .A...IEND. T.Y.I.NCFG <u>KNESTA.N.V</u> ...PLQADKE	217
*		
<b>B</b>		
H-BCDO	KQKGSPPWKHT EVFCSIPSRS LLSPSYHSE <u>GVTENYVIFL</u> EQPFRDLILK	259
M-Bcdo	.K...V..A .....S.....	259
C-Bcdo	.K...CF..L ..V..... .Q.....I...I..I	258
D-Bcdo	---FEDA H.VATL.C.W K.H.G.M.T. .L.DH.F.IV ...LSVSLTE	289
H-RPE65	---D.ISKS .IVVQF.CSD RFK...V...L.P...I..V .T.VKINLF.	263
*		
H-BCDO	MATAY-IRRM SWASCLAFHR EEKTYIHID QRTRQPVQTK FYTDAMVVFH	308
M-Bcdo	....-M.GV .....MS.D. .D..... .K..P... .P.....	308
C-Bcdo	L...-.GV N.....S.HK .D..WF.FV. RK.KKE.S...L.LY.	307
D-Bcdo	YIK.Q-LGGQ NLSA..KWEF DRP.LF.L.. RVSGKL...- YESE.FFYL.	337
H-RPE65	FLSSWSLWGA NYMD.FESNE TMGVWL..A. KKRKKYLNN. YR.SPFNL.	313
<b>C</b>		
H-BCDO	<u>HVNAYEEDGC</u> IVFDVIAYED -NSLYQLFY- -LANL--NQD FKE---NSRL	350
M-Bcdo	.....VL..... -S..... -...--K. .E---K..	350
C-Bcdo	.....H V...IV..R. -N...DM.. -..KK.--DK. .EV---NK.	349
D-Bcdo	<u>II.CF.R..H</u> V.V.ICS.RN PEMINCMYLE AI..MQT.PN YATLFRGRP.	387
H-RPE65	<u>IT.T.DN.F</u> LIV.LCCWKG FEFV.NYL.- -...--REN WE.VKK.A.K	359
*		

**FIG. 1.** Sequence comparison of human, mouse, chicken, and *Drosophila* BCDO and human RPE65. The most conserved regions are shaded and labeled **A-D**. H-BCDO, human BCDO; M-Bcdo, mouse Bcdo; C-Bcdo, chicken Bcdo; D-Bcdo, *Drosophila* Bcdo; H-RPE65, human RPE65. A period represents a residue that is identical to the human BCDO sequence, and a dash represents a site at which a space was introduced to maximize alignment. An asterisk indicates a position that is conserved in all Bcdo proteins but not in human RPE65. The sequence of the peptide used as an immunogen is shaded and underlined. The alignment was generated using GeneWorks 2.5.1 (Oxford Scientific) and modified to maximize homology.

range in size from 95 to 795 bp. Intron sizes, which were determined by PCR and confirmed by GenBank analysis, range from approximately 660 bp to 3 kb. All the 5'-donor and 3'-acceptor sites are consistent with the GT-AG consensus for pre-mRNA splicing recognition sequences.

Primer extension was used to identify the transcription start sites for human *BCDO*. Several potential initiation sites were identified, with the major site 216 bp upstream of the initiation methionine (Fig. 2). This is consistent with the existence of a typical TATA box 30 bp upstream of the transcription start site. Another

weaker start site was identified 185 bp upstream of the ATG translation start codon (data not shown).

#### *Human BCDO Gene Maps to 16q21-q23*

The chromosomal localization of human *BCDO* was determined using a combination of somatic cell and radiation hybrid analyses. The gene maps to chromosome 16q21-q23. This position is close to the BBS2 locus for Bardet-Biedl syndrome, an autosomal recessive disease associated with pigmentary retinopathy, mental retardation, polydactyly, obesity, and hypogen-

