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Human Apolipoprotein B Gene Intestinal Control Region†

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ABSTRACT: Recently, we reported that a 315 bp enhancer, located over 55 kilobases (kb) upstream of the transcriptional start site of the human apolipoprotein B (apoB) gene, was sufficient to direct high-level expression of human apoB transgenes in mice. In this report, we expand our analysis of the distant apoB intestinal control region (ICR), by examining the function of segments in the vicinity of the 315 bp intestinal enhancer (315 IE). DNaseI hypersensitivity (DH) studies of a 4.8 kb segment from the ICR revealed three new DH sites, in addition to the previously described DH1 region present within the 315 IE. DH2 mapped to a 485 bp segment (485 IE) immediately upstream of the 315 IE that exhibited strong intestinal enhancer activity in transient transfection experiments with intestine-derived CaCo-2 cells. Within the DH2 region, an HNF-4/ARP-1 binding site was demonstrated by gel retardation experiments. A 1.8 kb segment incorporating the 485 IE was capable of driving expression of human apoB transgenes in the intestines of mice. Additionally, a third component of the apoB ICR was found about 1.2 kb downstream of the 315 IE, within a 1031 bp segment (1031 IE) that also harbored two DH sites, DH3 and DH4. This segment did not display enhancer activity but was capable of driving transgene expression in the intestine. The three components of the ICR displayed a similar pattern of apoB mRNA expression along the horizontal axis of the intestine. The previously characterized in vivo liver-specific elements of the apoB gene, namely, the second intron enhancer and the 5′ upstream liver enhancer, did not play a role in intestinal expression of apoB transgenes in mice.

Recently, we demonstrated that co-microinjection of a 315 bp intestinal enhancer (315 IE)[1] with p158, a PAC clone encompassing the human apolipoprotein B (apoB) structural gene and containing an additional 19 kb of 5′ upstream sequence and 33 kb of 3′ downstream sequence, generated transgenic mice expressing human apoB mRNA at high levels both in the liver and in the intestine (*1*). The PAC insert alone was only expressed in the liver (*1*). Thus, whereas the apoB hepatic control elements are located relatively close to the transcriptional start site, the intestinal control region (ICR) is localized more than 53 kb upstream of it.

In previous studies with β-galactosidase (β-gal) reporter constructs, we demonstrated that high-level expression of human apoB transgenes in the liver required the promoter (nucleotides −898 to 121) and a 443 bp enhancer (nucleotides 621−1064) from the second intron of the gene as well as the segment from nucleotide −899 to −5262 of the gene. DNA sequences located 5′ of nucleotide −5262 are not required for liver expression of the human transgenes. Without the second intron enhancer, no liver expression is observed (*2*). Most of the enhancer activity is due to three liver-enriched transcription factors, namely, HNF-1α, C/EBPα, and a yet unidentified factor named protein II (*3*), binding to a 98 bp footprint within this enhancer and synergystically enhancing liver expression from the apoB promoter (*3*). The segment from nucleotide −898 to −5262 contains two nuclear matrix attachment regions (MARs) (*4*), and is required for maximal liver expression of the human transgenes (*2*). Thus, these elements define the requirements for liver expression of apoB transgenes.

Within the intestinal control region, a 315 bp intestinal enhancer (315 IE) is sufficient to confer intestinal expression upon human apoB transgenes (*1*). Functional binding sites for the intestine-enriched transcription factors HNF-3β, C/EBPβ, and HNF-4 reside within the 315 IE (*1*), and synergistic interaction between these proteins bound to their cognate sites is required for optimal functioning of this intestinal enhancer (*5*).

In this report, we show that the 315 IE confers intestinal expression in transgenic mice to apoB−β-gal fusion transgenes that contain all the in vivo liver-specific elements and that otherwise are expressed only in the liver. Furthermore, we have identified two additional elements from the apoB

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[1] Abbreviations: IE, intestinal element; apoB, apolipoprotein B; CAT, chloramphenicol acetyltransferase; HNF-3, hepatocyte nuclear factor 3; HNF-4, hepatocyte nuclear factor 4; ARP-1, apolipoprotein AI regulatory protein; C/EBP, CAAT enhancer binding protein; DH, DNaseI hypersensitive; ICR, intestinal control region; MAR, matrix attachment region; LCR, locus control region.

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intestinal control region (ICR) that are capable of independently conferring intestinal expression to human transgenes in mice. The first is a 485 bp segment located immediately upstream of the 315 bp IE and the second a 1031 bp segment situated 1.2 kb 3′ of the 315 bp IE.

Additionally, we assessed whether the DNA sequence elements required for liver expression are also required for intestinal expression of the human apoB transgenes in mice. Our results indicate that the key liver elements, namely, the second intron enhancer and the 5′ upstream region extending from nucleotide −899 to −5262, that are required for hepatic expression in vivo are not required for expression of the human transgenes in the intestine. Similarly, the intestinal control region is not required for hepatic expression of apoB. Therefore, hepatic and intestinal expression of the human apoB gene is controlled by two separate and distinct sets of regulatory regions.

EXPERIMENTAL PROCEDURES

Generation of Transgenic Mice. Plasmid pβ-gal5.2EM (5.2EM) has been previously described (*6*), and contains a 1020 bp *Pvu*II fragment representing the apoB promoter upstream of the β-gal gene. Additionally, a 704 bp *Pst*I fragment containing the apoB second intron enhancer is inserted immediately upstream of the apoB promoter, and upstream of the enhancer is the segment from nucleotide −899 to −5262 of the apoB gene, which includes the 5′ distal and the 5′ proximal MARs (*4*); 3′ of the β-gal gene is the 3′ MAR, a 1.2 kb *Xba*I fragment spanning nucleotides 43104−44329 of the human apoB gene (*4*). The 315 IE represents a 315 bp *Eco*RI−*Hin*dIII fragment situated 55 kb upstream of the transcriptional start of the human apoB gene (*1*). Plasmid pβ-gal5.2M (5.2M) is identical to pβ-gal5.2EM (5.2EM) except that the second intron enhancer has been removed. The 4.8 IE is the segment from the human apoB gene extending from −54 to −58.8 kb 5′ of the transcriptional start site. Plasmid pβ-galEP3 (EP3) is similar to 5.2EM, but it lacks the segment from nucleotide −899 to −5262. The 2.1 IE is the segment from the apoB gene extending from −56.7 to −58.8 kb; it contains the 1.8 IE and the 315 IE. The 1.8 IE is a 1.8 kb *Eco*RI fragment located immediately upstream of the 315 IE.

The inserts from plasmids 5.2EM, 5.2M, and EP3 were each co-microinjected with either the 315 IE, the 4.8 IE, or the 2.1 IE, into FVB/N fertilized mouse eggs to generate transgenic mice. Additionally, 5.2EM was co-microinjected with either the 1.8 IE, the 2.7 IE, the 1031 IE, or the 356 IE. The DNA fragments were microinjected in equimolar concentrations according to standard protocols (*1*). Transgenic mice were first identified by PCR using oligonucleotides B1 and B2 as primers to detect the human apoB gene. Cointegrant founder mice were then identified by Southern blot analysis of *Taq*I- or *Eco*RI-digested genomic DNA and hybridized with a 5′ MAR probe or with the second intron enhancer as a probe. All mice were weaned at 21 days, housed in a barrier facility with a 12 h light/dark cycle, and fed a chow diet containing 4.5% fat (Ralston Purina, St. Louis, MO).

