Identification and Characterization of a 315-Base Pair Enhancer, Located More than 55 Kilobases 5′ of the Apolipoprotein B Gene, That Confers Expression in the Intestine*

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We recently reported that an 8-kilobase (kb) region, spanning from −54 to −62 kb 5′ of the human apolipoprotein B (apoB) gene, contains intestine-specific regulatory elements that control apoB expression in the intestines of transgenic mice. In this study, we further localized the apoB intestinal control region to a 3-kb segment (−54 to −57 kb). DNaseI hypersensitivity studies uncovered a prominent DNaseI hypersensitivity site, located within a 315-base pair (bp) fragment at the 5′-end of the 3-kb segment, in transcriptionally active CaCo-2 cells but not in transcriptionally inactive HeLa cells. Transient transfection experiments with CaCo-2 and HepG2 cells indicated that the 315-bp fragment contained an intestine-specific enhancer, and analysis of the DNA sequence revealed putative binding sites for the tissue-specific transcription factors hepatocyte nuclear factor 3β, hepatocyte nuclear factor 4, and CAAT enhancer-binding protein β. Binding of these factors to the 315-bp enhancer was demonstrated in gel retardation experiments. Transfection of deletion mutants of the 315-bp enhancer revealed the relative contributions of these transcription factors in the activity of the apoB intestinal enhancer. The corresponding segment of the mouse apoB gene (located −40 to −83 kb 5′ of the structural gene) exhibited a high degree of sequence conservation in the binding sites for the key transcriptional activators and also exhibited enhancer activity in transient transfection assays with CaCo-2 cells. In transgenic mouse expression studies, the 315-bp enhancer conferred intestinal expression to human apoB transgenes.

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In recent years, valuable information regarding the molecular mechanisms regulating tissue-specific transcriptional control of mammalian genes has emerged (for a recent review see Ref. 1). For certain genes, such as the β-globin gene cluster, a large body of knowledge regarding their tissue-specific and developmental regulation has been gathered from transgenic mouse expression studies (2–4). However, for most other genes, mechanisms for tissue-specific transcriptional regulation are poorly understood. This is the case for genes expressed in the intestine (5), although specific DNA sequences important for intestinal gene expression have been identified for the fatty acid-binding protein genes (6, 7), the sucrase-isomaltase gene (8, 9), and the apolipoprotein AI/CIII/AIV genes (10, 11).

In this study, we sought to define the DNA sequences required for the intestine-specific regulation of the human apolipoprotein B (apoB)1 gene. This gene is transcribed almost exclusively in the liver and intestine and thus provides a good model system for studying tissue-specific transcriptional control. In the liver, the product of the apoB gene is apoB100 (12, 13), a 4536-amino acid protein that is important for the assembly of very low density lipoproteins. In the intestine, a single coding in the apoB transcript undergoes editing to produce a premature stop codon, resulting in the synthesis of a truncated protein, apoB48, which is 2152 amino acids in length (48% as large as apoB100) (14, 15). ApoB48 plays a critical role in the packaging of alimentary lipids into chylomicrons (16, 17). Within the intestine, the highest levels of apoB48 expression are found in the villus enterocytes with very low levels of expression detected in the crypts. There is also a gradient of expression along the length of the intestine, with high levels of expression in the duodenum and lower amounts in the jejunum and ileum (18).

Even though the function of apoB in the assembly of triglyceride-rich lipoproteins is identical in liver and intestine and even though both tissues arise from endoderm, it is clear that the chromatin domain of the apoB gene, as well as the regulatory elements themselves and their mechanism of action, differ in these two tissues. Earlier studies by our laboratory focused on the identification and characterization of the DNA sequences and nuclear proteins involved in liver-specific regulation of the human apoB gene (for review see Ref. 19). Both in

1 The abbreviations used are: apoB, apolipoprotein B; ICR, intestinal control region; kb, kilobase; DH, DNaseI hypersensitive; bp, base pairs; BAC(70,22), bacterial artificial chromosome clone; PCR, polymerase chain reaction; CAT, chloramphenicol acetyltransferase; HNF-3, hepatocyte nuclear factor 3; HNF-4, hepatocyte nuclear factor 4; C/EBP, CAAT enhancer-binding protein; IE, intestinal element.
hapatoma cells (HepG2) and transgenic mice, we have demonstrated that the hepatic regulatory elements are located relatively close to the structural gene (20–23). High level expression of the apoB gene in the liver requires the proximal promoter region (−898 to +1), an enhancer from the second intron (+346 to +1048), as well as a segment from −899 to −5262 5′ of the gene. The latter segment contains two nuclear matrix association regions (24); one of these matrix association regions (the 5′-distal matrix association region) was proposed to represent the 5′-boundary of the chromatin domain of the apoB gene in HepG2 cells (25).

