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Tissue-specific Expression in Transgenic Mice Directed by the 5′-Flanking Sequences of the Human Gene Encoding Interphotoreceptor Retinoid-binding Protein*

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Interphotoreceptor retinoid-binding protein (IRBP) is an extracellular protein that has been suggested to participate in the visual process as a carrier for visual retinoids. A chimeric gene composed of the human IRBP promoter fused to the bacterial reporter gene chloramphenicol acetyltransferase (CAT) was used to generate transgenic mice. Analysis of six transgenic families revealed that the CAT gene, concomitant with the endogenous IRBP gene, was expressed primarily in the retina and, to a lesser extent, in the pineal gland. These results establish that a 1.3-kilobase fragment from the 5′ end of the human IRBP gene is sufficient to direct transgene expression to a visual subdivision of the central nervous system.

IRBP[1] is a fatty acid-conjugated glycoprotein (1) that is expressed at high levels in vertebrate retinal photoreceptor cells (2) and, to a lesser degree, in pinealocytes (3). In the retina, IRBP is synthesized by the photoreceptor cells and secreted into the interphotoreceptor matrix where it binds retinoids. IRBP has thus been suggested to play a role in the visual process (reviewed in Refs. 4 and 5). In the developing mouse retina, IRBP is expressed when inner segments of the photoreceptors start to differentiate (6). Expression of IRBP is greatly reduced and its secretion impaired in mice with an inherited retinal degeneration *(rd)* (7). IRBP may also be reduced in humans with an autosomal dominant form of retinitis pigmentosa (8). Our long range goals are to understand how the IRBP gene is regulated during retinal development and in differentiated photoreceptor cells, and how retinal degenerations may be related to IRBP gene expression. As a first step toward these goals, we report here the use of gene transfer experiments in mice to identify a transcriptional regulatory region of the human IRBP gene.

EXPERIMENTAL PROCEDURES

Materials—The plasmid pTZCAT, MCAT, an extract of cultured embryonic chick primary myoblasts transfected with a CAT gene promoted by a chicken skeletal muscle actin gene promoter, and β-actin gene were gifts from K. L. Chow and Dr. R. J. Schwartz (Department of Cell Biology, Baylor College of Medicine). [^{14}C] Chloramphenicol (57 mCi/mmol) was obtained from Amersham International. Acetyl coenzyme A (lithium salt) was obtained from Pharmacia LKB Biotechnology Inc. Calf intestine alkaline phosphatase was from Boehringer Mannheim. Restriction enzymes, linkers, and RNA sizing standard (RNA Ladder) were from Bethesda Research Laboratories. Dye reagent used for protein assay and the nucleic acid blotting membranes (Zeta-Probe) were obtained from Bio-Rad.

Plasmid Construction—The 5′-flanking fragment of human IRBP gene S3 (9) was digetsed with *Pvu*II and the generated fragment of 1329 bp was ligated to *Hin*dIII linkers. This was then ligated to the 5′ end of a CAT gene (10) contained in the promoterless plasmid pTZCAT (11) to form pTZ-IRBPCAT.

Generation of Transgenic Mice—The procedure used to generate transgenic mice[2] in this study have been described (12). Single-cell stage FVB/N embryos were injected with the *Bst*UI fragment (Fig. 2) of IRBP-CAT at a concentration of 1 μg/ml in 10 mM Tris, 0.1 mM EDTA, pH 8.0. Transgenic mice were identified by isolation of tail DNA followed by dot blot and/or Southern hybridization to ^{32}P-labeled IRBP-CAT construct. A total of 250 FVB/N embryos were injected, yielding 40 newborns, 6 of which were transgenic.[3] All six F_0 mice were mated to C57BL/6J mice to generage F_1 offspring. FVB/N mice have a recessive mutation at the *rd* locus. C57BL/6J mice and C57BL/6J × FVB/N F_1 hybrids have normal retinas.