DNaseI Hypersensitivity Analysis. DNaseI hypersensitivity studies with nuclei from CaCo-2 cells were performed as described previously (*1*, *7*). Following digestions with DNaseI, DNA samples were digested with *Bgl*II or *Hin*dIII. The digestion products were separated by electrophoresis on 1.2% agarose gels, followed by Southern blot analysis. *Bgl*II blots were hybridized with a 215 bp probe corresponding to nucleotides 469−684 of the 4.8 IE that was amplified from 4.8 IE template DNA using oligonucleotides DHF and DHR as primers. The probe in the *Hin*dIII blots was a 591 bp *Hin*dIII−*Sty*I fragment located at the 5′ end of the 2.7 kb segment.

Ribonuclease Protection Analysis for Transgenic ApoB Expression. Tissue samples from duodenum, jejunum, and ileum were collected. Preparation of RNA from mouse tissues and RNase protection assays have been recently described (*1*). Quantification of expression levels was performed using a PhosphorImager and ImageQuant software.

Sequence Alignment and Search for Potential Transcription Factor Binding Sites. The sequences of the human apoB ICR (GenBank accession number AC010872) and that of the mouse apoB intestinal enhancer (GenBank accession number AF187728) were aligned using GeneWorks software. Sequence search analysis for putative binding sites for intestine-specific transcription factors was performed using the online TRANSFAC algorithm with the version 3.5 database (*8*).

Gel Retardation Experiments and Transfection Assays. Gel retardation experiments were carried out as described previously (*1*). Antibodies to human HNF-4 and ARP-1 were purchased from Santa Cruz Biotechnology. Transfections with CaCo-2 cells were performed at least twice and in duplicate, including as an internal control plasmid RSV-β-gal, to account for differences in transfection efficiencies between different plasmids and between experiments. The data were expressed as relative CAT activities, that is, as the value of CAT activity of the test plasmid divided by the β-gal activity of the internal control plasmid. Plasmid −85CAT has been previously described and contains the segment from nucleotide −85 to 121 of the human apoB promoter fused to the CAT gene. Plasmid 485CAT incorporates a 485 bp *Eco*RI fragment corresponding to the 3′-most segment of the 1.8 IE, upstream of the apoB promoter. The 2.7 CAT reporter constructs were made by ligation of the 2.7 kb *Hin*dIII fragment into the *Hin*dIII site of −85CAT followed by screening for the forward and reverse orientations. Subclones of the 2.7 kb IE were made by digestion of the 2.7 kb *Hin*dIII fragment with combinations of restriction enzymes. Thus, to make the 906 bp *Hin*dIII−*Bgl*II fragment, the 2.7 IE was digested with *Bgl*II. The 271CAT construct contains the proximal 271 bp *Bgl*II−*Sty*I fragment. The 1031 bp fragment is a *Sty*I−*Sty*I segment just 3′ of the 271 IE. The next 3′ fragment is a 356 bp *Sty*I−*Ssp*I segment, and the last piece is a 165 bp fragment which was made using restriction digestion of the 2.7 IE with *Ssp*I and *Hin*dIII. All fragments were gel-purified, filled in using Klenow DNA polymerase with 1 mM dNTPs, ligated into the filled-in *Hin*dIII site of −85CAT, and then screened for orientation using digestions with various restriction enzymes.

Oligonucleotides were as follows: HNF-4 consensus, CTACACAAATATGAACCTTGCC; ARP-1 consensus, TGAGCCCTTGACCCCT; 485 HNF-4/ARP-1 oligonucleotide, GGTTATGATCTTTCCACTTATACC; B1, GAAGAACTTCCGGAGAGTTGCAAT; B2, CTCTTAGCCC-

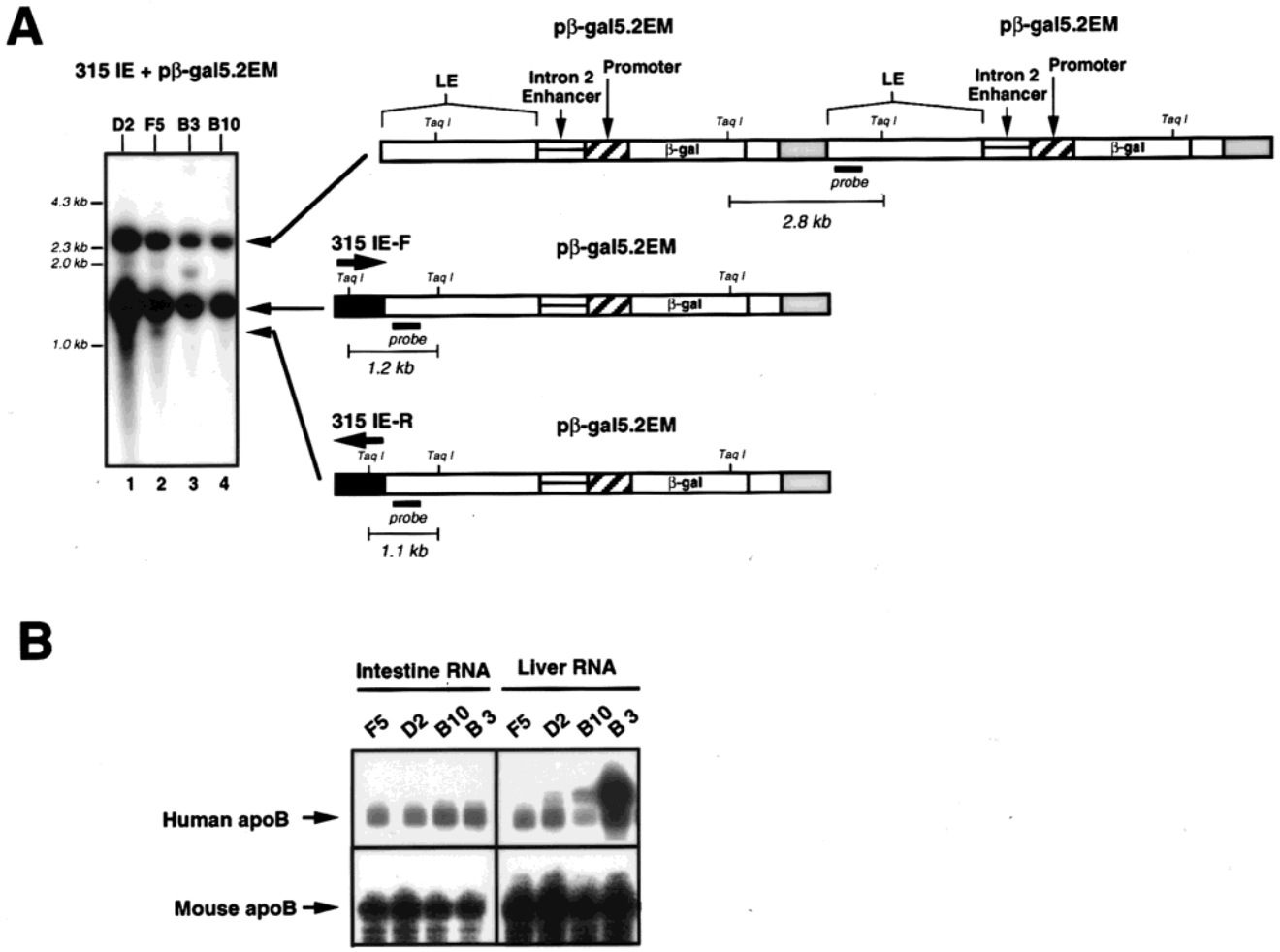

FIGURE 1: Transcriptional activities of transgenic mice co-injected with pβ-gal5.2EM and 315 IE DNA. Panel A displays a Southern blot of transgenic mice DNA co-injected with the two DNA inserts indicated above the blot and with the name of each mouse shown at the top of each lane. The numbers on the left reflect the mobilities of DNA size markers run in parallel in the same gel. The three different chromosomal integration events that are observed are illustrated on the right side of the blot. The sizes of the fragments analyzed and the location of the probe used in the blots are indicated below the map of each construct. Panel B shows the results of RNase protection assays, with the human transgene apoB RNA shown at the top and the mouse apoB RNA shown at the bottom. Each mouse designation is the same as that in the Southern blot.