The regulation of apoB gene expression in the intestine is very different. In transgenic mouse expression studies, we discovered that the control of human apoB gene expression in the intestine depends on very distant DNA sequence elements. The most recent of these studies suggested that the intestinal control region (ICR) of apoB is located between −54 and −62 kb 5′ to the structural gene (26). An earlier study revealed that the distant intestinal control sequences directed a spatially appropriate pattern of apoB gene expression in the intestine, with high expression levels in the duodenum and lower levels in the jejunum and ileum. Furthermore, in situ hybridization studies revealed that the human apoB transgene (as well as the endogenous mouse apoB gene) were expressed appropriately in the enterocytes of the intestinal villi but not in the crypts (26).

In this study, we sought to perform a thorough analysis of the ICR of the human apoB gene. In transgenic mouse experiments, we localized the ICR to −54 to −57 kb 5′ to the structural gene. DNaseI hypersensitivity (DH) studies of that segment revealed an intestine-specific DH site within a 315-bp EcoRI-HindIII fragment. This fragment harbors a potent intestine-specific enhancer that is sufficient to activate transcription of human apoB in the intestines of transgenic mice. The DNA sequence and distant spatial localization of this ICR has been conserved in humans and mice. Gel retardation studies and transactivation experiments allowed us to identify the most important transcription factors involved in the activity of the intestinal enhancer.

MATERIALS AND METHODS

**Generation of Transgenic Mice—**DNA from a P1 clone spanning the human apoB gene, p158, was prepared as described previously (26). A 6-kb EcoRI subclone (E27), spanning from −51 to −57 kb and a 3-kb EcoRI fragment from −59 to −62 kb 5′ to the structural gene were subcloned from a bacterial artificial chromosome clone, BAC(70,22), which contained 70 kb of 5′-flanking sequences (26). Another clone, p4.8IE, spanning sequences from −54 to −58.8 kb, was prepared from BAC(70,22) by long range PCR with the oligonucleotide primers: clone 7-T7 and clone 12-T3. A 3-kb clone, E3, spanning from −54 to −57 kb, represented an EcoRI fragment of p4.8IE. The 315-bp IE fragment is an EcoRI-HindIII segment of the p4.8IE. Fragments E3, E27, E7, or the 315-bp IE were co-microinjected with p158 into FVB/N fertilized mouse eggs to generate transgenic mice. The DNA fragments were microinjected in equimolar concentrations according to standard protocols. Transgenic mouse founders were first identified by PCR using oligonucleotide primers B1 and B2 as primers to detect the human apoB gene. Cointegrant founder mice were then identified through parallel Southern blot analysis of EcoRI-digested genomic DNA and hybridized with an exon 26 probe for the apoB structural gene and with probes representing the various co-injected fragments from the ICR. 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).

**Ribonuclease Protection Analysis for Transgenic apoB Expression—**

Total hepatic and duodenal RNA were isolated from transgenic mice using the totally RNA kit from Ambion, Inc. (Austin, TX). Expression of transgenes was evaluated with RNase protection assays using the Ambion RPA III kit. Antisense RNA riboprobes were transcribed in vitro with T7 RNA polymerase and [α-32P]UTP using the Ribobisc kit (Epizentrum Technologies, Inc., Madison, WI) and XbaI-linearized plasmids spanning either exon 1 of the mouse apob gene or exon 1 of the human apoB gene (24). The β-actin riboprobe spanned the mouse β-actin cDNA from nucleotides +480 to +559. The riboprobes were purified on 5% polyacrylamide, 8 μL urea gels. Typically, 2–4 × 10^5 cpm of eluted probe was used/hybridization reaction with 10–25 μg of sample RNA overnight at 50 °C, and then samples were digested using RNase A and RNase T1. RNase protection reactions were visualized with autoradiography.

**Plasmid Construction—**

The 5′-IE and 3′-IE-85CAT plasmids were made by digesting p4.8IE with HindIII, followed by purification of the 2.1-kb 5′-IE and the 2.7-kb 3′-IE fragments. These two fragments were then ligated separately into the HindIII site of −85CAT. Orientations of the EcoRI-IE fragments were determined by restriction digestion and DNA sequencing. To generate the 315-/−85CAT forward and reverse plasmids (315 IE-F and 315 IE-R), p4.8IE was digested with EcoRI and HindIII, and the 315-bp EcoRI-HindIII fragment was gel-purified. The −85CAT plasmid was digested with HindIII and purified in a similar manner. Both fragments of DNA were then incubated with 50 μM dNTPs and Klenow DNA polymerase for 20 min at 25 °C to fill in the single-stranded ends. The blunt-ended 315 fragment was then ligated into blunt-ended −85CAT. Transformants were screened for the 315-bp insert, and orientation was determined by restriction digests and DNA sequencing. The 315 TATA chloramphenicol acetyltransferase (CAT) plasmid was constructed by excising the 315-bp fragment from 315F/−85CAT with ClaI and XbaI and ligating it into the ClaI and XbaI sites of the −85CAT TATA CAT reporter plasmid (27).