H-BCDO	TSVPTLRRFA	VPLHVDKNAE	VGTNLIKVAS	TTATALKEED	GQVYCQPEFL	400
M-Bcdo	.....D..	..S..V..S.	.....K.	.H.....V.		400
C-Bcdo	. . I . . CK . . V	. . . QY . . D . .	. . S . . V . . L - P	. S . . V . . K .	. SI . . . . . I .	398
D-Bcdo	RF.LP.GTIP	PASIAKRGLV	KSFS.AGLSA	PQVSRMTKHS	VSQ.ADITYM	437
H-RPE65	APQ.EV..YV	L..NI..-D	T.K..VTLPN	.....ILCS.	ETIWLE..V.	408
H-BCDO	-YEG----L-	-ELPRVNY--	A-HNGKQYRY	VFATGVQWSP	IPTKIIKYDI	440
M-Bcdo	-----T-	.....T.---	..Y...P...	I..AE.....	V....L....	440
C-Bcdo	-C.-----I-	.....V.---	D-Y...K.K.	.Y.TE.....	V....A.LNV	438
D-Bcdo	PTN.KQATAG	E.S.KRDAKR	GRYEENLVN	LVTMEGSQAE	AFQGTNGIQL	487
H-RPE65	-FS.PRQAF.	..F.QI...--	QKYC..P.T.	AYGL.LNHF-	V.DRLC.LNV	452
<b>D</b>						
Human	LTKSSSLKWRE	DDCWPAEPLF	VPAP----GA	KDEDDGVILS	AIVSTDP-QK	485
Mouse	.....S.	ES.....T.	-----D.	RE..E..V.T	CV.VSE.-N.	485
Chicken	Q..EV.H.G.	.H...S...I.	...S.---D.	RE..E..V.T	CV.VSE.-N.	483
Drosopholia	RPEMLCD.GC	ETPRIYYERY	MGKNYRYFY.	ISS.VDAVNP	GTLIKVDVWN	537
Human RPE65	K..ETWV.Q.	P.SY.S...I.	.SH.---D.	LE.....V..	VV..PGAG..	498
H-BCDO	LPFLILIDAK	SFTELARASV	DVDMHMDLHG	LFITDMWDWT	KKQ--AASE-	532
M-Bcdo	.....	.....	.A...L....	...P.A..NA	V.--TPA.T	533
C-Bcdo	A.....	T.K..G...T.	N.E..L....	M..PQN.LGA	ETE-----	526
D-Bcdo	KSC.TWCEEN	VYPSEPIFVP	SP.PKSEDD.	VILAS.VLGG	LNDRYVGLIV	587
H-RPE65	PAY....N..	DLS.V...E.	EINIPVTF..	..-----	..S-----	533
H-BCDO	---EQRDRAS	D-----	-----CHGA	PLT		547
M-Bcdo	QEV.NS.HPT	.PTAPELSHS	ENDFTAG..G	SSL		566
C-Bcdo	-----	-----	-----	---		526
D-Bcdo	LCAKTMTELG	RCDFHTNGPV	PKCLHGWFAP	NAI		620
H-RPE65	-----	-----	-----	---		533

FIG. 1—Continued

italism (Beales *et al.*, 1997; Bruford *et al.*, 1997; Kwitek-Black *et al.*, 1993). Whether *BCDO* mutations are associated with some cases of Bardet-Biedl syndrome is currently under investigation.

*Human BCDO mRNA Is Highly Expressed in the RPE*

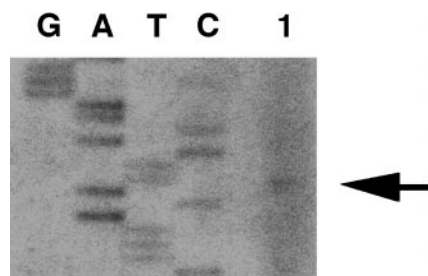
Northern analysis indicates that *BCDO* is highly expressed in human RPE as a 2.4-kb transcript (Fig. 3A), which is 0.7 kb shorter than the chicken *Bcdo* transcript. *BCDO* was not detected in retina, liver, brain, or testis in our human total RNA blot (Fig. 3A) nor in heart, brain, placenta, lung, liver, skeletal muscle, kidney, or pancreas in a Clontech human poly(A)<sup>+</sup>

RNA MTN blot (data not shown). To analyze further the expression pattern of human *BCDO*, RT-PCR was carried out with total RNAs from a variety of human tissues (Fig. 3B). Consistent with the Northern result, human *BCDO* is preferentially expressed in the RPE, but also expressed at lower levels in kidney, testis, liver, and brain. Since it is reported that chicken *Bcdo* is expressed primarily in duodenum, we also analyzed the expression of human *BCDO* mRNA in small intestine by RT-PCR. Although our RT-PCR is not quantitative, the level of *BCDO* in intestine seems similar to that in kidney and lower than that in RPE (Fig. 3B). We also performed RT-PCR for human *RPE65* and found that its expression pattern is more restricted