CAT Assays—CAT activities were assayed essentially according to Grichnik *et al.* (11), and the procedures are described as follows. Tissues were homogenized at 4 °C in Tris-HCl buffer (0.25 M, pH 7.8) with an OMNI Tissuemizer, heated to 65 °C for 6 min to inactive endogenous acetylases (12), and spun in a refrigerated microcentrifuge for 10 min at 15,000 rpm. The supernatants were assayed for CAT activity by incubation for 90 min at 37 °C with 0.5 μCi of [^{14}C] chloramphenicol (50–60 mCi/mmol, Amersham Corp.) and 0.5 mM acetyl-CoA (Pharmacia) in 0.25 M Tris-HCl buffer, pH 7.8. At 45 min, additional acetyl-CoA was added to make its final concentration 1 mM. Assays were stopped by extraction with ethyl acetate and were analyzed by ascending thin-layer chromatography (Kodak chromagram sheet) in chloroform:methanol (95:5) followed by autoradiography. Protein concentrations were determined with a dye-reagent (Bio-Rad).

Northern Blot Analysis—Hybridizations were at 42 °C in 50%

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) J05469.

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[1] The abbreviations used are: IRBP, interphotoreceptor retinoid-binding protein; CAT, chloramphenicol acetyltransferase; bp, base pairs); kb, kilobase(s); PE, retinal pigment epithelium; PECS, retinal pigment epithelium-choroid-sclera.

[2] All animals used were handled in accordance with the guidelines established by the Animal Protocol Review Committee at Baylor College of Medicine.

[3] The six families have been named 101 to 106 with full designation as: Tg (hg-IRBP, CAT, SV40) OVE101–OVE106.

formamide and 1 × BLOTTO (13) (0.36 M NaCl, 20 mM sodium phosphate, 2 mM EDTA, 2% sodium dodecyl sulfate, and 0.5% nonfat dry milk, pH 7.7). Final washes were in 0.5 × SSC (75 mM NaCl, 7.5 mM sodium citrate, pH 7.0) at 65 °C. Hybridization membranes were exposed to XAR-5 film (Kodak) with intensification screens at −80 °C.

RESULTS AND DISCUSSION

The structure of the human IRBP gene and its 5′-flanking region, IRBP-S3, was reported previously (9). As observed for many other eucaryotic genes (14), this fragment lacks TATA and CAAT boxes within conventional distances upstream of the transcription start site (9), and the putative promoter region appears to be GC-rich. To identify sequences in the IRBP-S3 that regulate transcription and confer tissue specificity, we chose a 1329-bp *Pvu*II fragment overlapping the transcription start site (Fig. 1) to be linked to the coding sequences of the bacterial CAT gene (Fig. 2). Transgenic mice were generated by microinjection of the fusion gene into one-cell stage FVB/N embryos (12). Six independent transgenic