Construction of the deletion mutants of the 315-bp IE was as follows: The 1–2 CAT and 1–2–3 CAT constructs were derived from a 203-bp fragment generated by PCR, extending from the 5′-end of the 315-bp IE (ClaI site) (PCR1-Cla primer) to the 3′-end of site 3 (3 site 3H primer). After gel purification of the 203-bp fragment harboring sites 1, 2, and 3, a portion of it was digested with ClaI and TaqI, followed by purification and cloning of this fragment into the ClaI site of −85CAT to generate plasmid 1–2 CAT. To make clone 1–2–3 CAT, the 203-bp fragment was digested with ClaI and HindIII and ligated to plasmid −85 CAT that had been cut with ClaI and HindIII. Constructs 2–3–4 CAT and 3–4 CAT were derived from a 235-bp PCR fragment made with primer site 2H and 3′XbaI. This fragment was digested with HindIII and XbaI and cloned into the HindIII and XbaI sites of −85CAT. For the 3′–4 CAT deletion mutant, the 225-bp fragment was digested with TaqI and XbaI, gel-purified, and cloned into the ClaI and XbaI sites of −85CAT. The 2–3 CAT deletion construct was made using PCR to generate a 110-bp fragment using as primers site 2H and site 3H oligonucleotides. This fragment was then digested with HindIII and ligated into the HindIII site of −85CAT. Orientation of all fragments was determined by restriction digestion and PCR screening.

**Identification and Localization of the Mouse apoB ICR—**DNA from a P1 clone spanning −31 to −110 kb 5′ to the mouse apoB gene was digested with various restriction enzymes. The resulting DNA fragments were separated on 1% agarose gels and transferred to a nylon membrane. The Southern blot was then hybridized with the human 315-bp IE (Clal site) (PCR1-Cla primer) to the 3′-end of site 3 (3 site 3H primer). After gel purification of the 203-bp fragment harboring sites 1, 2, and 3, a portion of it was digested with ClaI and TaqI, followed by purification and cloning of this fragment into the ClaI site of −85CAT to generate plasmid 1–2 CAT. To make clone 1–2–3 CAT, the 203-bp fragment was digested with ClaI and HindIII and ligated to plasmid −85 CAT that had been cut with ClaI and HindIII. Constructs 2–3–4 CAT and 3–4 CAT were derived from a 235-bp PCR fragment made with primer site 2H and 3′XbaI. This fragment was digested with HindIII and XbaI and cloned into the HindIII and XbaI sites of −85CAT. For the 3′–4 CAT deletion mutant, the 225-bp fragment was digested with TaqI and XbaI, gel-purified, and cloned into the ClaI and XbaI sites of −85CAT. The 2–3–CAT deletion construct was made using PCR to generate a 110-bp fragment using as primers site 2H and site 3H oligonucleotides. This fragment was then digested with HindIII and ligated into the HindIII site of −85CAT. Orientation of all fragments was determined by restriction digestion and PCR screening.

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co-transfected into cells in duplicate plates. Cell lysates were prepared after 48 h with three cycles of freeze-thaw and lyse clarification by centrifugation at 15,000 × g for 5 min at 5 °C. The levels of β-galactosidase activities, as well as levels of CAT activity, were assessed as described previously (23). The levels were quantitated with Phosphoimage analysis and Image Quant software (Molecular Dynamics, Inc., Sunnyvale, CA). All CAT activity values represent averages of at least three independent transfection experiments and are corrected for transfection efficiencies between plates by dividing the CAT activity levels by the β-galactosidase levels.

DNasel Hypersensitivity Analysis—DNasel hypersensitivity studies with nucleoli from CaCo-2 and HeLa cells were performed as described previously (32). Following digestions with DNasel, DNA samples were digested with Bg/II. The digestion products were separated by electrophoresis on 1.2% agarose gels, followed by Southern blot analysis, using a 215-bp 5′-fragment as a probe. The probe, corresponding to nucleotides +489 to +684 within the p4.8IE, was amplified from p4.8IE template DNA using oligonucleotides DHF and DHR as primers.

Gel Retardation Assays—COS cells were transfected (as above) with 10 μg of expression plasmids for HNF-3β, HNF-3α, C/EBPβ, or C/EBPα; cellular lysates enriched for these proteins were prepared as described previously (25). Nuclear extracts from CaCo-2 cells were prepared as described by Dignam et al. (33). Putative binding sites for the transcription factors HNF-3α, C/EBPβ, and for HNF-4 were identified within the human apoB gene ICR utilizing the TRANSFAC 3.5 data base sequence analysis algorithm (34). Sense and antisense oligonucleotides corresponding to consensus binding sequences for HNF-3β (27), C/EBPβ (28), and HNF-4 (35) sites within the 315-bp apoB intestinal control region were synthesized. The single-stranded oligonucleotides were annealed, purified, and end-labeled according to procedures previously described (21). Antibodies specific for C/EBPβ and HNF-3β were purchased from Santa Cruz Biotechnologies, Inc.; antibodies for HNF-4 were a generous gift from F. Sladek. Binding reactions and electrophoretic analyses of DNA-protein complexes were performed as described by Brooks et al. (21). Briefly, 1 ng of labeled double-stranded oligonucleotide probe was incubated with extracts (5–10 ng of protein) for 20 min at 25 °C. Competition assays were performed utilizing 200 ng of unlabelled double-stranded oligonucleotides. For supershift reactions, the binding reaction was preincubated at 25 °C for 15 min prior to the addition of 5–10 μg of antibody, and antibody binding was allowed to proceed for an additional 15 min at 25 °C.

