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Human Apolipoprotein B Transgenic Mice Generated with 207- and 145-Kilobase Pair Bacterial Artificial Chromosomes

EVIDENCE THAT A DISTANT 5′-ELEMENT CONFERS APPROPRIATE TRANSGENE EXPRESSION IN THE INTESTINE*

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We reported previously that ~80-kilobase pair (kb) P1 bacteriophage clones spanning either the human or mouse apoB gene (clones p158 and p649, respectively) confer apoB expression in the liver of transgenic mice, but not in the intestine. We hypothesized that the absence of intestinal expression was due to the fact that these clones lacked a distant DNA element controlling intestinal expression. To test this possibility, transgenic mice were generated with 145- and 207-kb bacterial artificial chromosomes (BACs) that contained the human apoB gene and more extensive 5′- and 3′-flanking sequences. RNase protection, *in situ* hybridization, immunohistochemical, and genetic complementation studies revealed that the BAC transgenic mice manifested appropriate apoB gene expression in both the intestine and the liver, indicating that both BACs contained the distant intestinal element. To determine whether the regulatory element was located 5′ or 3′ to the apoB gene, transgenic mice were generated by co-microinjecting embryos with p158 and either the 5′- or 3′-sequences from the 145-kb BAC. Analysis of these mice indicated that the apoB gene's intestinal element is located 5′ to the structural gene. Cumulatively, the transgenic mouse studies suggest that the intestinal element is located between −33 and −70 kb 5′ to the apoB gene.

The B apolipoproteins apoB48 and apoB100 play central roles in lipoprotein metabolism and are components of all lipoproteins considered to be atherogenic (1, 2). ApoB48 synthesis in intestinal enterocytes is required for the assembly of chylomicrons (1, 3), whereas apoB100 synthesis in liver hepatocytes is required for the generation of very low density lipoproteins (1). Of note, the basic role of apoB, to serve as the structural protein in the assembly of triglyceride-rich lipoproteins, is essentially the same in both the liver and the intestine.

Even though apoB plays the same functional role in hepatocytes and intestinal enterocytes and even though both cell types arise from primitive gut tissue during embryonic development (4), there is strong evidence to suggest that the genetic control of apoB gene expression in the liver is distinct from that in the intestine. To assess the effect of apoB overexpression on lipid metabolism, we generated human apoB transgenic mice with an 80-kb[1] P1 bacteriophage clone (p158) that spans the entire human apoB gene and contains 19 kb of 5′-flanking sequences and 17.5 kb of 3′-flanking sequences (5). Remarkably, the human apoB transgene was expressed at high levels in the liver, but not at all in the intestine. This pattern of expression was observed in many transgenic lines in our laboratory and was also documented in independent studies by another laboratory (6). Moreover, in human apoB transgenic rabbits that we generated with p158, transgene expression was high in the liver, but undetectable in the intestine (7). This absence of intestinal expression with the large p158 transgene was unexpected because transient transfection assays in intestinal or liver cell lines using apoB promoter-reporter gene constructs had suggested that as few as 260 bp of the apoB gene promoter were sufficient for apoB expression in the intestine and the liver (8). Moreover, we found the transgenic mouse expression data intriguing simply because the absence of intestinal transgene expression contrasted so sharply with the normal hepatic expression pattern. While transgene expression in the intestine was undetectable even by the most sensitive techniques (*i.e.* reverse transcription-polymerase chain reaction) (9), expression in the liver was robust and completely homogeneous (not variegated) and appeared to be copy number-dependent and position-independent (5, 6), all indications that this large clone contained all of the sequences required for appropriate gene expression.

