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Retina-Specific Expression from the IRBP Promoter in Transgenic Mice Is Conferred by 212 bp of the 5'-Flanking Region*

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IRBP is a photoreceptor-specific glycoprotein that has been suggested as a retinoid carrier in the visual process. Previous research has shown that 1.3 kb of 5'-flanking sequence from the human IRBP gene is sufficient to promote photoreceptor-specific expression of reporter genes in transgenic mice. To define more narrowly the sequences that promote tissue-specific expression, chimeric constructs with shorter promoters were used to generate transgenic mice. The bacterial CAT gene was fused to fragments of 706 bp or 212 bp from the 5' end of the human IRBP gene. Analysis of the three transgenic families bearing the 706 bp IRBP promoter revealed that CAT expression was confined to the neuro-retina and the pineal gland. Analysis of the four transgenic families bearing the 212 bp IRBP promoter revealed the same tissue-specific CAT expression in three families. These results establish that tissue-specific expression of IRBP can be regulated by a short 212 bp promoter which has been conserved between humans and mice.

Interphotoreceptor retinoid-binding protien (IRBP) is a photoreceptor- and pinealocyte-specific glycoprotein that is implicated as a retinoid carri er in the visual process and during photoreceptor development (reviewed in Refs. 1 and 2). In the developing mouse retina,

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The abbreviations used are: IRBP, interphotoreceptor retinoid-binding protein; CAT, chloramphenicol acetyltransferase; bp, base pairs; TSS, transcription start site; PE, retinal pigment epithelium; PECS, retinal pigment epithelium-choroid-sclera.

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IRBP expression begins when inner segments of the photoreceptors start to differentiate (3,4). Expression of IRBP is greatly reduced and its secretion impaired in mice (5) and cats (6) with inherited retinal degenerations. As a first step toward understanding how the human IRBP gene is regulated, we have previously tested a 1329-bp Pvu II fragment from the 5' end of the human IRBP gene that overlapps the tran scription start site (TSS) or the cap site. The 1329 bp promoter was sufficient to direct the expression of reporter genes to the neuro-retina and the pineal gland (7), and specifically to the photoreceptor cells of the neuro-retina (8). In this report we describe experiments that used chimeric con structs containing shorter promoters in order to define more narrowly the sequences required for tissue-specific expression.

MATERIALS AND METHODS

Materials-- The plasmid pTZCAT was from K. L. Chow (Yeshiva University, New York, NY); pBluescript II KS was from Stratagene (La Jolla, CA). [^{14}C]Chloramphenical (55 mCi/mmol) was from ICN, acetyl CoA from Pharmacia LKB Biotechnology Inc, restriction enzymes from New England Biolabs and Stratagene, dye reagent for protein assay from Bio-Rad, and thin-layer chromatography sheets from Kodak.

Generation of Transgenic Mice--The plasmid pTZ-IRBP-CAT (7) was digested with Ava II or HgiA I to generate shorter versions of the human IRBP promoter (Figure 1). The fusion genes were isolated from the vector sequences by further digestion with BamH I to separate the insert and the vector followed by agarose gel electrophoresis and purification with glass powder (GENECLEAN, BIO 101). Single-cell stage FVB/N embryos were injected with the purified DNA at a concentration of 1 ug/ml in 10 mM Tris, 0.1 mM EDTA, pH 8.0 (9). Transgenic mice[1] were identified by isolation of tail DNA followed by dot blot hybridization to ^{32}P-labeled IRBP-CAT (7). A total of 164 FVB/N embryos were injected, yielding 44 newborns, three of which harbor the Ava II version IRBP-CAT construct, and four of which harbor the HgiA I version. All F_0 mice were mated to C57BL/6 mice to generate F_1 offspring.

CAT Assays--CAT activities were assayed as described previously (10).

Mouse IRBP Promoter Isolation and Sequencing --Mice were sacrificed by cervical dislocation, the retinas removed, frozen in liquid nitrogen, and stored in RNase-free microcentrifuge tubes. Total retina RNA was isolated by the extraction procedure of Chirgwin et al. (11). Polyadenylated RNA was selected by oligo (dT)-cellulose chromatography (12), and was used to make a cDNA library in Lambda ZAP II (Stratagene, La Jolla, CA).

The mouse retina cDNA library was screened with human IRBP cDNA clones H4 (American Type Culture Collection No. 59198) and H12 (13) and five overlapping mouse IRBP cDNA clones (M1-M5) were obtained. Clones M1, M3 and H12 were subsequently used to screen a mouse genomic library in Lambda Fix (from Dr. Steve J. Pittler, Baylor College of Medicine) and three overlapping clones, each with 15 to 17 kb insertions, designated as MIRBP#1, #4 and #5 were isolated. MIRBP#4, which spans the 5'-flanking and coding regions of the mouse IRBP gene, was further analyzed by subcloning and dideoxynucleotide sequencing (14-16).