Oligonucleotides—The various oligonucleotides used are listed below: DHF, GGA ATA CTA ATT CAG CAG AC; DHR, CAG ACG ACA GGT GAC ATC GC; clone 7-T7, TGC TGT GGC AGA AGG GGCA-Xba, GCAGCAACCGAGAAGGGCA-GAG AGT TGC AAT; B2, CTC TTA GCC CCA TTC AGC TCT GAC; TGCAGATTGCGCAATCTGCA; HNF-3 consensus, GTTGACTAAGT-CAT CCC CTA ACC CCA AAA AAC AAT TTA; C/EBP consensus, TCA GAA TTC CTT GAA GTA TAC AGA AGG TAG GGA AGG GAA; GTT GAC ATA GC; clone 7-T7, TGC TGT GTG AGC ACT GAC AGT with E7 (extending from 59 to 74 kb) did not express the transgene in the intestine as well as in the liver. E27 is a 6-kb fragment extending from 51 to 57 kb of the human apoB gene, whereas E3 extends from 54 to 57 kb. Transgenic mice produced with co-microinjections of p158 and with E7 (extending from 59 to 62 kb) did not express the transgene in the intestine. Representative examples of RNase protection assays used with these mice are shown in Fig. 1C.

RESULTS

Distant DNA Sequences, Located from −54 kb to −57 kb Upstream from the apoB Gene, Confer Intestinal apoB Expression in Transgenic Mice—Recently, we examined human apoB gene expression in a collection of transgenic mice produced from Reca-assisted restriction endonuclease cleavage-modified BAC clones (26). Each of these BAC clones spanned the human apoB gene but contained different lengths of 5′ and 3′-flanking sequences. These studies revealed that DNA sequences between −54 to 62 kb 5′ to the human apoB gene are essential for apoB gene expression in the intestine. In this study, to further localize the intestinal control region, we co-microinjected fertilized mouse eggs with three smaller segments from this region (E7, E27, and E3; see Fig. 1A) and the human apoB P1 clone, p158. p158 spans the entire human apoB gene, along with 19 kb of the 5′-flanking sequences and 17.5 kb of the 3′-flanking sequences. Because p158 lacks the distant intestinal control region, it does not confer intestinal expression in transgenic mice. As illustrated in Fig. 1B, transgenic mice produced with clone p158 exhibit human apoB expression in the liver but not in the small intestine. Co-microinjection of p158 with either E27 or E3 produced transgenic mice that expressed human apoB in the intestine as well as in the liver. E27 is a 6-kb fragment extending from −51 to −57 kb of the human apoB gene, whereas E3 extends from −54 to −57 kb. Transgenic mice produced with co-microinjections of p158 and with E7 (extending from 59 to 62 kb) did not express the transgene in the intestine. Representative examples of RNase protection assays used with these mice are shown in Fig. 1C.

DNasel Hypersensitivity Studies of the Intestinal Control Region—To localize the intestinal control region of the human apoB gene, DH assays were performed. DH sites reflect an open chromatin structure, which could facilitate binding of transcriptional activators. We first focused on a 2.6-kb BgIII fragment from the intestinal control region (−58.6 to −56 kb). Progressive digestion of the parental BgIII fragment with DNasel revealed a broad hypersensitive region (designated DH1) in transcriptionally active CaCo-2 cells but not in transcriptionally inactive HeLa cells (Fig. 2, top panel). DH1 mapped almost entirely within a 315-bp EcoRI-HindIII fragment (Fig. 2). Further DH analysis of the BgIII-HindIII segment located immediately 3′ of the 2.6-kb BgIII fragment revealed no additional strong hypersensitive areas.

The 315-bp Fragment Is an Intestine-specific Enhancer—Next, the potential for transcriptional activation or repression of the apoB promoter by DNA sequences in the vicinity of DH1
ApoB Intestinal Control Elements

**Fig. 2.** DNaseI hypersensitivity in the intestinal control region. The top panel shows autoradiograms from DH studies with nuclei from CaCo-2 and HeLa cells. The amounts of DNase used are indicated on top of the blots. The hypersensitive site DH1 is indicated by arrows on the left side of the autoradiograms, and its location within the intestinal control region is shown above the restriction map in the lower panel. The location of the probe used is shown below the map. Key restriction sites are indicated.

was tested (see map, top panel of Fig. 3). For these experiments, we employed an apoB promoter-CAT gene construct, −85CAT, that contains the proximal apoB promoter sequences (from −85 to +121) (Fig. 3). This construct exhibits a transcriptional activity about 6 times weaker than that of the full apoB promoter (−898 to +121) (36) and therefore is well suited for assays of potential activator elements. Various segments from the intestinal control region were inserted in both the forward (F) and the reverse (R) orientations upstream of −85CAT, and transfections were performed in intestine-derived CaCo-2 cells and in liver-derived HepG2 cells.