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GCTGCCTACTGAGGCACACAGGGGCGCCTGCCTGCTGCCCGCTCAGCCAAGGCGGTGTTG -1247
CTGGAGCCAGCTTGGGACAGCTCTCCCAACGCTCTGCCCTGGCCTTGCGACCCACTCTCT
GGGCCGTAGTTGTCTGTCTGTTAAGTGAGGAAAGTGCCCATCTCCAGAGGCATTCAGCGG
CAAAGCAGGGCTTCCAGGTTCCGACCCCATAGCAGGACTTCTTGGATTTCTACAGCCAGT
CAGTTGCAAGCAGCACCCATATTATTTCTATAAGAAGTGGCAGGAGCTGGATCTGAAGAG
TCAGCAGTCTACCTTTCCCTGTTTCTTGTGCTTTATGCAGTCAGGAGGAATGATCTGGAT  -947
TCCATGTGAAGCCTGGGACCACGGAGACCCAAGACTTCCTGCTTGATTCTCCCTGCGAAC
TGCAGGCTGTGGGCTGAGCCTTCAAGAAGCAGGAGTCCCCTCTAGCCATTAACTCTCAGA
GCTAACCTCATTTGAATGGGAACACTAGTCCTGTGATGTCTGGAAGGTGGGCGCCTCTAC
ACTCCACACCCTACATGGTGGTCCAGACACATCATTCCCAGCATTAGAAAGCTCTAGGGG
GACCCGTTCTGTTCCCTGAGGCATTAAAGGGACATAGAAATAAATCTCAAGCTCTGAGGC  -647
TGATGCCAGCCTCAGACTCAGCCTCTGCACTGTATGGGCCAATTGTAGCCCCAAGGACTT
CTTCTTGCTGCACCCCCTATCTGTCCACACCTAAAACGATGGGCTTCTATTAGTTACAGA
ACTCTCTGGCCTGTTTTGTTTTGCTTTGCTTTGTTTTGTTTTGTTTTTTGTTTTTTTGT
TTTTTAGCTATGAAACAGAGGTAATATCTAATACAGATAACTTACCAGTAATGAGTGCTT
CCTACTTACTGGGTACTGGGAAGAAGTGCTTTACACATATTTTCTCATTTAATCTACACA  -347
ATAAGTAATTAAGACATTTCCCTGAGGCCACGGGAGAGACAGTGGCAGAACAGTTCTCCA
AGGAGGACTTGCAAGTTAATAACTGGACTTTGCAAGGCTCTGGTGGAAACTGTCAGCTTG
TAAAGGATGGAGCACAGTGTCTGGCATGTAGCAGGAACTAAAATAATGGCAGTGATTAAT
GTTATGATATGCAGACACAACACAGCAAGATAAGATGCAATGTACCTTCTGGGTCAAACC
ACCCTGGCCACTCCTCCCCGATACCCAGGGTTGATGTGCTTGAATTAGACAGGATTAAAG  -47
GCTTACTGGAGCTGGAAGCCTTGCCCCAACTCAGGAGTTTAGCCCCAGACCTTCTGTCCA   14
                                    +1
CCAGC
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FIG. 1. **Nucleotide sequence of the *Pvu*II fragment of IRBP-S3, the 5′-flanking region of the human IRBP gene.** Numbers on the *right* indicate nucleotide positions starting with the transcription start site as position 1.

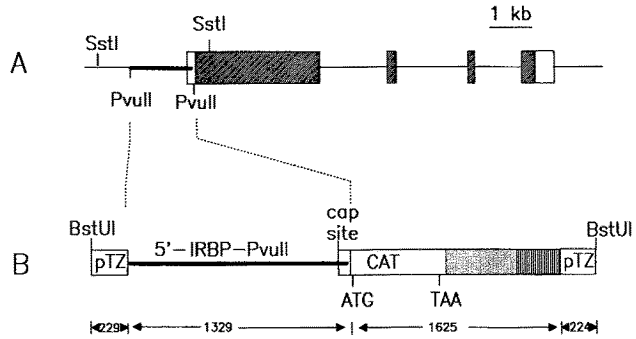

FIG. 2. **Human IRBP gene and construction of the IRBP-CAT fusion gene.** *A*, the distribution of exons (*boxes*), introns (*lines*), and flanking regions of the human IRBP gene (9). The *hatched boxes* represent the protein coding regions. *B*, IRBP-CAT fusion gene. A 1329-bp *Pvu*II fragment containing IRBP sequences from −1311 to +19 bp was fused with *Hin*dIII linkers to the 5′ end of a CAT gene (10) in the promoterless CAT plasmid pTZCAT (11). The CAT gene (1625 bp) consists of the CAT coding and noncoding sequences, an SV40 fragment containing the small t-antigen intron (*stippled box*), and an SV40 fragment containing the early region polyadenylation site (*box with vertical lines*) (10). The 3407-bp *Bst*UI restriction fragment was used for microinjections.

TABLE I

The copy number, transmission rate, and CAT activity in the transgenic mouse families

Copy numbers in F_1 animals were determined by dot blot hybridization to ^{32}P-labeled IRBP-CAT construct and scintillation counting of positive dots. Copy number per haploid genome was estimated by comparison to standards containing known amounts of IRBP-CAT DNA mixed with nontransgenic mouse DNA. Percent transmission was determined by screening the offspring of F_0 × wild type matings by means of dot blot and/or Southern hybridizations to tail DNA. CAT activities were assayed as described (11), except that the incubation time was 30 min and the amount of soluble protein per assay was adjusted to ensure a linear range of acetylation (40% or lower). Radioactive spots were excised and counted. One unit of CAT activity was defined as the amount of enzymatic activity catalyzing acetylation of 1 μmol of chloramphenicol/min at 37 °C (16). PECS, retinal pigment epithelium accompanied by choroid and sclera. NT, not tested.