More recently, we excluded the possibility that the absence of human apoB expression in the intestine resulted from our attempt to express a *human* transgene in another species. A P1 clone spanning the *mouse* apoB gene (including 33 kb of 5′-flanking sequences and 11 kb of 3′-flanking sequences) yielded high levels of expression in transgenic mouse liver, but absolutely none in the intestine, as judged by a sensitive transgene-specific RNase protection assay (10). In the latter study, the absence of intestinal transgene expression was underscored by mating the transgenic mice with apoB knockout mice. Mouse apoB transgenic mice that were homozygous for a knockout mutation in the endogenous apoB gene expressed the apoB

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[1] The abbreviations used are: kb, kilobase pair(s); bp, base pair(s); BAC, bacterial artificial chromosome; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

transgene in the liver, but lacked all apoB synthesis in the intestine, leading to a massive accumulation of fat within intestinal enterocytes (10). The results of these experiments, together with the earlier transgenic experiments with p158, suggested that distant DNA sequences, perhaps located >33 kb 5′ or >17.5 kb 3′ to the gene, might be required for the expression of apoB in the intestine. In this study, we sought to test the hypothesis that appropriate expression of apoB in the intestine is controlled by a distant regulatory element.

MATERIALS AND METHODS

BAC and P1 Bacteriophage Clones—Two BAC clones spanning the human apoB gene were identified by polymerase chain reaction screening of libraries of human genomic DNA (5, 11). A 207-kb clone, designated BAC(120,35) because it had 120 kb of 5′-flanking sequences and 35 kb of 3′-flanking sequences, was identified in a library constructed in pBAC108L; a 145-kb clone, designated BAC(70,22) because it had 70 kb of 5′-flanking sequences and 22 kb of 3′-flanking sequences, was identified in a library constructed in pBeloBAC11. The BAC clones were mapped by a combination of restriction endonuclease digestion, pulsed-field gel electrophoresis, Southern blot analysis, and automated DNA sequencing (10, 12, 13). Two *Not*I fragments of BAC(70,22) (a 70-kb fragment spanning from the 5′-polylinker *Not*I fragment in the BAC to the *Not*I site within intron 1 of the apoB gene and a 66-kb fragment spanning from the *Not*I site within intron 1 to the 3′-polylinker *Not*I site in the BAC) were purified from *Not*I-cleaved BAC(70,22) DNA and ligated into a *Not*I-cleaved and dephosphorylated P1 bacteriophage vector to generate two new P1 clones, P1–70 and P1–66 (see Fig. 1).

Preparation of DNA for Microinjection and Generation of Transgenic Mice—To prepare DNA for microinjection, BAC DNA was cleaved with *Nru*I or *Bss*HII (see Fig. 1), and P1 bacteriophage DNA was cleaved with *Nru*I or *Mlu*I. These enzymes cleave the vectors twice, but do not cleave the insert. The cleaved DNA was size-fractionated on a 1% low melting point pulsed-field agarose gel (Seaplaque GTG, FMC Corp. BioProducts, Rockland, ME). The segment of the gel containing the large DNA fragment was excised and digested with gelase (Epicentre Technologies Corp., Madison, WI) (14). The DNA solutions were adjusted to 3 ng/μl and used to microinject fertilized mouse eggs (C57BL/6J × SJL); co-injected fragments were 3 ng/μl each.

To identify transgenic mice, mouse plasma samples were screened with a radioimmunoassay specific for human apoB (5). Transgenic lines were established by mating founder animals with C57BL/6J mice. To generate transgenic mice lacking expression of the endogenous mouse apoB gene, BAC(70,22) transgenic mice were bred with heterozygous apoB knockout mice ($apoB^{+/-}$) (15). Transgenic mice that lacked mouse apoB expression (BAC(70,22)$apoB^{-/-}$) were identified by Southern blot analysis of tail DNA and by Western blot analysis of mouse plasma. Slot blot analysis with a ^{32}P-labeled 1857-bp *Bam*HI-*Eco*RI fragment from exon 26 of the human apoB gene (corresponding to apoB cDNA nucleotides 4650–6507) was used to assess transgene copy number.

To evaluate whether the ends of the BAC transgenes had integrated into the genome of transgenic mice, a 272-bp fragment of the BAC vector at the 5′-end of the linearized transgene was amplified by polymerase chain reaction with oligonucleotides 5′-GTATTCAGTGTCGCTGATTTG-3′ and 5′-CATTTAGTTATGACGAAGAAG-3′. In parallel, a 322-bp fragment of the BAC vector at the 3′-end of the transgene was amplified with oligonucleotides 5′-GTAATATCCAGCTGAACGGTCTG-3′ and 5′-CTGTGACGGAAGATCACTTCGCAG-3′. Finding both ends of the BAC vector in genomic DNA provided suggestive (but not definitive) evidence that the BAC transgene was intact within the chromosomal DNA.