CATTCAGCTCTGAC; DHF, GGAATACTAATTCAGCAGAC; and DHR, CAGAGCACAGTTGACATAGC.

RESULTS

The 315 bp IE Confers Intestinal Expression upon Human ApoB β-Galactosidase Fusion Transgenes. Having recently established that the 315 bp IE conferred intestinal expression upon p158, a human apoB transgene extending from −19 kb 5′ of the transcriptional start site to 33 kb 3′ of the poly(A) addition site, we tested whether this intestinal element would function with pβgal5.2EM, a reporter construct containing the apoB promoter (P) fused to the β-galactosidase (β-gal) gene and containing the DNA sequence elements required for high-level liver expression of the apoB gene, namely, the second intron enhancer (E), the segment from nucleotide −899 to −5262 (LE), and the apoB 3′ MAR (M) (nucleotides 43104−44329) (for a map, see Figure 1A). Plasmid pβgal5.2EM includes only 5 kb of upstream sequence and 2 kb of downstream sequence, thus allowing us to ask whether the additional 5′ and 3′ sequences contained within p158 are required for intestinal expression.

Co-microinjections of 5.2EM and the 315 IE generated several founder mice; four representative examples are depicted in Figure 1. Three major types of integration events were evident and are displayed on the Southern blot in Figure 1A. The main integration product corresponded to the intestinal enhancer (315 IE-F) upstream of the 5.2EM region in the forward orientation. Mice D2 and F5 also contained inserts in which the 315 IE was in the reverse orientation (315 IE-R) upstream of 5.2EM. Tandem arrays of 5.2EM were also evident.

RNA was prepared from liver and intestine of each of these mice, and RNase protection assays were carried out using a mouse probe and a human probe to detect the endogenous mouse apoB mRNA and the human transgene RNA, respectively. All four lines of mice expressed the human transgene RNA in both the liver and the intestine (Figure 1B). These data demonstrate that the 315 bp IE confers intestinal expression upon pβgal5.2EM and, therefore, that the segment from −19 to −5 kb upstream of the promoter and the segment from 2 to 33 kb downstream of the apoB gene are **not** required for intestinal expression of human apoB transgenes.

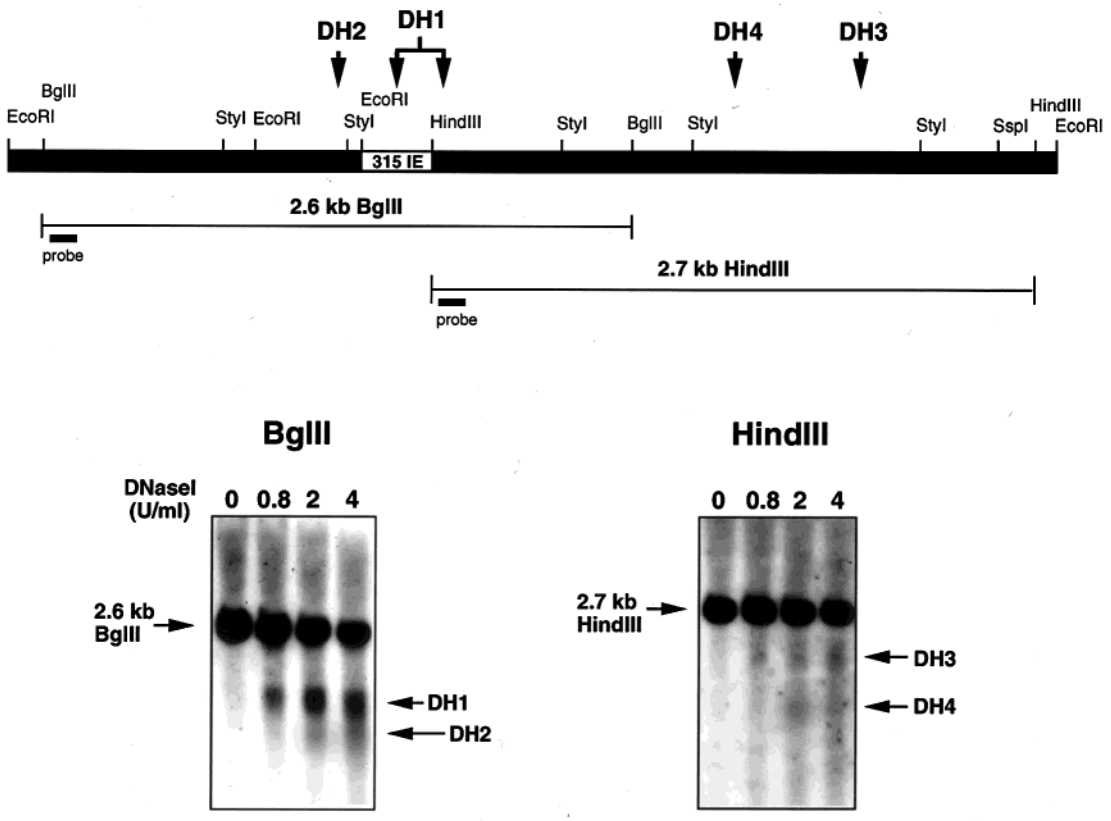

FIGURE 2: DNaseI hypersensitivity of the human 4.8 kb intestinal control region (ICR). The top portion shows a partial restriction map of the 4.8 kb ICR with the location of the 315 IE shown in a white box. The positions of four DH sites are indicated above the map and are derived from the data displayed below. The locations of the probes used in the analyses are shown below the map with the fragments of interest. The amounts of DNaseI that are used are indicated above the autoradiograms in the lower panel, and the positions of the *Bgl*II (2.6 kb) or the *Hin*dIII (2.7 kb) parental fragments, as well as those of the DH sites generated from CaCo-2 nuclei, are indicated at either side of the autoradiograms.

DNaseI Hypersensitive Sites within the 4.8 IE in CaCo-2 Cells. To search for additional regulatory elements in the regions flanking the 315 bp IE, we performed DNaseI hypersensitive (DH) assays using intestine-derived CaCo-2 cell nuclei by examining two segments within a 4.8 kb region (Figure 2). The first segment is a 2.6 kb *Bgl*II fragment containing the 315 bp IE and an additional 1.6 kb of upstream sequence and 0.7 kb of downstream sequence, and the second segment is a 2.7 kb fragment situated immediately downstream of the 315 bp IE. Three separate DNaseI experiments were performed, and representative results are illustrated in Figure 2. Within the 2.6 kb *Bgl*II fragment, two DH sites were evident: DH1, corresponding to the previously identified DH region within the 315 bp IE (*1*), and the second, less prominent DH site (DH2), residing approximately 200 bp upstream of DH1, within a 1.8 kb *Eco*RI fragment. DNaseI hypersensitivity analysis of the 2.7 kb *Hin*dIII segment revealed two additional DH sites, both residing within a 1031 bp *Sty*I−*Sty*I fragment.