Family	Copy number	Transmission		CAT activity			
		Percent	Number screened	Neuro-retina	PECS	Pineal	Cerebrum
				milliunits/mg protein			
101	30	53	19	1350	33	NT	0.2
102	3	35	17	4	0.3	NT	<0.01
103	20	53	53	674	25	NT	0.04
104	5	22	9	700	17	75	<0.01
105	100	22	32	200	93	14	NT
106	30	14	37	933	14	103	<0.01

founder (F_0) animals, five females and one male, were identified by Southern hybridization (data not shown). Two of the F_0 mice transmitted the IRBP-CAT sequences to approximately 50% of their offspring. The remaining four F_0 mice had lower transmission rates (14 to 35%), suggesting that these mice were mosaic. The copy numbers of the integrated DNA ranged from 3 to about 100 in the F_1 mice (Table I).

F_1 mice from all six families were screened to determine the tissue specificity of the IRBP-CAT gene expression (Fig. 3). Retinas were separated into two components, the neuro-retina, and retinal pigment epithelium (PE) accompanied by choroid-sclera (PECS). The retinal PE cells and photoreceptor outer segments interdigitate (15) rendering their quantitative separation difficult. In all six families, high levels of CAT activity were present in both neuro-retina and PECS. Much lower activities were detected in the brains (Fig. 3*A*). In order to evaluate the pattern of CAT activity in the brain, cerebrum, cerebellum, and pineal gland of three families were dissected. CAT activity was found in the pineal glands of all three families (Fig. 3*B*). Very little CAT activity was found in the cerebellum or cerebral cortex in these families. CAT activity was not detected in other tissues (Fig. 3*C*). These results indicate that the human IRBP promoter functions appropriately in transgenic mice and directs gene expression specifically to the mouse tissues that contain endogenous IRBP.

To compare the levels of CAT activity in neuro-retina, PECS, pineal gland, and cerebrum, quantitative enzyme assays were done on one F_1 mouse in each family (Table I). The neuro-retinas showed the highest levels of CAT activity in all six families. Purified CAT enzyme from *Escherichia coli* has a specific activity of about 100 units/mg (16). Assuming that the intrinsic activity of the enzyme is unmodified in the retina, the specific activity of 1.3 units/mg in the retina of family 101 indicates that the CAT protein constitutes about 1% of the soluble protein in the retina. CAT activities measured in the pineal glands of the three families tested were around 10%