Plasma Lipoprotein Analysis—Human apoB and mouse apoB in mouse plasma were detected by Western blot analysis of SDS-polyacrylamide gels (16). To detect human apoB, we used an antiserum to human apoB that had been absorbed against mouse apoB by passing it over a mouse apoB-Sepharose 4B column. To prepare the column, apoB-containing mouse lipoproteins ($d < 1.040$ g/ml) were coupled to CNBr-activated Sepharose 4B (Pharmacia Biotech Inc.) according to the manufacturer's instructions. An antiserum specific for mouse apoB was prepared by immunizing a human apoB transgenic rabbit (7) with purified mouse apoB (17).

RNase Protection Assays—Total cellular RNA was isolated from mouse tissues with the Totally RNA kit (Ambion Inc., Austin, TX). T7 RNA polymerase was used to prepare antisense mRNA transcripts from linearized plasmids. One human apoB probe spanned the first 121 nucleotides of exon 1 of the apoB gene; another probe was a 220-bp fragment of exon 26 (human apoB cDNA nucleotides 4487–6507). The mouse apoB probe was a 245-bp *Xba*I-*Msc*I fragment of exon 26 of the mouse apoB gene (17). A 316-bp mouse GAPDH probe was purchased from Ambion Inc. The RNase protection experiments were performed with the RPA II ribonuclease kit from Ambion Inc.; electrophoresis was performed on a 6% polyacrylamide gel containing 8.3 M urea (Boehringer Mannheim). Protected RNA fragments were visualized by autoradiography or with a PhosphorImager.

Immunocytochemistry—Mouse tissues were fixed in 4% paraformaldehyde and embedded in paraffin. Sections (4 μm thick) were mounted on glass slides. After deparaffinization in xylene, the sections were rehydrated; embedded in 10 mM citrate buffer, pH 6.0;, and boiled in a microwave oven for 3 min. The slides were incubated with phosphate-buffered saline, pH 7.4 (Sigma), with 0.5% casein and 0.1% Tween 20 for 30 min, followed by incubations first with an antiserum to mouse apoB (1:250), an antiserum to human apoB (1:250), or preimmune rabbit serum (undiluted); then with biotinylated goat anti-rabbit antibody (1:200; Vector Laboratories, Inc., Burlingame, CA); and finally with streptavidin-alkaline phosphatase (BioGenex Labs, San Ramon, CA). Finally, the slides were incubated with Vector Red (Vector Laboratories, Inc.) for 15 min and counterstained with hematoxylin.

In Situ Hybridization—Plasmid pBSSK*sma*K/X (18) was linearized with *Xba*I and *Kpn*I, respectively, and human apoB ^{35}S-riboprobes were synthesized with T7 (antisense probe) and T3 (sense probe) RNA polymerases (Promega, Madison, WI). To prepare the 245-bp mouse apoB ^{35}S-riboprobes, plasmid pBSmB245 (17) was linearized with *Xba*I and *Xho*I, respectively, and ^{35}S-riboprobes were synthesized with T7 (antisense probe) and T3 (sense probe) RNA polymerases.

In situ hybridizations of the ^{35}S-riboprobes were performed as described (19). Briefly, tissue sections were deparaffinized in xylene, followed by hydration and a 10-min fixation in 4% paraformaldehyde. After treatment with proteinase K (1 μg/ml in 500 mM NaCl and 10 mM Tris-HCl, pH 8.0) for 10 min, followed by a 10-min wash in 0.5 × SSC, tissue sections were prehybridized at 55 °C for 1–3 h in a buffer containing 50% formamide, 0.3 M NaCl, 20 mM Tris, pH 8.0, 5 mM EDTA, 1 × Denhardt's solution (Sigma), 10% dextran sulfate (Sigma), and 20 mM dithiothreitol. ^{35}S-Riboprobe and tRNA (300,000 cpm and 20 μg/100 μl of the prehybridization buffer, respectively) were then added and incubated at 55 °C for 14–18 h. Unbound ^{35}S-riboprobe was removed by washes with 2 × SSC containing 10 mM β-mercaptoethanol and 1 mM EDTA, followed by RNase A (Sigma) treatment. The slides were then further washed two times in 2 × and 0.1 × SSC, respectively. For autoradiography, the slides were dipped in Eastman Kodak NTB2 nuclear emulsion (diluted 1:1 with H_2O and prewarmed at 42 °C), air-dried for 2 h, and exposed for 4–8 weeks at 4 °C. The slides were developed at 15 °C using reagents from Kodak and stained with hematoxylin and eosin.