TABLE 1  
Exon/intron Boundaries of the Human *BCDO* Gene

Exon	Exon size (bp)	Intron	Intron size (kb)	5' donor	3' acceptor
1	282	A	0.63	TGACAGgtgagc	tctcagGCAAGA
2	129	B	1.4	GAGACCgtgaga	ttgcagGTGAAG
3	130	C	2.4	TTCCAAGtact	ccccagAGCTTT
4	148	D	2.4	GAGAAGgtatca	ttgcagGTTGAT
5	148	E	2.8	TACCAAGgtagc	ttgcagAGGGCA
6	224	F	2.2	GAGAAGgtgagg	tttcagACTTAT
7	258	G	1.1	GACAAGgtaatg	tttcagAATGCA
8	106	H	2.0	ATGAAGgtaaaa	ttgtagGCTTAG
9	95	I	0.68	ACCAAGgtaactg	gtacagATAATA
10	112	J	2.7	ATGACGgtaaga	ctaaagGAGTAA
11	795				

Note. The exon sequences are shown in uppercase letters, and the intron sequences are displayed in lowercase letters. The gt-ag consensus sequences in the splicing sites are shown in boldface type.



**FIG. 2.** Primer extension analysis of the *Bcdo* transcription start site. The arrow indicates the position of the band in lane 1 corresponding to the major transcription start site, at 216 bp upstream of the initiation methionine. Sequencing lanes that were used for orientation are shown.

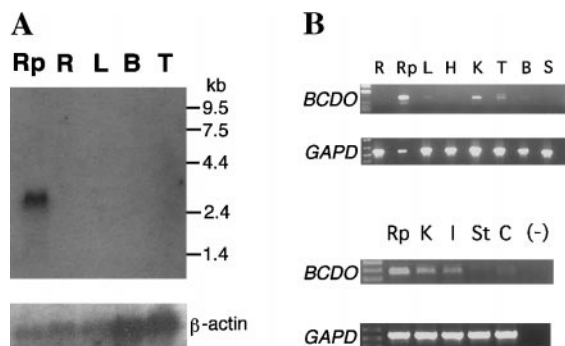
than that for *BCDO* (data not shown), consistent with the fact that so far *RPE65* expression outside the RPE has been reported only in transformed kidney cells and cone photoreceptors (Ma *et al.*, 1998, 1999).

#### Bovine RPE Expresses Bcdo Protein

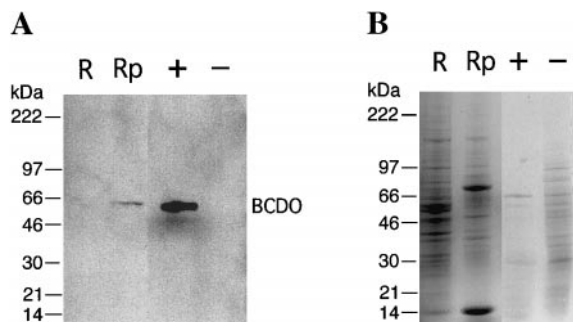
To confirm at the protein level that Bcdo is expressed in the RPE, we generated a polyclonal anti-Bcdo antibody. The antibody was raised against a Bcdo synthetic peptide containing a sequence that is 100% conserved between human and mouse Bcdo and is minimally homologous to RPE65 (see Fig. 1). As a positive control, the antibody detected a 65-kDa band in total cell extract from BCDO expressing Sf9 cells (see below), but not in an extract from Sf9 cells infected with an empty bacmid vector (Fig. 4). As expected, a band with similar mobility was detected with bovine RPE extract. No immunoreactivity was seen with bovine retinal extract.

#### Human BCDO Has Dioxygenase Activity

To study the biochemical characteristics of human BCDO, the enzyme was expressed using a baculovirus



**FIG. 3.** Human *BCDO* gene is highly expressed in the RPE. (A) Northern analysis of human total RNA probed with labeled human *BCDO* cDNA. Lanes: **Rp**, RPE; **R**, retina; **L**, liver; **B**, brain; and **T**, testis.  $\beta$ -actin probe (**bottom**) was used to control for the amount of RNA loaded. (B) Expression of human *BCDO* gene analyzed by RT-PCR. The *BCDO* primers yielded a 364-bp fragment, and the control *GAPD* primers yielded a 556-bp fragment. Lanes: **R**, retina; **Rp**, RPE; **L**, liver; **H**, heart; **K**, kidney; **T**, testis; **B**, brain; **S**, spleen; **I**, small intestine; **St**, stomach; **C**, colon; and (-), negative control.