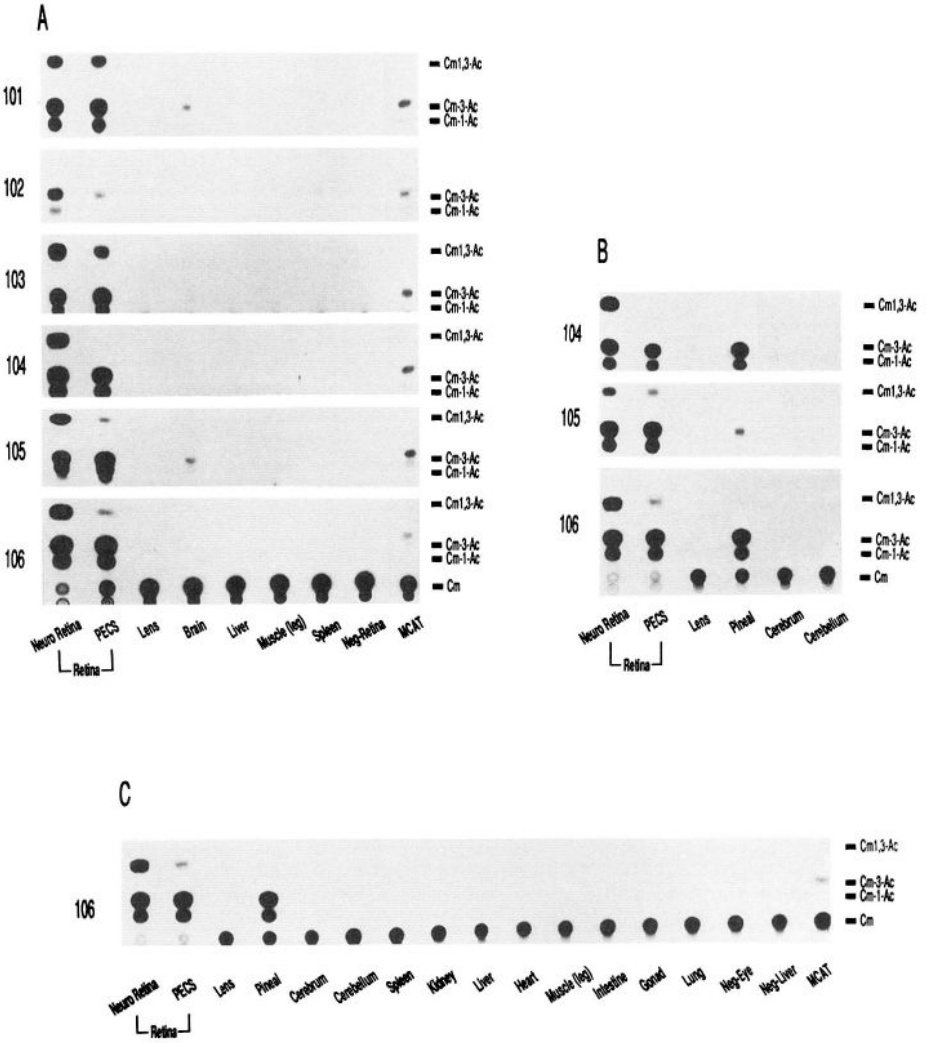

FIG. 3. **CAT activities.** Various tissues of F_1 mice were assayed as described earlier (11). Assay conditions for the retinal tissues are nonlinear. Five μg of soluble protein were used in each assay, except for pineal, for which only 1.5 μg of protein was available. The unacetylated (*Cm*), monoacetylated (*Cm-1-Ac* and *Cm-3-Ac*), and diacetylated (*Cm-1,3-Ac*) forms of chloramphenicol are shown for family 106. Only the mono and diacetylated forms of chloramphenicol are shown for the other families. PECS, retinal pigment epithelium accompanied by choroid and sclera. *A*, CAT activities in a variety of tissues for the six families. In these assays whole brain homogenates were used. *Neg-Retina*, retinas from a nontransgenic littermate. *MCAT*, an extract of cultured embryonic chick primary myoblasts transfected with a CAT gene promoted by a chicken skeletal muscle actin gene promoter (a gift from K. L. Chow). *B*, CAT activities in the ocular and brain tissues of families 104, 105, and 106. In these families the pineal gland, cerebral cortex, and cerebellum were isolated by dissection. *C*, CAT assays were repeated on family 106 to include other tissues that were not assayed in *A* or *B*. *Neg-Eye* and *Neg-Liver*, tissue homogenates from a nontransgenic littermate.

of the activities in the neuro-retinas. CAT activities in the cerebral cortexes were near background levels.

IRBP-CAT expression in the pineal gland is not surprising. It is generally accepted that a major population of mammalian pineaolocytes evolved from pineal photoreceptor cells, which are photosensitive in lower vertebrates (17). During evolution, these pineal photoreceptor cells appear to have been progressively transformed into endocrine secretory cells. However, mammalian pinealocytes and retinal photoreceptors still share a number of common proteins, including IRBP, S-antigen, or 48-kDa protein, rhodopsin kinase, and opsin (reviewed in Ref. 3). It is thought that in higher vertebrates the pineal gland may play a role in the regulation of circadian rhythms (18).