CAT activities of the various constructs were expressed relative to that of the promoter alone, which was assigned an activity level of 1.0 (Fig. 3, bottom panel). The 5′-IE fragment when in the forward orientation enhanced transcription from the apoB promoter by 5-fold in CaCo-2 cells but not in HepG2 cells. This enhancer activity was not observed when the 5′-IE was placed upstream of the promoter in the reverse orientation (Fig. 3); instead, a 75% reduction in CAT activity was observed in CaCo-2 cells but not in HepG2 cells. The 3′-IE segment exhibited a weak repressor activity in both orientations in CaCo-2 cells (less than 50%) but a slight enhancer effect in the forward orientation in HepG2 cells.

We then tested the 315 IE segment. It fragment enhanced transcription from the apoB promoter by 5–6-fold in both orientations in CaCo-2 cells and in the reverse orientation in HepG2 cells. Similar results were obtained when fragments from the IE region were cloned upstream of the heterologous SV40 promoter (data not shown). These results establish the presence of a strong transcriptional enhancer in the 315-bp EcoRI-HindIII fragment.

**Fig. 3.** Transcriptional activity of various segments from the intestinal control region. The top panel shows a restriction map of the 4.8-kb intestinal control region. Some restriction sites are indicated above the map. The position of the DH1 site is also indicated by arrows. The 315-bp EcoRI-HindIII segment is hatched. A scale is shown above the map. Below the map, the two HindIII fragments used in the transfections are shown in the lower panel; they were designated 5′-IE (shown in white) and 3′-IE (shown in gray). The left portion of the bottom panel shows the constructs used in the transfection experiments and the right portion shows the relative CAT activities of each construct in CaCo-2 and HepG2 cells ± S.D. The number in parenthesis indicates the number of independent transfection assays performed with each construct. "F" after a construct name means the forward orientation and "R" denotes the reverse orientation of the IE. The value of 1.0 was assigned to the activity of the apoB promoter alone in each cell type. The CAT activities can only be compared within each cell line.

Various Intestine-enriched Transcription Factors Bind to the 315-bp Intestinal Enhancer—Analysis of the DNA sequence of this enhancer revealed putative binding sites for the intestine-enriched transcription factors HNF-3β, C/EBPβ, and HNF-4; a schematic drawing is shown in Fig. 4. Gel retardation experiments were performed to determine whether these transcription factors indeed bind to the apoB enhancer. First, binding of HNF-3β to site 1 was examined with a double-stranded site 1 oligonucleotide. In lanes 1–6 of Fig. 5A, we show binding experiments with an HNF-3 consensus oligonucleotide (representing a high affinity HNF-3 binding site that can be bound by each of the three major HNF-3 isoforms, α, β, and γ). In lanes 7–12, the probe was the site 1 oligonucleotide. Two specific complexes were formed by the HNF-3 consensus oligonucleotide and proteins from a COS cell extract enriched in HNF-3β (lane 1). Specificity of binding is shown in lane 2. The upper complex is HNF-3β and the lower complex represents HNF-3γ (see Paulweber et al. (27)). A 200-fold excess of nonradioactive site 1 oligonucleotide competes well for binding of the HNF-3 consensus probe for the upper HNF-3β complex (lane 3). When a CaCo-2 nuclear extract was used, three retarded complexes were observed (lane 5), representing binding of the three HNF-3 isoforms to the consensus probe. A HNF-3β-specific antibody supershifted the HNF-3β complex (lane 6).

The site 1 oligonucleotide formed one specific complex with
well for binding of the consensus probe to C/EBP increasing amounts of an expression vector for C/EBP. The functional significance of C/EBP binding to sites 2 and 4 of the apoB intestinal enhancer. Therefore, binding of C/EBP to sites 2 and 4 is illustrated in Fig. 6. The first four lanes show that a consensus C/EBP oligonucleotide forms a specific retarded complex with C/EBPα (lanes 1 and 2), which is competed for by sites 2 and 4 oligonucleotides (lanes 3 and 4, respectively). Specific binding of C/EBPα to site 2 is depicted in lanes 5–7 and binding to site 4 is shown in lanes 8–10. Therefore, we conclude that C/EBPα, like C/EBPβ, can also bind to sites 2 and 4 within the 315 IE. Co-transfection of the 315F TATA CAT construct with increasing amounts of the C/EBPα expression vector repressed transcription of this construct but not that of the control, enhancer-less construct TATA CAT (Fig. 6F), suggesting that C/EBPα competes with C/EBPβ for binding, and in doing so, inhibits its transcriptional activation through sites 2 and 4.