RESULTS

A 207-kb BAC that spanned the coding regions of the human apoB gene and contained 120 kb of 5′-flanking sequences and 35 kb of 3′-flanking sequences (BAC(120,35)) (Fig. 1) was used to generate human apoB transgenic mice. In one transgenic line, BAC vector sequences on both the far 5′- and 3′-ends of the transgene could be amplified from transgenic mouse tail DNA (Fig. 2). To investigate whether sequences within BAC(120,35) conferred intestinal expression of the apoB gene, RNA was prepared from the liver and duodenum of F1 transgenic mice and analyzed by RNase protection assays. In contrast to transgenic mice generated with the ~80-kb P1 bacteriophage clone (p158), which manifested transgene expression only in the liver, the BAC(120,35) transgenic mice expressed human apoB in both the liver and the duodenum (Fig. 3).

To localize further the DNA sequences that direct intestinal expression of apoB, we isolated a BAC that contained 70 kb of 5′-flanking sequences and 22 kb of 3′-flanking sequences (BAC(70,22)) (Fig. 1) and used it to generate additional lines of human apoB transgenic mice. A high-expressing line, with ~13 copies of the human apoB transgene integrated into the mouse genome, was chosen for extensive analysis. Both ends of the BAC(70,22) transgene could be amplified from mouse genomic DNA (Fig. 2). Moreover, Southern blot analysis of mouse genomic DNA that had been digested with *Not*I and size-fractionated on a pulsed-field agarose gel also indicated that the