RESULTS AND DISCUSSION

Construction of the Transgenes and Generation of Transgenic Mice--Our previous experiments demonstrated that a 1329-bp Pvu II fragment from the 5' end of the human

[1]All animals used were handled in accordance with the guidelines established by the Committee on Animal Use for Research and Education at Medical College of Georgia.

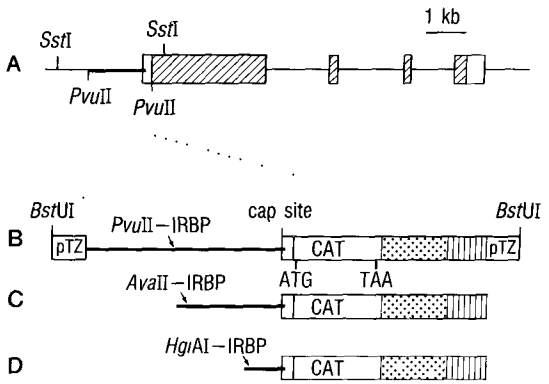

Fig. 1. Construction of the IRBP-CAT fusion genes. A, B: The structure of the human IRBP gene (13) and the construction of the initial IRBP-CAT fusion gene (7) were described previously. **C, D**: The IRBP promoter was shortened by digestion with Ava II and HgiA I. **C**, Ava II-IRBP-CAT, containing 706 bp of the 5'-flanking fragment. **D**, HgiA I-IRBP-CAT, containing 212 bp of the 5'-flanking fragment. In **A**, the boxes represent exons, unfilled boxes, non- coding and diagonal hatching, coding region of the IRBP gene. In **B-D**, the dotted and vertically lined boxes represent intronic and polyadenylation sequences from the early region of SV40, and pTZ indicates plasmid sequences.

IRBP gene overlapping TSS or the cap site (-1311 to +19 bp) was sufficient to direct the expression of reporter genes to the neuro-retina (7), specifically to the photoreceptor cells (8). In an attempt to localize the tissue-specific regulatory sequences in this long fragment, we have delet ed sequences from the 5' end of the promoter by digestion with Ava II and HgiA I. Two new fusion genes were generated: Ava II-IRBP-CAT containing 706 bp of sequences upstream from the cap site and HgiA I-IRBP-CAT with 212 bp from the cap site (Figure 1). Transgenic mice were generated by microinjection of these fusion genes into one-cell stage FVB/N embryos (9). Independent trans genic founder animals (F_0) were identified by dot blot hybridization. The three founders generated from Ava II-IRBP-CAT (Ava II-IRBP) injected embryos were designated as 4474, 4477 and 4483. Four founders were generated with HgiA I-IRBP-CAT (HgiA I-IRBP) (designated as 4487, 4491, 4500 and 4504). Transmission rates and the copy numbers of the transgenic families are given in Table 1.

CAT Expression in Transgenic Mice--F_1 mice from all families were screened to determine the tissue specificity of CAT gene expression. As in our previous experiment (7), retinas were separated into neuro-retina and retinal pigment epithelium -choroid-sclera (PECS). CAT assays were performed on various tissues for all transgenic families. CAT activity was detected in both the neuro-retina and the pineal gland in both the Ava II-IRBP and the HgiA I-IRBP families, with the HgiA I-IRBP families typically showing lower level of activity (data not shown). CAT assays on tissue extracts of one Ava II-IRBP mouse (family 4474) and one HgiA I-IRBP mouse (family 4500) are shown in Figure 2. CAT activity was

Table 1. Copy numbers, transmission rates, and specific CAT activities in the transgenic mouse families. Copy numbers in F_1 animals were determined by dot blot hybridization to ^{32}P-labeled, Ava II or HgiA I IRBP-CAT constructs, followed by scintillation counting of positive dots. Copy number per haploid genome was estimated by comparison to standards containing known amounts of the Ava II or HgiA I IRBP-CAT DNAs mixed with nontransgenic mouse DNA. Percent transmission was determined by screening the offspring of F_0 x wild type matings. CAT activities were assayed as described (10) and expressed after the background CAT activity was subtracted. The background CAT activity for each tissue was obtained by assaying the activity of tissue homogenates from a nontransgenic littermate. The amount of soluble protein per assay was adjusted so that a linear range of acetylation (40% or lower) was obtained. One unit of CAT activity was defined as the amount of enzymatic activity catalyzing acetylation of 1 umol of chloramphenicol/min at 37°C (18). ND, not determined.