Transgenic Mice Studies Reveal Additional Intestinal Regulatory Regions within the 4.8 IE. To assess whether the additional DH sites identified in CaCo-2 cells represented in vivo intestinal regulatory elements, transgenic mice were generated with pβgal5.2EM in combination with the 1.8 IE or the 2.7 IE (Figure 3). Additionally, two smaller subfragments within the 2.7 IE, namely, a 1031 bp fragment (1031 IE) and a 356 bp fragment (356 IE), were also tested in co-microinjections with pβgal5.2EM (Figure 3). The copy number of the cointegrant transgenes determined by quantitation of Southern blot signals was 5 copies/cell for the 4.8 IE cointegrants, 5−10 copies/cell for the 1.8 IE cointegrants, 10−20 copies/cell for the 315 IE cointegrants, 15−30 copies/cell for the 2.7 IE cointegrants, 20−25 copies/cell for the 1031 IE cointegrants, and 10−15 copies/cell for the 356 IE cointegrants.

RNase protection analysis of intestinal and liver tissues from 1.8EM transgenic mice (Figure 3) revealed that the 1.8 IE, located just upstream of the 315 IE, does confer intestinal expression upon the human transgenes. Similarly, the 2.7 IE also conferred intestinal expression (Figure 3). These results demonstrate the presence of additional intestine-specific regulatory elements in the 1.8 IE and the 2.7 IE, which are functional in transgenic mice.

To further localize the intestinal elements within the 2.7 IE, two separate fragments from this region were used in co-microinjection experiments. Transgenic mice carrying the 1031 bp *Sty*I−*Sty*I subfragment (1031 IE) expressed the transgenes in the intestine at levels similar to those exhibited by the 2.7 IE mice. On the other hand, a 356 bp *Sty*I−*Ssp*I fragment (356 IE) located just 3′ of the 1031 IE did not confer intestinal expression (Figure 3). These experiments further localized in vivo intestinal elements to a 1031 bp *Sty*I segment within the 2.7 IE.

Further Analysis of the 1.8 IE. We have recently found that a CTCF-binding site exhibiting functional properties of an insulator resides within the 3′-most 485 bp of the 1.8 IE (see ref *9*). This insulator blocks the activity of the 315 bp IE when placed between this enhancer and the apoB

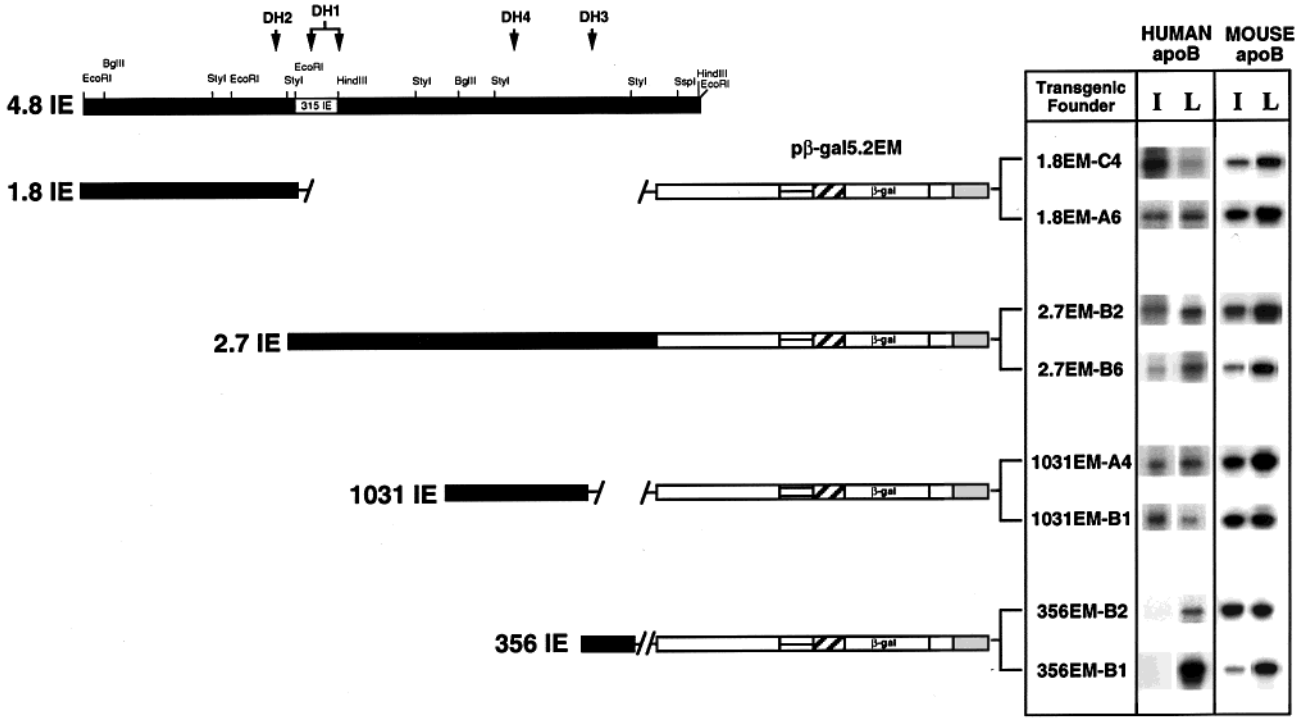

FIGURE 3: Intestinal and hepatic apoB expression levels of mice co-microinjected with fragments from the human 4.8 kb ICR and pβ-gal5.2EM. The top left shows a map of the 4.8 kb ICR, and below it are depictions of the fragments used in co-microinjections. ApoB expression levels in intestine (I) and liver (L) are shown to the right of each cointegrant transgene.

promoter, and its physiological role may be to prevent the apoB intestinal elements from acting upon genes located 5′ of this regulatory region (*9*). This implies that the most likely location of the intestinal elements within the 1.8 kb fragment is 3′ of the insulator. On the basis of this supposition, we asked whether the 485 bp *Eco*RI segment corresponding to the 3′ portion of the 1.8 IE would enhance transcription from the apoB promoter in intestine-derived CaCo-2 cells. To this end, the 485 bp IE was cloned upstream of the apoB promoter−reporter gene construct −85CAT (*1*), and transfections with CaCo-2 cells were performed. The results, depicted in Figure 4A, demonstrate that the 485 bp segment enhanced transcription of the apoB promoter by 15-fold, thus revealing the presence of a robust enhancer.

To localize intestine-enriched transcription factor binding sites within the 485 IE, we first focused on the area around DH site 2 because in past studies DH sites have revealed the locations of important regulatory elements (*7*, *10*, *11*). Comparison of the DNA sequence in the vicinity of DH2 between the human and mouse apoB genes revealed a high degree of sequence similarity (63%) (Figure 4B), and sequence analysis of this region using TRANSFAC (*8*) revealed a potential binding site for the tissue-restricted transcription factors HNF-4 (*12*) and ARP-1 (*13*). Outside of the DH2 region are putative binding sites for transcription factors GATA4, -5, and -6 (*14*) and NFY (*15*).