Binding of HNF-4 to site 3 was then evaluated. In lane 1 of Fig. 7A, we illustrate binding of a consensus HNF-4 oligonucleotide to a CaCo-2 nuclear extract. Specificity is validated in lane 2; lane 3 shows that the site 3 oligonucleotide competes for binding of HNF-4 to the consensus HNF-4 probe; lane 4 shows that an HNF-4 antibody can supershift the complex formed with the consensus probe. Site 3 forms a similarly complex with the CaCo-2 nuclear proteins (lane 5). That complex is abolished by an excess of either site 3 oligonucleotide (lane 6) or HNF-4 consensus oligonucleotide (lane 7). Furthermore, the HNF-4 antibody supershifts the site 3 complex, thereby demonstrating binding of HNF-4 to site 3. Transactivation experiments similar to those employed with HNF-3β and C/EBPβ were performed with increasing levels of an HNF-4 expression vector. As demonstrated in Fig. 7B, increasing amounts of HNF-4 stimulate transcription of the 315F TATA CAT construct but not of the TATA CAT construct, confirming a functional role of HNF-4 in the 315 IE enhancer activity.

To further evaluate the role of sites 1–4 in the activity of the 315-bp IE, deletion mutants were made, each lacking one or two of these sites. The results of transfection experiments with CaCo-2 cells are illustrated in Fig. 8. Deletion construct 1–2, containing the 5’ most 113 bp of the 315-bp IE and including the HNF-3β and the first C/EBPβ site, exhibited about one half of the activity of the wild-type enhancer. Similarly, deletion 3–4, containing the 3’ 195 bp of the 315 IE and including sites 3 and 4, enhanced expression by 51% compared with the wild-type IE. Construct 2–3 also displayed 51% of the activity of the wild-type enhancer. When only site 4 was deleted, as in construct 1–3, 80% of the enhancer activity was retained, and when only site 1 was deleted, enhancer activity of the 2–3–4 construct was 56% of the activity of the wild-type construct.

The Mouse apoB Gene Contains an Intestinal Control Region Similar to That of the Human Gene—Important regulatory elements tend to be evolutionarily conserved. Because our ultimate goal is to understand the molecular mechanisms involved in intestinal transcription of the apoB gene in our trans-
genic mouse model system, it was of interest to ask whether a similar distant ICR was present in the mouse apoB gene. Earlier studies demonstrated that a mouse apoB genomic clone extending 233 kb upstream from the structural gene was not expressed in the intestines of transgenic mice (37), suggesting that the mouse intestinal control sequences resided more 5' than 233 kb from the structural gene (18). Accordingly, a mouse genomic P1 clone containing the segment of the mouse apoB gene from 230 to 2100 kb was subjected to digestion with various restriction enzymes followed by Southern blot analysis and hybridization with the human 315-bp intestinal element as a probe. The results are shown in Fig. 9A. The smallest fragment, a 690-bp HindIII-PstI fragment, was cloned and sequenced. Mapping experiments localized this element between 240 and 283 kb 5' of the mouse apoB promoter, in approximately the same location as that of the corresponding human sequences (data not shown).

The potential functional role of the 690-bp mouse IE, m690, was tested by incorporating this segment in both orientations upstream of the 85CAT apoB construct. Maps of the constructs are depicted in Fig. 9B. Transient transfections with CaCo-2 cells revealed that the m690 element displayed strong enhancer activity in both orientations (Fig. 9B).

The Key Transcription Factor Binding Sites in the apoB Intestinal Enhancer Have Been Conserved between the Human and the Mouse Gene—Comparative analysis of the DNA sequence of the m690 IE and the 315 IE was performed (Fig. 10). DNA sequence conservation was striking at or near the binding sites for HNF-3β, C/EBPβ, and HNF-4. The high degree of conservation surrounding these sites (85%) suggests that this region is indeed an important part of the in vivo intestinal control region. Another conserved segment was located between sites 2 and 3 of the human IE. Two oligonucleotides corresponding to this region were synthesized, and gel retardation experiments were performed with CaCo-2 nuclear extracts. Two specific retarded complexes were observed whose identities remain unknown (data not shown).

The 315 IE Directs Intestinal Expression of Human apoB Transgenes—The ultimate proof of the functional relevance of the intestinal enhancer is its ability to function in vivo. This issue was tested in co-microinjection experiments with 315 IE and p158 (the 80-kb clone that confers apoB expression in the liver but not the intestine) (Fig. 1) (18). Southern blot analysis was used to select transgenic mice that incorporated both the apoB and the 315 IE segments. RNase protection analysis of RNA derived from the liver and small intestine of transgenic mice is shown in Fig. 11. The left panel shows the reactions with the mouse probe to detect the endogenous apoB mRNA, and the right panel depicts the reactions with the human probe to detect the transgene RNAs. Three separate founders, namely 6M1, 6M4 and 8M3, were analyzed for liver and intestine expression. As expected, all three animals expressed the