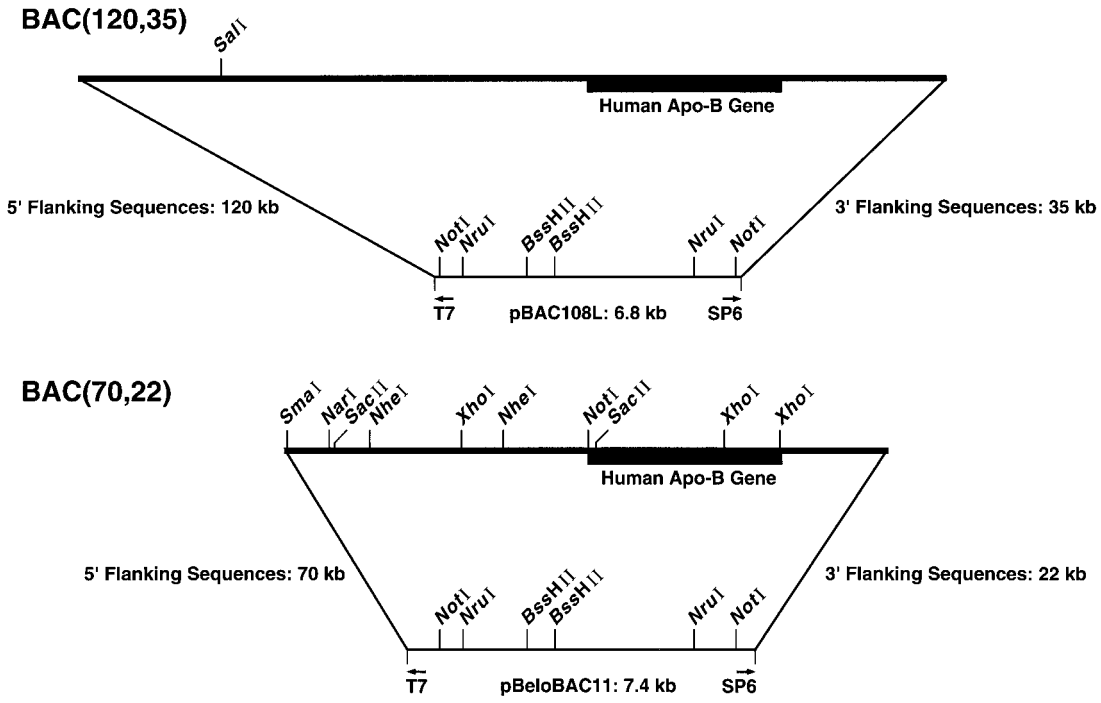

FIG. 1. **Map of two BAC clones spanning the human apoB gene.** BAC clones were mapped by a combination of restriction endonuclease digestion, pulsed-field gel electrophoresis, Southern blot analysis, and direct DNA sequencing.

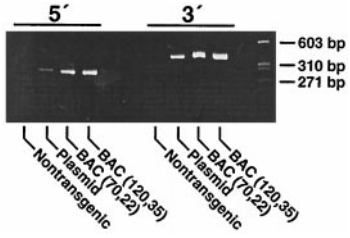

FIG. 2. **Assessment of the intactness of BAC transgenes within the genomic DNA of a transgenic mouse.** A 272-bp fragment at the 5′-end of the transgene and a 322-bp fragment at the 3′-end of the transgene were amplified from genomic DNA of BAC(70,22) and BAC(120,35) transgenic mice with BAC vector-specific primers. Amplification of these sequences from BAC(70,22) plasmid DNA was used as a positive control.

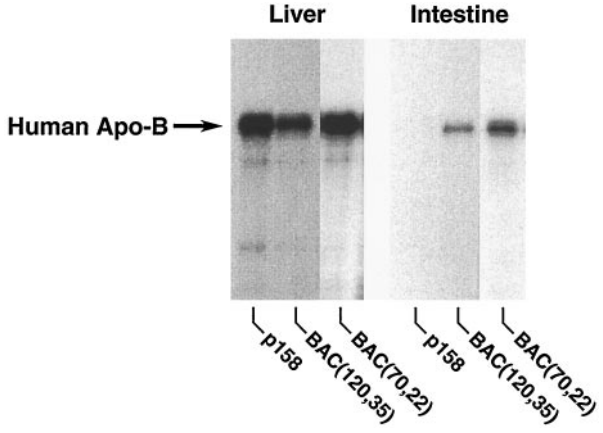

FIG. 3. **RNase protection assay illustrating human apoB expression in the liver and duodenum of BAC(120,35) and BAC(70,22) transgenic mice.** The RNase protection assay was performed with a 121-bp human apoB riboprobe and revealed that both of the BAC transgenes conferred expression in the liver (5 μg of RNA) and the intestine (5 μg of RNA), whereas the 80-kb P1 clone (p158) yielded expression only in the liver.

BAC(70,22) transgene was intact.[2] On a chow diet, this transgenic line had human apoB levels of ~60 mg/dl and high levels of low density lipoprotein cholesterol, as judged by fast-phase liquid chromatography analysis (Fig. 4). Furthermore, transgene expression in the liver hepatocytes was uniform, as judged by both *in situ* hybridization and immunohistochemistry (data not shown), indicating that the BAC(70,22) transgene directed fully appropriate apoB expression in the liver without transgene variegation.

RNase protection analysis of intestinal and liver RNAs from BAC(70,22) transgenic mice revealed abundant amounts of human apoB mRNA in both tissues (Fig. 3). To assess whether the BAC(70,22) transgene directed a spatially appropriate pattern of apoB gene expression in the intestine, the stomach-to-colon and the crypt-to-villus expression patterns of both the mouse and human apoB genes were examined. RNase protection assays demonstrated that the human apoB transgene was expressed at high levels in the duodenum and the jejunum and at lower levels in the ileum; transgene expression was not detectable in the colon or the stomach (Fig. 5*A*). This expression pattern was identical to that of the endogenous mouse apoB gene (Fig. 5*B*). As judged by a quantitative analysis of RNase protection assays with a PhosphorImager, the amounts of human apoB mRNA in the duodenum and the jejunum were 55 and 61% of that in the liver, respectively, whereas the corresponding amounts of endogenous mouse apoB mRNA in the duodenum and the jejunum were 56 and 51% of that in the liver, respectively.