Hepatocyte nuclear factor-4 (HNF-4) and ARP-1 are members of the orphan receptor family of transcription factors and recognize the same DNA sequence (*12*). To determine whether these proteins bind to the 485 bp IE, gel retardation experiments were performed with CaCo-2 nuclear extracts (Figure 4C), and with extracts from COS cells that had been transfected with expression vectors for HNF-4 or ARP-1 (Figure 4D). Lane 1 of panel C shows binding of a known HNF-4 oligonucleotide probe to CaCo-2 nuclear proteins, with competitions for binding by unlabeled HNF-4 and apoB probes displayed in lanes 2 and 3 and supershifting by an HNF-4 antibody shown in lane 4. Lane 5 in panel C shows that the apoB probe formed a complex identical to that of the consensus probe with the HNF-4 protein from a CaCo-2 extract, which was competed with an excess of the apoB probe, by the ARP-1 probe, and by the HNF-4 probe (lanes 6−8, respectively). The retarded complex formed by the apoB IE probe was also supershifted by the HNF-4 antibody (lane 9) and by the ARP-1 antibody (lane 10). These data demonstrate binding of the apoB IE probe to HNF-4 and ARP-1 present in CaCo-2 extracts. Additional controls are shown in lanes 11−13. Thus, in lane 11 are shown the complexes formed by the ARP-1 consensus probe and CaCo-2 nuclear proteins; competitions by the ARP-1 and apoB oligonucleotides are shown in lanes 12 and 13, respectively, whereas supershifting by the ARP-1 antibody is shown in lane 14. Similarly, in panel D of Figure 4, lanes 1−3 depict the complexes formed by the HNF-4 consensus probe and COS extracts enriched in ARP-1 (lane 1), HNF-4 (lane 2), or a mixture of both (lane 3); lanes 4−6 illustrate that the apoB oligonucleotide probe bound to ARP-1 and HNF-4, and lanes 7−9 revealed complexes between the ARP-1 consensus probe and the two proteins.

The combined results presented in Figure 4 indicate that a strong intestine-specific enhancer resides in a 485 bp segment positioned immediately upstream of the 315 bp IE. Within the 485 bp region there is a segment that exhibits hypersensitivity to DNaseI (DH2), whose DNA sequence exhibits similarities between the human and mouse apoB genes. This conserved segment harbors a binding site for transcription factors HNF-4 and ARP-1.

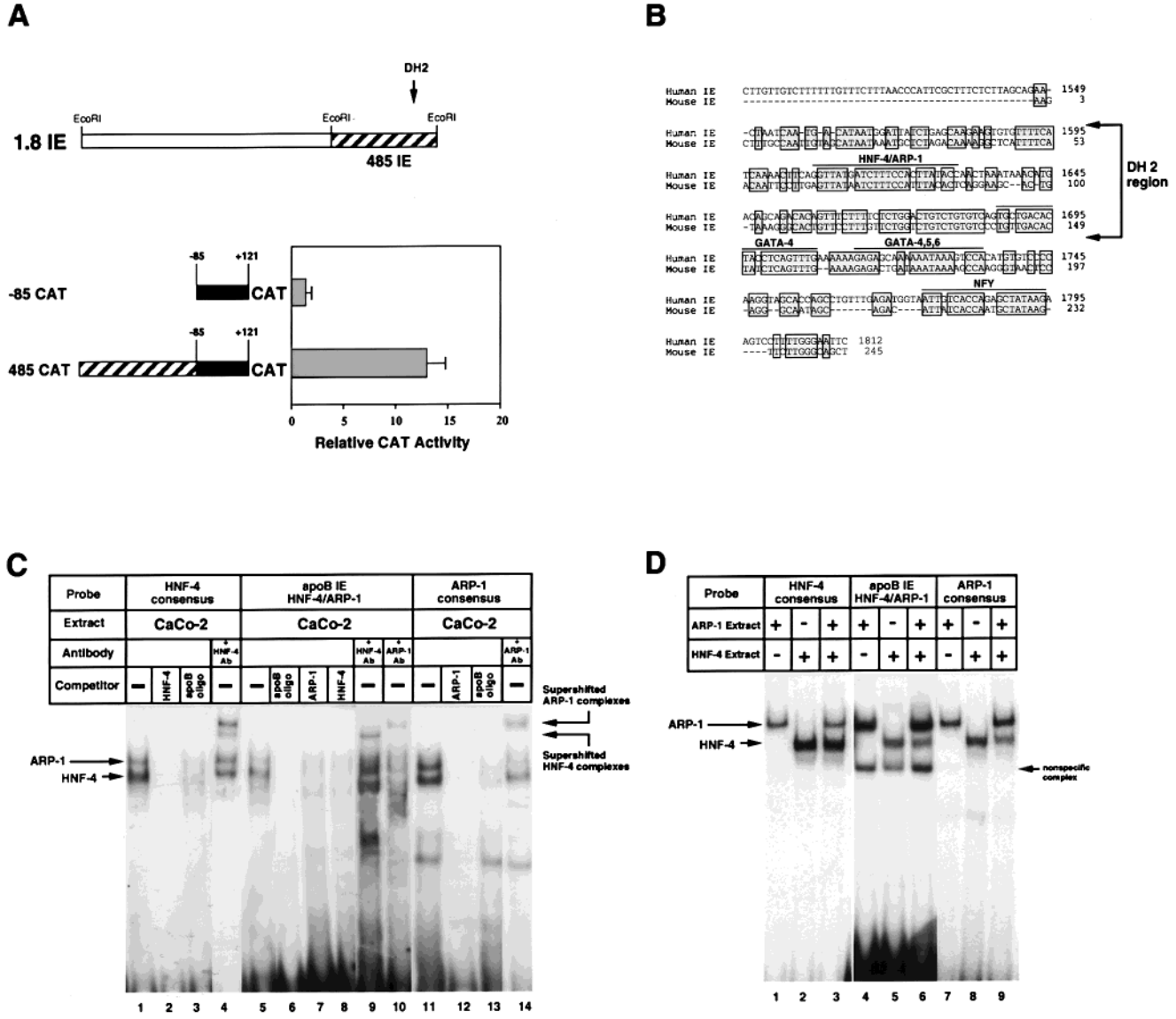

FIGURE 4: Intestinal enhancer within the 1.8 IE. The top of panel A shows a map of the 1.8 IE, indicating the locations of the 485 IE and DH2. Below the map are the constructs used in transfection experiments with CaCo-2 cells and the results of the transfections in histogram form. Panel B displays a sequence alignment of a portion of the 485 IE harboring DH2, and of a fragment from the analogous region from the mouse apoB gene. Nucleotides which are identical between the human and mouse apoB genes are shaded, and homologous segments are boxed. The location of DH2 is indicated by arrows. The locations of transcription factor binding sites are indicated by a black bar above the sequence. Panels C and D display data from gel shift experiments. The reagents added in each lane are indicated above the gel, and the retarded complexes are indicated on the left side of the gels.

Further Analysis of the 2.7 IE. Results from transgenic mice experiments in Figure 3 indicated that the 2.7 IE also conferred intestinal expression upon the human apoB−β-gal fusion transgenes. Furthermore, DNaseI hypersensitivity studies (Figure 2) revealed two DH sites in this region, within a 1031 bp *Sty*I segment. These observations prompted us to search for an enhancer activity, in transient transfection experiments with CaCo-2 cells. To this end, the entire 2.7 IE, as well as various fragments derived from it, was cloned upstream of apoB promoter CAT plasmid −85CAT, and transfections were performed. A map of the 2.7 IE region is shown at the top of Figure 5. The transcriptional activity of the promoter alone was set at 1.0. As previously observed (*1*), the 2.7 IE did not elevate the level of transcription but rather decreased it by a factor of 2, a result consistent with the notion that the 2.7 IE may contain both positive and negative elements. Further analysis of the components of the 2.7 IE revealed that the 5′-most 906 bp segment as well as the downstream 271 bp segment (constructs 906CAT and 271CAT) did not contain an enhancer. Surprisingly, the 1031 IE that functioned well in transgenic mice (Figure 3) did not display an enhancer activity, suggesting that this segment may contain neighboring or overlapping positive and negative elements whose activities neutralize one another in our cell culture assay, but function properly when the transgenes are integrated into the mouse genome in a chromatin context. Indeed, this is exactly what occurs with the apoB liver element located in the region from nucleotide −899 to −5262 of the human gene. This segment is required for high-level hepatic expression of apoB transgenes in mice; however, in transient transfection experiments with liver-derived HepG2 cells, it causes a 2-fold decrease in transcriptional activity, similar to that of the 2.7 IE (*2*, *6*, *16*). A 2-fold enhancer activity was observed with the 356 IE, but this segment did