The presence of CAT activity in the PECS was unexpected, since the endogenous IRBP gene is expressed specifically in the photoreceptor cells of the neuro-retina (2). To determine whether the IRBP-CAT gene is transcribed in both neuro-retina and retinal PECS, poly(A^+) RNAs were isolated from these tissues of an F_1 mouse of family 101 and analyzed by Northern hybridization (Fig. 4*A*). Transcripts encoding IRBP (5.7 kb, Ref. 5), opsin (transcripts of 1.75–4.9 kb, Ref. 19) (data not shown) and the CAT transgene (1.9 kb) were detected exclusively in the neuroretina. Overexposure of the autoradiograph shown in Fig. 3*A* failed to show any trace of a CAT transcript in the PECS (data not shown). The CAT activity that is detected in the PECS may therefore be the consequence of the normal phagocytosis of photoreceptor outer segments by the retinal PE cells (15). Alternatively, the CAT activity in the PECS may be attributed to incomplete separation of PECS from photoreceptor outer segments which may contain significant amounts of CAT enzyme.

To determine whether the integration of the IRBP-CAT construct altered the endogenous IRBP transcription, retinal poly(A^+) RNAs from F_1 mice of family 101 and family 105 were analyzed. Both families have high copy numbers of the transgenic DNA (30 and 100 copies per haploid genome, respectively) (Table I). The endogenous 5.7-kb IRBP mRNA was present at similar levels in transgenic and nontransgenic mice as judged by comparison with the expression of the β-actin gene (20) (Fig. 4*B*). In addition, electroretinogram patterns in representative transgenic mice appear to be normal, an indication of normal retinal function (data not shown).

These studies using transgenic mice demonstrate that a 1.3-kb fragment from the 5′ end of the human IRBP gene is sufficient to promote tissue-specific gene expression *in vivo*. The promoter is active in the neuro-retina and the pineal gland. Based on the pattern of expression of the endogenous IRBP gene, it is likely that the IRBP promoter is active specifically in the photoreceptor cells within the neuro-retina. To our knowledge, this is the first demonstration of a promoter that can direct transgene expression to the visual

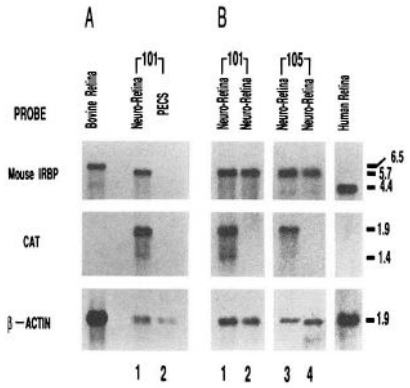

FIG. 4. **Northern analysis of poly(A$^+$) RNAs.** Isolations of mouse neuro-retina and PECS poly(A$^+$) RNA were carried out according to the method described by Badley *et al.* (21); bovine and human neuro-retina poly(A$^+$) RNA was isolated as described by Chirgwin *et al.* (22). RNA samples of 2 μg were separated on formaldehyde denaturing gels and then blotted to Zeta-Probe. Hybridizations and post-hybridization washes were carried out as described previously (13). Hybridization probes are indicated on the *left*: *Mouse IRBP*, a 722-bp mouse IRBP cDNA, corresponding to human IRBP cDNA positions 3089 to 3810 (G. I. Liou, unpublished results); *CAT*, the IRBP-CAT construct; and *β-ACTIN*, the chicken cytoplasmic β-actin cDNA (20). *A*, neuro-Retina (*lane 1*) and PECS (*lane 2*) poly(A$^+$) RNAs from a transgenic F_1 mouse of family 101. *B*, neuro-retina poly(A$^+$) RNAs from transgenic families 101 and 105; *lanes 1* and *3*, transgenic mice; *lanes 2* and *4*, nontransgenic littermates. The sizes of the hybridizing transcripts (in kilobases) were estimated by comparison to an RNA ladder. The CAT transcript is about 1.9 kb in size and may have a large poly(A$^+$) tail attached to the 1.5-kb predicted transcript, since neither a 600-bp IRBP promoter fragment upstream of the cap site nor a 400-bp fragment downstream of the polyadenylation site showed hybridization to the CAT transcript (data not shown). The bovine and human IRBP transcripts are the expected sizes of 6.5 kb (23) and 4.4 kb (5), respectively.

subdivision of the nervous system. The identification of this retina/pineal-specific promoter represents a significant advance for studies of IRBP gene regulation, retinoblastoma formation, and retinal degeneration. In addition, the promoter potentially provides a valuable tool for future transgenic studies of photoreceptor cell development, differentiation, and function.

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