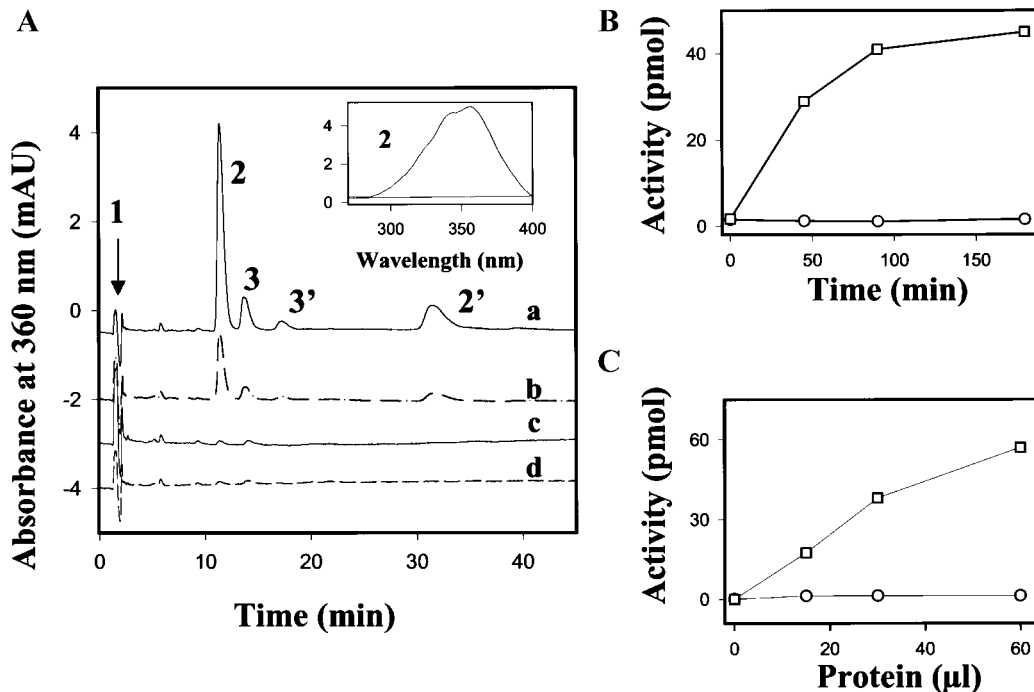


**FIG. 4.** BCDO protein is expressed by bovine RPE. (A) Immunoblot analysis of Bcdo expression. Ten micrograms of protein from bovine retina ("R") and RPE ("Rp") was loaded in the indicated lanes. The apparent molecular mass of the band in the RPE lane representing Bcdo is approximately 65 kDa, and its position is indicated on the right side of the panel. As positive and negative controls, respectively, Sf9 cell extract (0.2  $\mu$ g protein total) from cells infected with BCDO expressing baculovirus ("+") and baculovirus without insert ("-") were also run. (B) Coomassie blue stained protein gel containing the same samples as in "A," except that 2  $\mu$ g Sf9 extract was loaded in lane "+."

system. The majority (>75%) of BCDO activity was extracted without detergent, suggesting that the enzyme is a soluble protein. To improve delivery of  $\beta$ , $\beta$ -carotene, extracts were supplemented with Triton X-100. The crude 0.1% Triton X-100 soluble fractions were then tested for BCDO activity using a HPLC assay. Extracts from cells containing the BCDO recombinant baculovirus (Fig. 5A, a), but not from control cells containing the empty bacmid (Fig. 5A, d), demonstrated conversion of the  $\beta$ , $\beta$ -carotene substrate into a major peak representing (*syn*) all-*trans*-retinal oxime and minor peaks representing (*anti*) all-*trans*-retinal oxime, (*syn*) 13-*cis*-retinal oxime, and (*anti*) 13-*cis*-retinal oxime. The retinoids were identified by their characteristic absorption spectra and elution times of authentic standards. For example, the absorption spectrum of peak 2 (Fig. 5A, inset) is consistent with all-*trans*-retinal, and peak 3 represented 13-*cis*-retinal oximes (data not shown). The activity was significantly reduced by the addition of EDTA (Fig. 5A, b) and eliminated by prior boiling of the extract (Fig. 5A, c). As expected, the enzyme activity was also time- (Fig. 5B) and concentration-dependent (Fig. 5C).