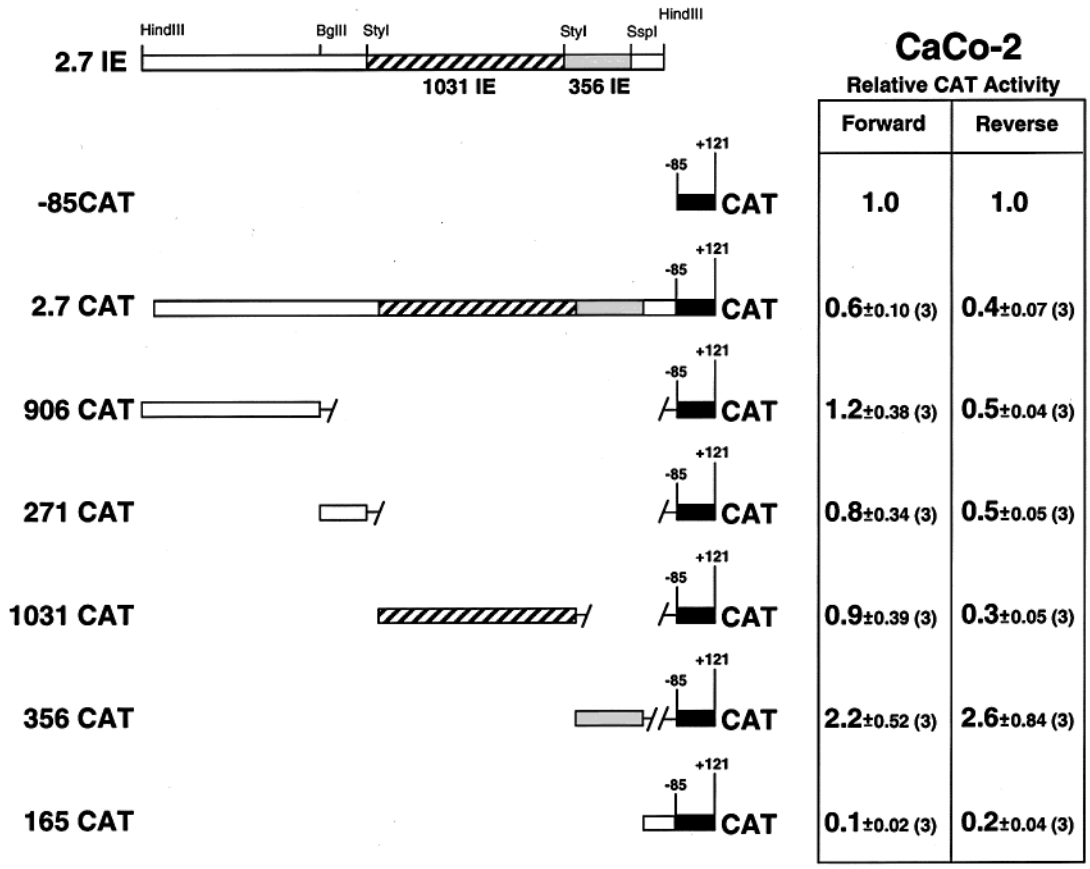

FIGURE 5: Transfection experiments with segments from the 2.7 IE region. A map of the 2.7 IE is shown at the top of the figure, with relevant restriction sites indicated as well as the sizes of the internal segments that are analyzed. Below the map are shown the various constructs used in the transfection experiments with CaCo-2 cells, and the results of the transfections are displayed on the right, expressed as relative CAT activities ± the standard deviations, with the number of independent transfections performed shown in parentheses. Forward and reverse refer to the orientation of the test fragments situated upstream of the promoter.

not confer intestinal expression upon transgenic mice (Figure 3). Finally, the 3′-most 165 bp segment reduced promoter activity by 10-fold (Figure 5), suggesting that it harbors negative regulatory elements. Essentially identical results were observed when the various IE segments were cloned upstream of the SV40 promoter (data not shown).

Transgenic ApoB Expression along the Longitudinal Axis of the Intestine. Both the mouse and the human apoB genes are expressed at high levels in the three portions of the small intestine: the duodenum, the jejunum, and the ileum. Having uncovered three separate sets of elements capable of driving intestinal expression of human apoB transgenes, we asked whether these three separate elements, namely, the 1.8 IE, the 315 IE, and the 1031 IE, participate to different extents in the expression of the transgenes in the three segments of the intestine. To this end, we prepared RNA from duodenal, jejunal, and ileal tissues from mice containing either 1.8+5.2EM, 315+5.2EM, or 1031+5.2EM cointegrant transgenes and analyzed mRNA levels using RNase protection assays (Figure 6). Transgene expression was detected in all three tissues for all three co-microinjected fragments, although some variations in expression levels were observed, as determined by the ratio of the amount of human RNA to the amount of mouse RNA, corrected for differences in the specific activities of the two probes, with the highest levels of transgene mRNA detected in the duodenum, followed by the ileum and the jejunum.

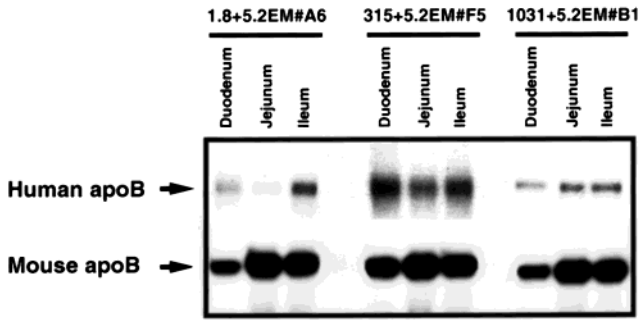

FIGURE 6: Duodenal, jejunal, and ileal expression analysis of cointegrant transgenes. Autoradiograms representing RNase protection assays of RNA from mice harboring the human cointegrant transgene construct 1.8+5.2EM, 315+5.2EM, or 1031+5.2EM are shown. The cointegrant DNA fragments used in each case, as well as the mouse designation numbers, are indicated above the autoradiograms. The RNase protection products are labeled on the left.