Family		Transmission			CAT Activity, mU/mg			
Transgene	ID	Copy #	%	# Screened	Neuro-retina	Pineal	Cere-brum	Liver
*Hgi*AI-IRBP	4487	1	20	5	0	0	0	0
	4491	1	40	5	0.01	0.01	0	0
	4500	5	27	11	98	10	0	0
	4504	2	14	14	0.01	0.01	0	0
*Ava*II-IRBP	4474	2	50	8	702	746	0	7
	4477	ND	43	7	ND	ND	ND	ND
	4483	1	32	25	ND	ND	ND	ND

not detected in any tissues other than neuro retina and pineal gland in family 4474. CAT activity was detected only in neuro retina in family 4500 (Figure 2), but was also detected in the pineal gland by overexposing the thin layer chromatograph (data not shown). These

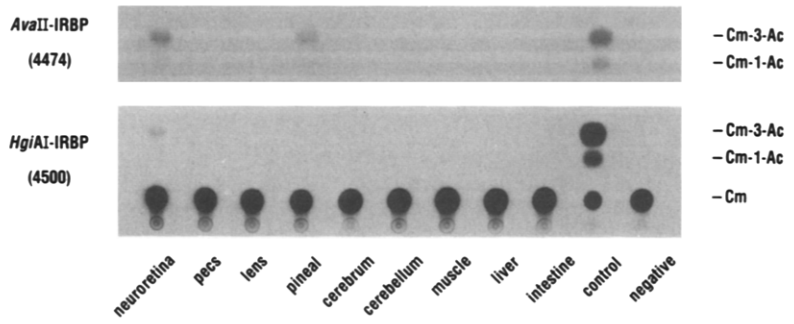

Fig. 2. CAT activities. Various tissues of F_1 mice of the Ava II- IRBP family 4474 and the HgiA I-IRBP family 4500 were assayed as described (10). Five µg of soluble protein was used in each assay, except for pineal, for which only 1.5 µg of protein was available. The unacetylate (Cm) and monoacetylated (Cm-1-Ac and Cm-3-Ac) forms of chloramphenicol are shown for family 4500. Only the monoacetylated forms are shown for family 4474.