The formation of small amounts of 13-*cis*-retinal is consistent with an equilibrium between all-*trans*-retinal and 13-*cis*-retinal. The formation of the equilibrium is accelerated by the presence of membranes, and standard all-*trans*-retinal was converted to 13-*cis*-retinal even in the presence of boiled membranes. Similarly, the formation of 13-*cis*-retinal has been observed in similar assays carried out with the *Drosophila* enzyme (von Lintig and Vogt, 2000a).

The substrate specificity of BCDO was explored using other carotenoids and found to be specific toward  $\beta$ -carotene. No detectable activity was observed with the  $\beta$ , $\beta$ -carotene-related compounds lutein (hydroxylated form) or lycopene, using the same HPLC assay under similar conditions as used for  $\beta$ , $\beta$ -carotene (data



**FIG. 5.** BCDO protein has  $\beta,\beta$ -carotene 15,15'-dioxygenase activity. (A) The assay and HPLC conditions were as described under Materials and Methods. Numbered peaks represent: 1,  $\beta,\beta$ -carotene; 2, (*syn*) all-*trans*-retinal oxime; 2', (*anti*) all-*trans*-retinal oxime; 3, (*syn*) 13-*cis*-retinal oxime; and 3', (*anti*) 13-*cis*-retinal oxime. (Inset) UV spectrum of peak 2. Triton X-100 (0.1%) solubilized extract (66  $\mu$ g protein) was assayed for  $\beta,\beta$ -carotene 15,15'-dioxygenase at 37°C for 70 min with 10  $\mu$ M FeSO<sub>4</sub>, 10 mM ascorbic acid, and 40  $\mu$ M  $\beta,\beta$ -carotene (a) and in the presence of 5 mM EDTA (b). The same assays were also performed after boiling (c) and with 0.1% Triton X-100-soluble fraction from control cells infected with an empty bacmid (d). (B) The recombinant 0.1% Triton X-100-solubilized  $\beta,\beta$ -carotene 15,15'-dioxygenase (66  $\mu$ g protein, top trace) and control (empty bacmid) (108  $\mu$ g protein, bottom trace) extracts were assayed at 37°C for the indicated times with 10  $\mu$ M FeSO<sub>4</sub>, 10 mM ascorbic acid, and 40  $\mu$ M  $\beta,\beta$ -carotene. Enzymatic activity was expressed as picomoles of (*syn*) all-*trans*-retinal oxime produced. (C) Increasing amounts of recombinant 0.1% Triton X-100-solubilized  $\beta,\beta$ -carotene-15,15'-dioxygenase (2.2  $\mu$ g protein/ $\mu$ l, top trace) and control (empty bacmid) (3.6  $\mu$ g protein/ $\mu$ l, bottom trace) extracts were assayed at 37°C for 65 min with 10  $\mu$ M FeSO<sub>4</sub>, 10 mM ascorbic acid, 1 mM DTT, and 40  $\mu$ M  $\beta,\beta$ -carotene.

not shown). These results are similar to those observed with the *Drosophila* enzyme (von Lintig and Vogt, 2000a) and with crude preparations of rat liver and intestine (Nagao and Olson, 1994).

## DISCUSSION

Since mammals cannot synthesize vitamin A or other retinoids *de novo*, the major source of new retinoids is dietary intake of plant-derived C<sub>40</sub> carotenoids, with subsequent oxidative cleavage. Although the ability of certain mammalian tissues to convert  $\beta,\beta$ -carotene into vitamin A has been known for well over 50 years, its mechanism has been controversial, with an active debate about whether the cleavage was central or eccentric (Sivakumar, 1998). Recently, however, there were significant developments in this field with the first cloning of invertebrate (*Drosophila*) (von Lintig and Vogt, 2000a) and vertebrate (chicken) (Wyss *et al.*, 2000) Bcdos and demonstration that the *Drosophila* enzyme works via a central cleavage mechanism.

In this paper, we describe the first cloning of a mammalian *Bcdo*. Based on their high level of sequence and biochemical similarity with chicken *Bcdo* (Wyss *et al.*,

2000), we suggest that the human and murine genes are orthologues of the chicken gene and probably also orthologues of the *Drosophila* gene (von Lintig and Vogt, 2000a). Like chicken and *Drosophila* Bcdo, human BCDO uses a central cleavage mechanism at the 15,15' double bond, to produce two molecules of retinal from each molecule of  $\beta,\beta$ -carotene, and is highly restricted in terms of substrate specificity. Also, like chicken and *Drosophila* Bcdo, the mammalian enzyme is soluble rather than membrane-associated, as shown by the solubility of the baculovirus expressed human BCDO as well as by immunoblot analysis of membrane-associated and soluble fractions of bovine RPE (data not shown).