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**Fig. 5.** A functional HNF-3β binding site in the apoB 315-bp element. A shows gel retardation experiments performed with an HNF-3 consensus oligonucleotide probe and with an apoB ICR site 1 probe. The source of protein extract is indicated above the gels as well as the nature of the competing oligonucleotides. The retarded complexes are shown on either side of the gel. In B, the abscissa shows the quantities (in µg) of the pCMV HNF-3β expression plasmid used in the co-transfection assays with the 315F TATA CAT and TATA CAT constructs, and the ordinate shows the mean value of the relative CAT activities observed in CaCo-2 cells with the TATA CAT (■) and the 315F TATA-CAT (□) construct.
mouse apoB mRNA both in the liver and the intestine (left panel, lanes 4–6 and 8–10). Similarly, all three co-microinjected transgenic founders expressed the human transgenes in the liver (right panel, lanes 4–6) and in the intestine (lanes 8–10). As anticipated, mice carrying only p158 transgenes had very high levels of expression in the liver but did not exhibit intestinal expression of the transgenes (both panels, lanes 3 and 7). These data demonstrate that the 315 IE is necessary and sufficient to confer intestinal expression to human apoB transgenes.
DISCUSSION

We have identified a 315-bp intestine-specific transcriptional enhancer within a segment of the human apoB gene extending from −54 to −57 kb 5′ of the transcriptional start. This 3-kb region directs intestinal expression of human apoB transgenes in mice (Fig. 1). Two experimental approaches were undertaken to further localize the intestinal element. The first method involved DNaseI hypersensitivity and revealed a DH site that mapped within this 315-bp segment in transcriptionally active CaCo-2 cells but not in transcriptionally inactive HeLa cells. The second approach involved testing the effects of various fragments from the ICR upon the transcriptional activity of the apoB promoter in transfection assays with CaCo-2 and HepG2 cells. This approach revealed a strong intestine-specific enhancer activity associated with the 315-bp EcoRI-HindIII segment that displayed hypersensitivity to DNaseI. Functional binding sites for the tissue-specific transcription factors HNF-3β, C/EBP-β, and HNF-4 were demonstrated within the 315-bp enhancer. The 315-bp human enhancer was sufficient to confer intestine-specific expression of the apoB gene in transgenic mice. A similar distal intestinal enhancer was isolated from the mouse apoB gene; it exhibited a high degree of sequence conservation in the region of the binding sites for the key transcriptional activators, suggesting that the mechanism for intestinal control of apoB transcription, as well as the distal location of the regulatory elements, have been evolutionarily conserved.

An interesting observation that emerged from these studies is that although the 315 IE exhibited similar enhancer activity in both orientations, the 5′-IE construct did not enhance when in the reverse orientation. We propose that this may be because of the presence of a boundary insulator element 5′ of the 315 IE within the 2.1-kb HindIII fragment and that the 3-fold repression observed is a manifestation of the enhancer blocking capability of the insulator when placed between the enhancer and the promoter (38). Using enhancer-blocking assays, we have
ATTCGCCAAT. Most C/EBP sites contain a well conserved half-site and a more divergent one. The most abundant and well characterized members of this family are C/EBPα, -β, -δ, and -γ; they can form homodimers or heterodimers in vitro. C/EBP proteins are only detected in differentiated hepatocytes, adipocytes, intestinal epithelial cells, pregranulocyte, and myeloblastic cell lines (40).

C/EBPβ bound to sites 2 and 4 of the 315-bp IE and increased the activity of the apoB enhancer. In contrast, although C/EBPα bound to sites 2 and 4, it repressed the activity of the intestinal enhancer. Our data are supported by the work of Oesterreicher, et al. (42), demonstrating that C/EBPα exerts a negative effect on transcription of apoB in the intestines of mice and that either a total absence of C/EBPα (such as that observed in null mice), or reduced levels (as in heterozygotes), leads to increased expression of apoB. The repressive effect may be caused by competition between C/EBPβ and C/EBPα for binding to sites 2 and 4. Our data in Fig. 6 indicate that C/EBPβ is the intestinal activator. Therefore, C/EBPα can either displace C/EBPβ from its two binding sites or it may form heterodimers with C/EBPβ, therefore impairing the activity of the enhancer. Competition for binding to sites 2 and 4 by these two C/EBP isoforms may reflect an in vivo developmental role for these proteins in the expression of apoB in the intestine. In rats, intestinal apoB mRNA levels vary during development. Thus, in fetal intestine, apoB mRNA levels are low until the last (21st) day of gestation, when it increases sharply. A large decrease is observed during the late suckling and weaning periods, followed by an increase to adult levels, to a level similar to that encountered at birth (43). A second mechanism for the repression of the enhancer activity by C/EBPα, observed in our in vitro studies with CaCo-2 cells, may involve competition between the two C/EBP isoforms for limiting coactivators such as P300, a known coactivator of C/EBPβ (44).