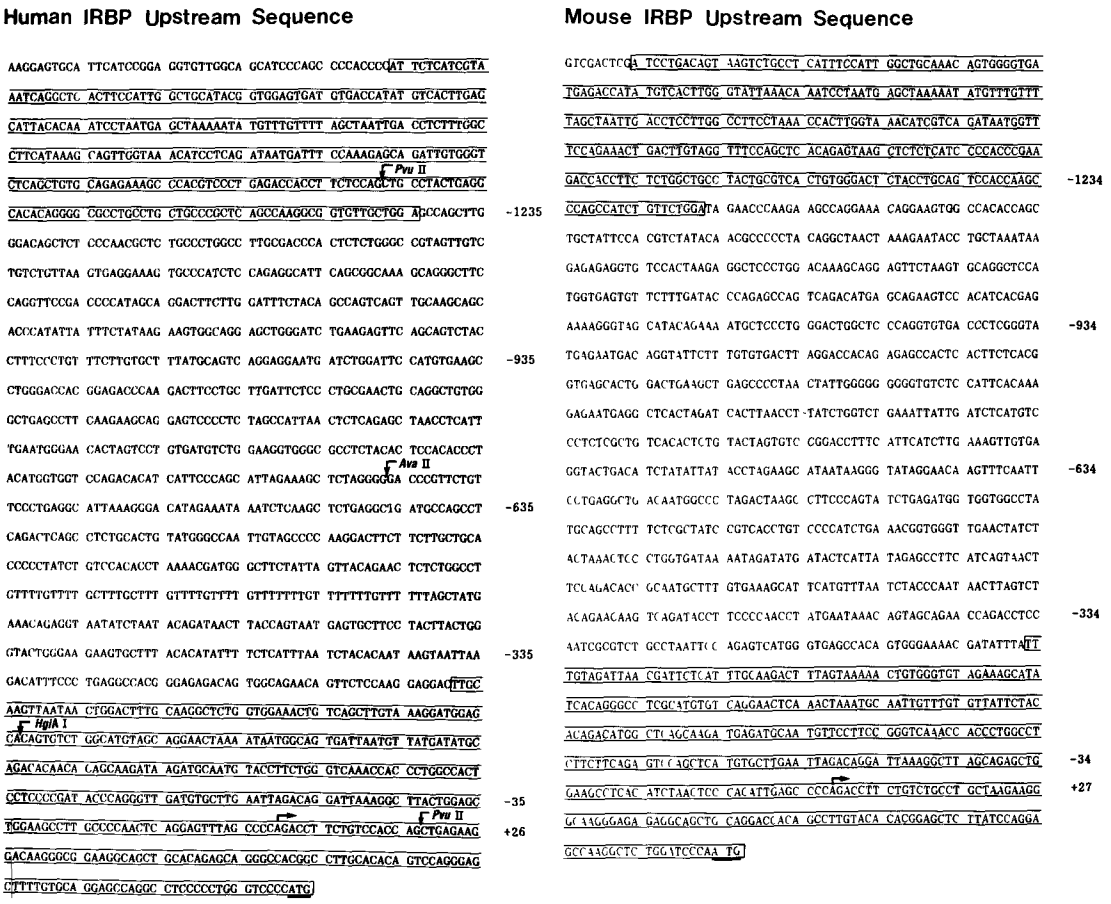

Fig. 3. DNA sequences of the upstream regions of the human and mouse IRBP genes. For the human gene the nucleotide sequence is shown from 1594 bp upstream to the protein initiation codon (13). The cleaving sites of Pvu II, Ava II, and HgiA I involved in the original transgene construct (7) and the new constructs are indicated. In mouse the sequence is shown from 1533 bp upstream to the protein initiation codon, which is underlined. The cap site is indicated by an arrow. Both the protein initiation codon and the cap site of the mouse IRBP gene were deduced by sequence comparison with the human IRBP gene. The two homologous regions are boxed in each sequence.

observations demonstrate that retina-specific regulatory elements reside within the HgiA I-IRBP construct, which contains a short 212 bp promoter from the human IRBP gene.

Quantitative CAT enzymatic assays (7) were performed to compare the levels of CAT activity in the neuro-retina, pineal gland, cerebrum and liver in the F_0 and F_1 mice of all of the HgiA I-IRBP families and one of the Ava II-IRBP families (Table 1). In the Ava II-IRBP family 4474 and three of the four HgiA I-IRBP families CAT activity was detected in the neuro-retina and in the pineal gland, although CAT activities were very low in the HgiA I-IRBP families 4491 and 4504. A low CAT activity was also detected in the liver in family 4474 (Table 1). The low levels of CAT gene expression in most of the HgiA I-IRBP

families may be due to inhibitory influences that originate in the chromosomal structure at the transgenic integration sites (17). These chromosomal influences may become stronger when shorter promoter sequences are used. Alternatively, it is possible that within the deleted upstream regions there are sequences that enhance gene expression.

Comparison of IRBP Gene Sequences--The mouse IRBP gene, including 1534 bp of the 5'-flanking region and 1938 bp of the coding region downstream of the cap site, was sequenced. The DNA sequences of the 5' end of mouse and human IRBP genes are shown in Figure 3. Comparison of the 5'-flanking region of the mouse and human IRBP gene revealed two highly conserved regions (Figure 4). The first one is a 310 bp fragment of 79 % identity between -1526 to -1217 bp, and the second one with 76 % identity extends from -277 bp to the TSS. The function of the conserved sequence found in the most upstream part of the promoter requires further investigation.

Our studies of IRBP promoter function and sequence homology have identified and localized regions for future research toward the eventual determination and characterization of the cis- and trans-acting transcription elements which are essential for the understanding of IRBP gene regulation.

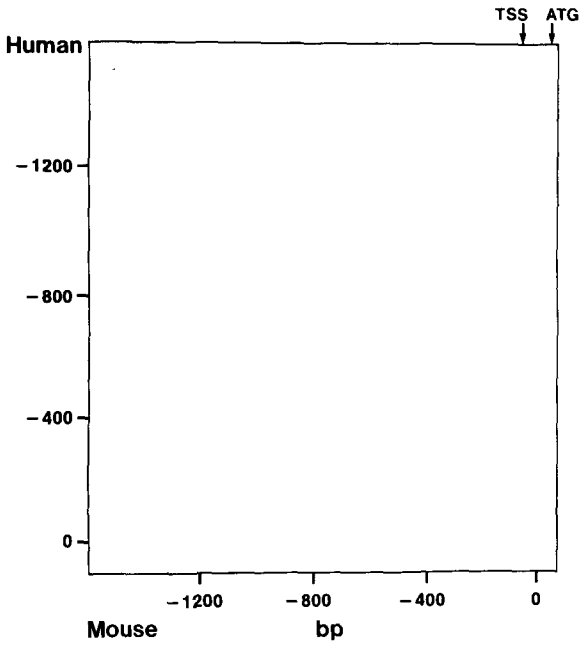

Fig. 4. Upstream sequence homology between human and mouse IRBP genes. The upstream sequences of the human and mouse IRBP genes are compared by dot matrix analysis (Microgenie release 5, Beckman Instruments). The parameters were set at a window of 18 nucleotides with 78% stringency, so that a dot was scored when 14 out of 18 residues matched. The positions of the transcription start site (TSS, or cap site) and the protein initiation codon, ATG, are indicated by arrows.

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