The appropriateness of the BAC(70,22) transgene expression along the crypt-to-villus axis in the intestine was evaluated by analyzing the expression of both the human apoB transgene

[2] *Not*I cleaves BAC(70,22) in the vector sequences at the far 3′- and 5′-ends of the transgene and within intron 1 of the apoB gene, generating a 70-kb 5′-fragment and a 66-kb 3′-fragment (Fig. 1). In Southern blot experiments, we used a ^{32}P-labeled 1.1-kb *Hin*dIII-*Stu*I fragment from the apoB gene promoter region (*i.e.* 5′ to the intron 1 *Not*I site) (48) and a ^{32}P-labeled 2.7-kb *Hin*dIII fragment from exon 26 of the human apoB gene (*i.e.* 3′ to the intron 1 *Not*I site) (5) to confirm the presence of the two *Not*I fragments in genomic DNA of BAC(70,22) transgenic mice (data not shown).

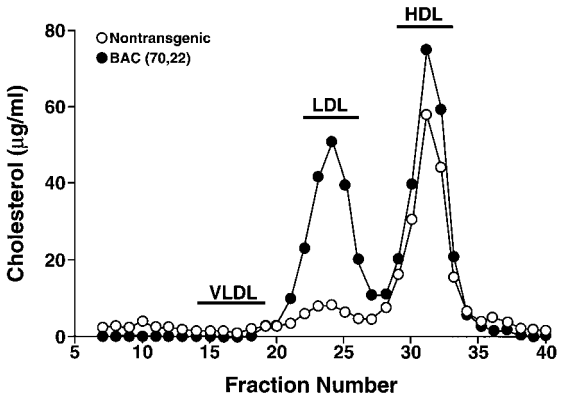

FIG. 4. **Distribution of cholesterol in the plasma of BAC(70,22) transgenic mice.** Plasma samples (200 μl) from a BAC(70,22) transgenic mouse and a nontransgenic mouse were subjected to fast-phase liquid chromatography on a Superose B6 10/50 column (Pharmacia Biotech Inc.) (5). The cholesterol content in plasma fractions was determined enzymatically (cholesterol high performance diagnostic system, Abbott). *VLDL*, very low density lipoprotein; *LDL*, low density lipoprotein; *HDL*, high density lipoprotein.

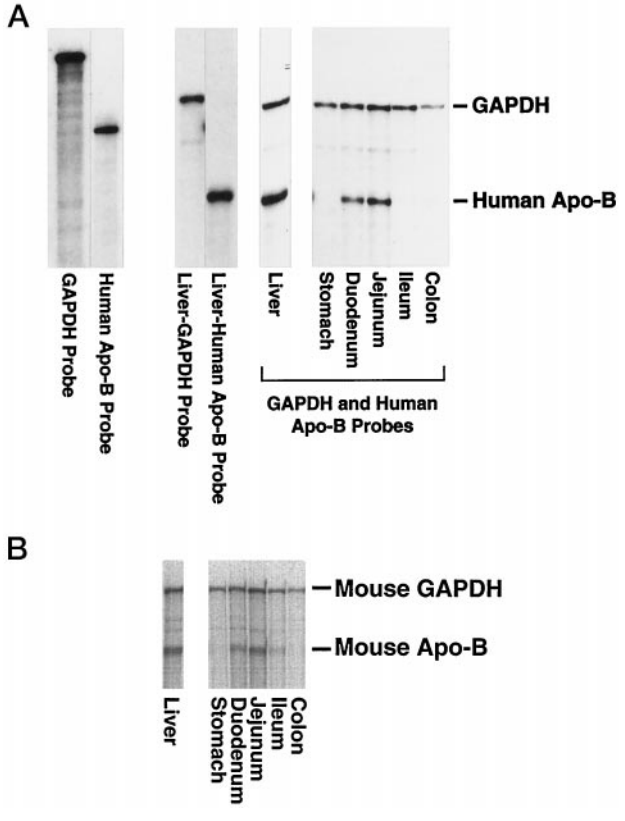

FIG. 5. **Expression of the human and mouse apoB genes along the stomach-to-colon axis.** *A*, RNA (10 μg), prepared from liver and intestinal sections of a BAC(70,22) mouse, was used in an RNase protection assay with a 220-bp human apoB riboprobe and a 316-bp mouse GAPDH riboprobe. *B*, RNA (10 μg), prepared from liver and intestinal sections of a nontransgenic mouse, was used in an RNase protection assay with a 245-bp mouse apoB riboprobe and a 316-bp mouse GAPDH riboprobe.