Results with the deletion mutants in Fig. 8 demonstrate a role for the HNF-3β, C/EBPβ, and HNF-4 binding sites in the activity of the 315-bp enhancer. This finding is reminiscent of the situation with the liver element of the human apoB gene. The hepatic core enhancer functions by synergetic binding and transactivation of three liver-enriched transcription factors, namely: HNF1α, C/EBPα, and protein II (23). Both the apoB liver and intestinal enhancers show a high degree of interspecies DNA sequence conservation, suggesting that the mechanisms of transcriptional activation by these enhancers have also been conserved. In the case of the intestinal element, the spatial location of the region with respect to the promoter has also been preserved between humans and mice, implying similar long range chromatin interactions. It is of interest to note that the high degree of sequence identity (85%) between the human ICR and the mouse ICR is restricted only to the core binding sequences for the transcription factors described here and that sequences immediately flanking this region diverge greatly. Although the 315 IE represents the only intestinal enhancer detected in transient transfection assays with segments from the −59 to −54-kb region, the possibility that other in vivo intestinal regulatory sequences may be present in the vicinity of this region cannot be ruled out.

Fragmentary information is available to date regarding all of the DNA elements and transcription factors involved in the intestinal transcription of other genes and of their mechanisms of interaction with each other and with the basal transcriptional machinery. Two issues merit consideration; the first is the question of whether a small group of intestine-enriched transcription factors are implicated in basal intestinal transcription as is the case for liver-specific genes, where various combinations of a few families of transcription factors (namely

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2 T. Antes and B. Levy-Wilson, unpublished results.
HNF-1, C/EBP, HNF-3, HNF-4, and HNF-6), in conjunction with various ubiquitous transcription factors, appear to be responsible for liver-specific transcription of a large number of genes. The second question is whether in vivo intestinal elements are often distant from the structural gene, as in the case of the apoB IE. For these comparisons, we discuss four intestine-specific genes: the sucrase isomaltase gene, the fatty acid-binding protein gene, and the apoAI/apoAIV genes. Work with the sucrase isomaltase gene has established that an intestine-specific transcription factor, Cdx-2, a member of the caudal family of homeodomain proteins (9), acts in conjunction with HNF-1α and HNF-1β. They bind to nearby sites within the proximal promoter region and play key roles in intestine-specific transcription of the sucrase isomaltase gene (45). For this gene, whose expression is confined exclusively to the intestine, the promoter alone is able to direct transcription in the intestine in vivo (46). In the case of the rat intestinal I-fatty acid-binding protein gene, again it would appear that promoter sequences extending up to −1200 bp upstream of the transcriptional start site are sufficient for correct, high level tissue-restricted expression of the gene in transgenic mice (7). The principal transcription factors involved in enterocyte transcription of I-fatty acid-binding protein are HNF-4 and ARP-1 (6). HNF-4 also plays a key role in the IE of the apoAI gene (47). Expression of apoAI human transgenes in the intestines of mice requires a 260-bp element localized at positions −780 to −520 of the apoAI gene (10). This fragment can function in either orientation to direct intestinal expression when it is placed 1.7 kb 3′ to the last exon of the apoAI gene. Finally, this same segment of the apoCIII enhancer that is implicated in apoAI intestinal expression is needed in combination with the −700 to −310 segment of the apoAIV promoter to direct a pattern of gene expression in transgenic mice similar to that of the endogenous apoAIV gene (11). Therefore, by comparison with the examples described above, we conclude that some of the same tissue-specific factors participate in intestinal expression of various genes; however, the apoB ICR is unique in that it is localized much further away from the structural gene than are the IEs for these other four genes.

The distant location of the apoB ICR instantly brings to mind the classical example of the human β-globin locus control region, localized at a similar distance from the globin gene locus. In this case, however, five erythroid-specific genes are arranged along the chromosome in the order in which they are expressed during development. Upstream of the cluster there are five DNaseI hypersensitive sites within a 20-kb region (β-locus control region). Each DNaseI hypersensitive site corresponds to the core region of separate elements containing numerous binding sites for ubiquitous and erythroid-restricted trans-acting factors. Each of the five separate elements of the
$\beta$-globin locus control region is responsible for activating one of the genes in the locus at the correct developmental stage in the correct cell type (for review see Ref. 48). This and other examples of distant locus control region suggest that some spatial separation between the structural genes and their regulatory elements may be advantageous, in the case of gene clusters, and whose components must be either differentially (2) or coordinately (49) expressed.

To date, there is no evidence of the presence of other genes in the vicinity of the apoB gene. Preliminary sequencing efforts have not uncovered other genes between the apoB gene and the ICR. However, earlier we demonstrated that deletion of the segment from 25 to 247 kb did not alter intestinal expression of the human apoB transgenes (26), implying that additional intestinal control elements are not present within this segment. Such a long segment of DNA between the ICR and the apoB gene may be required for the formation of a chromosomal loop that would bring the ICR in close proximity to the promoter. Thus, the apoB gene represents an intriguing model for studying the influence of chromatin structure on long range enhancer/promoter interactions, which mediate tissue-specific gene regulation.

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Identification and Characterization of a 315-Base Pair Enhancer, Located More than 55 Kilobases 5' of the Apolipoprotein B Gene, That Confers Expression in the Intestine

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