Are the Two Key Hepatic Regulatory Elements of the Human ApoB Gene Also Required for Intestinal Expression of Human ApoB Transgenes? As previously mentioned, the second intron enhancer is absolutely required for liver expression of apoB transgenes (*2*). To determine whether the second intron enhancer is also required for intestinal expression, we co-microinjected plasmid 5.2M, which is similar to 5.2EM but lacks the second intron enhancer, with 4.8 IE, a larger segment from the human apoB intestinal

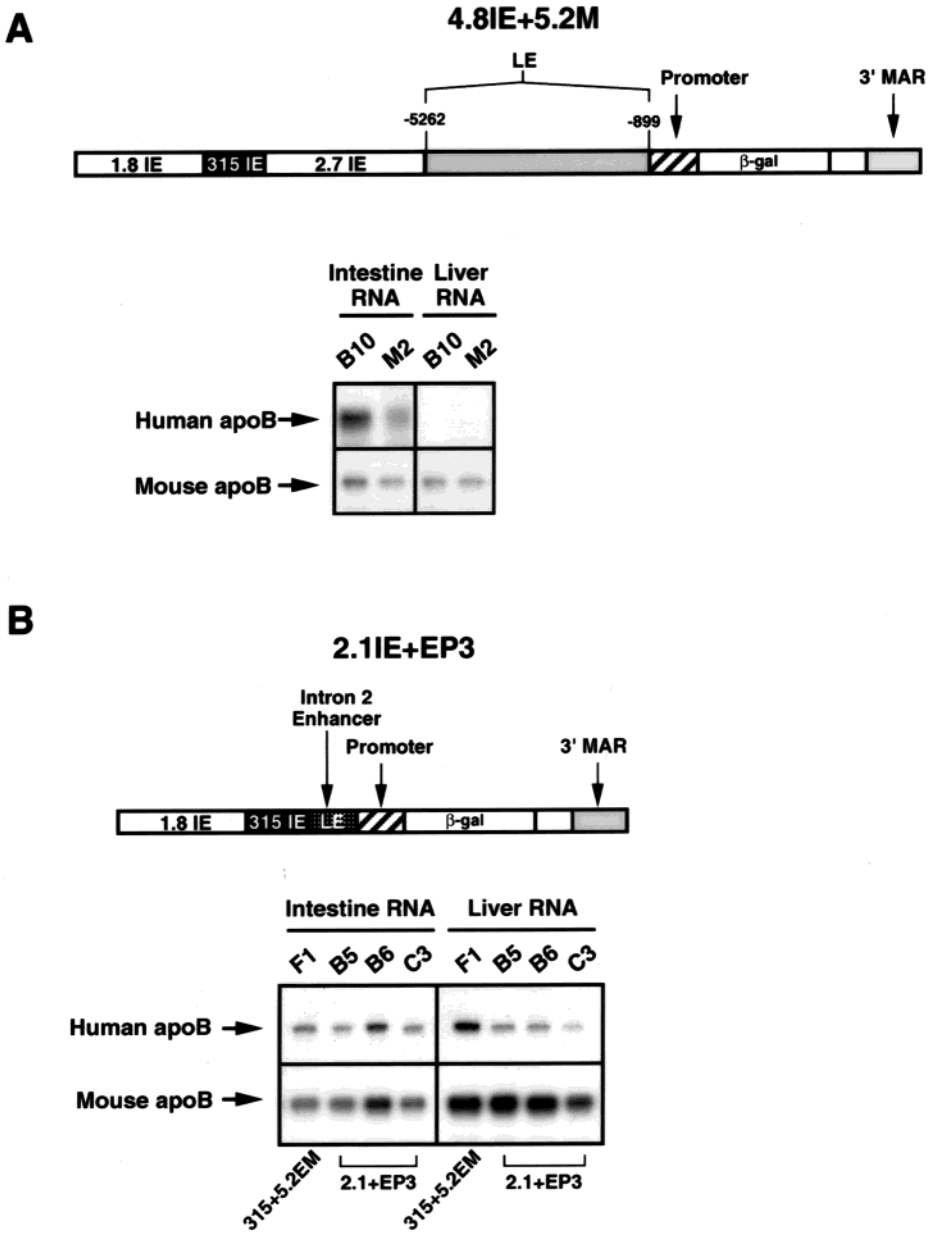

FIGURE 7: RNase protection assays of mice co-microinjected with 4.8 IE and 5.2M DNA or 2.1 IE and EP3 DNA. The structure of the cointegrant DNA segments is shown above the autoradiograms, with the RPA results below. The positions of the human and mouse apoB RNase protection products are shown on the left side of the autoradiograms. The source of tissue RNA and the transgenic mouse designations are at the top of each lane. In panel B, two control 315+5.2EM mice are also included.

control region that includes the three regions of the IE described above. Two lines of mice, named B10 and M2, were generated (Figure 7A). As predicted, the human transgenes lacking the second intron enhancer were not expressed in the livers of these mice, in agreement with our earlier work (*2*). Nevertheless, both mice expressed the transgenes in the intestine, at levels comparable to those exhibited by the endogenous mouse apoB mRNA, thus demonstrating that the second intron enhancer is not required for intestinal expression of the human apoB gene in transgenic mice.

Next, we asked whether the segment from nucleotide −899 to −5262, which confers high-level liver expression upon human apoB transgenes (*2*), also played a role in intestinal expression. To this end, we co-microinjected EP3, a plasmid similar to 5.2EM but lacking the segment from nucleotide −899 to −5262 of the human apoB gene, with the 2.1 IE, a segment from the ICR that includes both the 315 IE and the 1.8 IE. Previously, we determined that EP3, when injected alone, yields only low-level liver expression of the transgenes but no intestinal expression (B. Levy-Wilson, unpublished data). Three lines of mice harboring cointegrant transgenes were selected and analyzed for expression in liver and intestine (Figure 7B). As predicted, the three lines expressed the transgenes in the liver at low levels compared to the level exhibited by a control mouse (F1) carrying 315+5.2EM transgenes. However, the intestinal expression levels seen with these mice lacking the upstream hepatic enhancer were similar to the levels observed with the control mice carrying 315+5.2EM transgenes. Furthermore, the ratio of intestine to liver expression of the 2.1IEP3 transgenes was higher than that exhibited by the 315+5.2EM transgenes, thus demon-

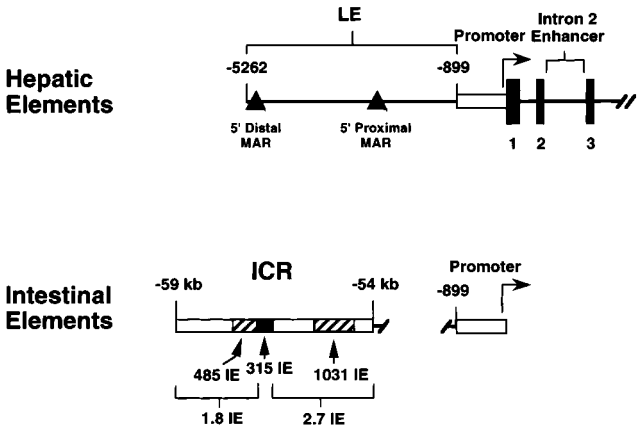

FIGURE 8: Summary of the human apoB gene intestinal and hepatic regulatory elements. The top panel shows a schematic illustration of the 5′ end of the apoB gene, from nucleotide −5262 to intron 3. The locations of the promoter (white box), the second intron enhancer, and the in vivo 5′ LE as well as the two MARs (black triangles) are indicated. The arrow represents the transcriptional start site. The first three exons are shown as black boxes. The bottom panel shows the segment from −59 to −54 kb of the human apoB gene containing the ICR, with its various components indicated below the bar. Also shown is the promoter region.

strating that the upstream liver enhancer segment extending from nucleotide −899 to −5262 is not required for intestinal expression of the human apoB transgenes. The combined data in Figure 7 show that the human apoB hepatic and intestinal regulatory elements are separate and distinct from one another, sharing only the promoter.

A schematic summary of the key in vivo hepatic and intestinal control elements of the human apoB gene is shown in Figure 8. The key in vivo liver-specific elements are located close to the transcriptional start site and include, in addition to the promoter, the second intron enhancer and the segment from nucleotide −899 to −5262. In contrast, the intestinal control elements are situated more than 50 kb 5′ of the start site and reside in three separate fragments that are in apposition, namely, a central 315 bp element (315 IE), a second upstream element, within a 485 bp IE, and a third element within a 1031 bp region located 1.2 kb 3′ of the 315 IE.