Interestingly, the homology between human BCDO and human RPE65 is at least as high as, and probably higher than, that between human BCDO and *Drosophila* Bcdo, suggesting the possibility that RPE65 might have Bcdo activity. However, direct testing of *in vitro* expressed RPE65 protein failed to identify any such activity (von Lintig and Vogt, 2000a). Perhaps related to this lack of Bcdo activity, there is a central asparagine in the sequence of region A that is conserved in all homologous dioxygenases that is changed to a cysteine in all known RPE65s (Fig. 1).

Although the function of Bcdo is at this point unclear, we suggest that it may, in part, act as a component of a local supply pathway to supplement the visual cycle in providing 11-*cis*-retinal to photoreceptors. Such a mechanism would be particularly important under bright-light conditions when the ability of the visual cycle to provide sufficient substrate might be limited. As a precedent demonstrating that some retinoid metabolizing enzymes might be important mainly in times of stress, 11-*cis*-retinol dehydrogenase null mice appeared to have normal dark adaptation kinetics under typical light conditions, but when subjected to a strong bleach they demonstrate significantly delayed dark adaptation kinetics (Driessen *et al.*, 2000). Perhaps also of relevance to the situation with Bcdo, 11-*cis*-retinol dehydrogenase is expressed in a number of tissues outside the eye, but no extraocular phenotypes have been reported in the knockout mice.

The human *BCDO* gene maps to chromosome 16q21–q23, which is near the recessive BBS2 locus for Bardet–Biedl syndrome (Beales *et al.*, 1997; Bruford *et al.*, 1997; Kwitek-Black *et al.*, 1993). *BCDO* is a reasonable candidate gene for BBS, not only because of its map location, but also because several of the characteristics of the syndrome, such as retinal degeneration and polydactyly, could be related to abnormalities of the retinoid metabolism. In addition, *BCDO* could be considered as a candidate gene for a number of other retinal diseases both because of the earlier mentioned association with the *Drosophila ninaB* phenotype (von Lintig *et al.*, 2000b) and because many retinal degenerations have been associated with mutations in genes involved in the visual cycle and retinoid metabolism and transport. For example, mutations in ABCR have been associated with Stargardt's disease (Allikmets *et al.*, 1997b) and possibly, but controversially, with age-related macular degeneration (Allikmets, 2000; Allikmets *et al.*, 1997a; Dryja *et al.*, 1998; Stone *et al.*, 1998); mice null for interphotoreceptor retinoid binding protein (IRBP) develop an early retinal degeneration (Liou *et al.*, 1998) (although surprisingly the visual cycle appears to function fairly normally in the absence of IRBP) (Palczewski *et al.*, 1999; Ripps *et al.*, 2000); mutations in 11-*cis*-retinol dehydrogenase have been reported in patients with fundus albipunctatus (Gonzalez-Fernandez *et al.*, 1999; Yamamoto *et al.*, 1999); mutations in CRALBP have been reported in cases of autosomal recessive retinitis pigmentosa (Maw *et al.*, 1997) and retinitis punctata albescens (Burstedt *et al.*, 1999; Morimura *et al.*, 1999); mutations in RPE65 cause a childhood retinal degeneration (Gu *et al.*, 1997; Marlhens *et al.*, 1997); and mutations in serum retinol-binding protein are associated with night blindness and RPE atrophy (Biesalski *et al.*, 1999; Seeliger *et al.*, 1999). These last two findings are potentially the most relevant to *BCDO* because of its homology to RPE65 and because, even though serum retinol-binding protein is widely expressed outside the eye, the clinical phenotype almost exclusively affects

the eye. Also of potential interest, carotenoids have been implicated in age-related macular degeneration (Seddon *et al.*, 1994).

The cloning of the human *BCDO* gene allows direct testing of whether mutations in this gene are associated with retinal diseases or other diseases. Availability of the mouse gene makes analysis of Bcdo function possible through generation of null mice. In addition, the ability to express the protein *in vitro* will allow detailed studies of enzyme kinetics, mechanism, and structure–function relationships to be performed.

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