DISCUSSION

The human apolipoprotein B gene is expressed predominantly in human liver and intestine. Whereas the liver-specific enhancers responsible for in vivo expression of this gene are relatively close to the promoter region (for a review, see ref *17*), the intestinal control elements are located more than 53 kb upstream of the promoter (*18*). The main goals in this study were to identify all of the individual components of the human apoB gene distant intestinal control region (ICR) and to determine whether the key in vivo liver-specific elements of this gene also participate in intestinal expression. Recently, we identified a 315 bp fragment (315 IE) that exhibits enhancer activity in transient transfection experiments with intestinal cells and confers intestinal expression to the human apoB gene in transgenic mice (*1*). In this report, we show that the 315 IE is capable of conferring intestinal expression upon a human apoB−β-gal fusion transgene construct that is otherwise expressed at high levels only in the liver (Figure 1). Furthermore, we identified two additional segments of the ICR capable of independently driving apoB expression in the intestines of transgenic mice. Thus, the apoB ICR is composed of three separate sets of regulatory elements, each capable of functioning independently in transgenic mice.

The first of these elements is represented by the 315 IE. The second intestinal element resides within a 485 bp segment (485 IE) located immediately upstream of the 315 IE, and the third intestinal element resides in a 1031 bp segment situated 1.2 kb downstream of the 315 IE. There are similarities and differences among these three components of the ICR. All three coincide with DH sites, suggesting the chromatin in these segments is open and that nucleosomes within these segments may be absent or unfolded to allow binding of transcription factors. The 485 IE and the 315 IE function as powerful intestine-specific enhancers in transient transfection assays. The 1031 IE does not, suggesting that it may contain both positive and negative elements that neutralize one another in our assays or that the 1031 IE may contain a chromatin opening activity that remodels chromatin, thus facilitating transcription. This situation is reminiscent of earlier results with the segment of the liver element from nucleotide −899 to −5262, demonstrating that this segment lacks an enhancer, even though it is required for high-level liver expression of apoB transgenes in mice (*2*, *6*). However, this upstream liver regulatory region requires the second intron enhancer to confer hepatic expression to the transgenes, whereas the 1031 IE fragment can confer intestinal expression on its own. Given that CaCo-2 cells are poorly differentiated, one may suggest that the 1031 IE may play a role in developmental regulation of intestinal expression, perhaps repressing expression early in development and stimulating expression in the adult.

DNA sequence comparisons among the three intestinal elements revealed some similarities; for example, there is a 50% overall similarity between the 5′-most 180 bp of the 315 IE and the 3′ portion of the 485 IE, with the similarity toward the end of the DH2 region. However, there is a slightly less than 50% DNA sequence identity between the important HNF-3β and C/EBPβ binding sites of the 315 IE and the corresponding segment from the 485 IE, suggesting that if these elements arose via a gene duplication event, there has been significant drift. Comparison of the DNA sequences from the 315 IE and the 1031 IE also revealed some 50% sequence homology with a segment of the 1031 IE located between DH sites 3 and 4. Other duplicated LCRs include hepatic control regions (HCRs) 1 and 2, as well as the macrophage/adipocyte LCRs of the human apolipoprotein E (apoE) gene (*19*−*21*), although in these examples, the DNA sequence homology is over 80%.

A key participant in the enhancer activity of the 485 IE appears to be the conserved HNF-4/ARP-1 binding site shown in Figure 4. HNF-4 appears to play a pivotal role in the expression of many intestine-specific genes, particularly of other apolipoprotein genes. Thus, an HNF-4 binding site localized between positions −780 and −520 of the human apolipoprotein CIII gene plays a key role in the intestinal expression of apoCIII in transgenic mice (*22*). Similarly, HNF-4 controls transcription of the apoAIV gene via an interaction with the proximal promoter region (*23*), and that of the apoAI gene, by binding to its intestinal enhancer (*24*). Additionally, HNF-4 when bound to the human apoB 315

bp IE interacts synergistically with HNF-3β and C/EBPβ to enhance apoB gene transcription in CaCo-2 cells (*1*, *5*). Examination of the sequence of the DH3 region revealed strong similarities with the binding sites for intestine-enriched transcription factors C/EBPβ (two sites) and GATA-4 (three sites). Similarly, the DH4 region contained two potential binding sites for C/EBPβ , with a GATA-4 site between them.

The existence of three independent sets of intestinal control elements, each capable of driving apoB gene expression in transgenic mice, raises the question of whether these elements may play slightly different roles in apoB intestinal expression. Like other genes expressed in intestinal tissue (*22*, *25*−*27*), the apoB gene is regulated during development (*28*), as well as spatially along the cephalocaudal or horizontal axis and also along the vertical, crypt to villus axis, with high levels of expression observed in the villus enterocytes of the duodenum and the jejunum and no expression detectable in the colon or the crypt cells (*29*). At present, it is not known whether each of the three elements of the human apoB ICR has the same or different functions during development in humans. However, our results in Figure 6 suggest that the three apoB IEs exhibit a similar expression pattern along the horizontal axis of the digestive tract. Whether these elements behave similarly along the intestine's vertical axis remains unknown; nevertheless, the entire ICR region directs expression of human apoB transgenes exclusively in the villus enterocytes, the correct site of expression (*29*).

Multiple-component LCRs have been described in several gene loci, such as in other apolipoprotein genes (*19*−*21*, *30*), the β-globin gene loci (for reviews, see refs *31* and *32*), the adenosine deaminase locus (*33*), and others. Comparative analysis of many elements that appear to meet the criteria of LCR function has revealed common structural features of LCRs. Thus, LCRs contain varying numbers of tissue-specific DNaseI hypersensitive sites, consisting of a central core of 150−300 bp, enriched in transcription factor binding sites (*1*, *34*, *35*). In the case of the β-globin LCR, the four DH sites are clustered in one contiguous segment of DNA. Other LCRs are a collection of elements, with different numbers of DH sites distributed over large distances (*32*). Interesting data regarding LCR function have come from studies of the human CD2 LCR, which consists of three DH sites localized within 2 kb of 3′ flanking DNA (*36*). DH1 functions as a transcriptional enhancer, but the two other DH sites have no enhancer activity. DH3 plays a role in the establishment and/or maintenance of an open chromatin domain (*37*). The apoB ICR may be analogous to the former example, with DH sites 3 and 4 within the 1031 IE playing a role in the maintenance of an open chromatin structure, while DH sites 1 and 2 within the 485 IE and the 315 IE function as intestine-specific transcriptional enhancers. LCRs are distinct from promoters, classical enhancers, insulators, and matrix attachment regions (MARs), but they incorporate the functions of enhancers and can also contain MARs. The apoB ICR is flanked at its 5′ end by an insulator, which is situated within the 485 IE, immediately upstream of the enhancer (see ref *9*). Upstream of the insulator resides a MAR, and we have proposed that the insulator and the MAR represent the functional and structural 5′ boundary of the human apoB chromatin domain in intestinal cells (*9*).

Finally, we have demonstrated that the two elements that are required for expression of human apoB transgenes in the livers of mice, namely, the second intron enhancer and the segment from nucleotide −899 to −5262 of the apoB gene, do not participate in intestinal expression. Similarly, the intestinal control elements described above are not required for liver expression of apoB. Therefore, two very different mechanisms of transcriptional control that include different sets of regulatory elements in separate spatial locations operate to control expression of the human apoB gene in its two major sites of expression, the liver and the intestine.

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