and the endogenous mouse apoB gene by *in situ* hybridization. Both the human and mouse apoB genes were expressed in enterocytes of the intestinal villi, but not in the crypts (Fig. 6).

To determine whether the absolute level of human apoB protein expression in the BAC(70,22) transgenic mice was physiologically appropriate, we mated those mice with heterozygous apoB knockout mice and ultimately generated transgenic mice lacking mouse apoB gene expression (BAC(70,22)*apoB*$^{-/-}$). The absence of mouse

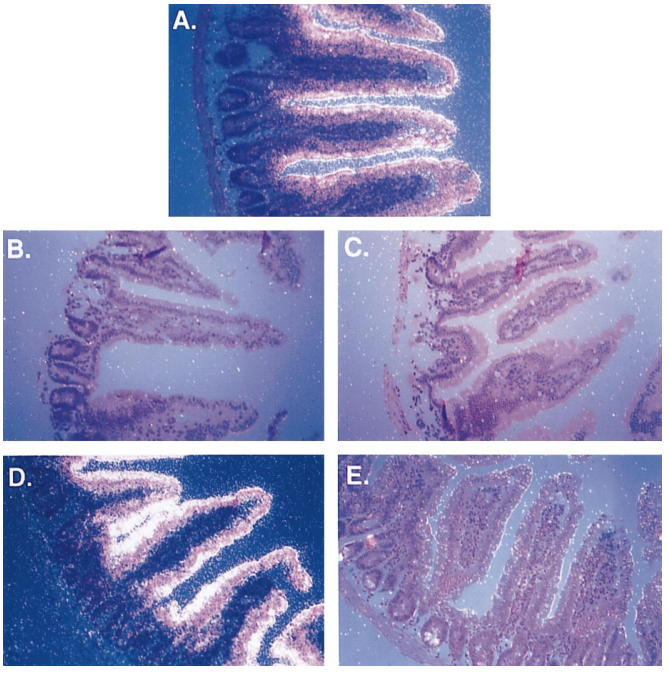

FIG. 6. **Crypt-to-villus expression patterns of the BAC(70,22) human apoB transgene and the endogenous mouse apoB gene.** *A* and *B*, *in situ* hybridizations of human apoB antisense and sense riboprobes, respectively, onto duodenal sections from a BAC(70,22) transgenic mouse; *C*, *in situ* hybridization of a human apoB antisense riboprobe onto a duodenal section from a nontransgenic mouse; *D* and *E*, *in situ* hybridizations of mouse apoB antisense and sense riboprobes, respectively, onto duodenal sections from a BAC(70,22) transgenic mouse.

apoB expression was established by Western blot analysis of mouse plasma with antibodies specific for human or mouse apoB (Fig. 7). In control experiments, we generated p158*apoB*$^{-/-}$ mice, which lacked all apoB expression (mouse or human) in the intestine. The p158*apoB*$^{-/-}$ mice had growth retardation and manifested a massive accumulation of fat within the villus enterocytes (Fig. 8). In contrast to the p158*apoB*$^{-/-}$ mice, the BAC(70,22)*apoB*$^{-/-}$ mice grew normally, and microscopic analysis of the intestines showed normal histology and no evidence of intestinal fat accumulation (Fig. 8). As expected from the results of the *in situ* hybridization analysis, immunohistochemical staining of intestinal sections of the BAC(70,22)*apoB*$^{-/-}$ mice revealed expression of human apoB in the villus enterocytes. Mouse apoB expression was undetectable (Fig. 9).

The fact that BAC(70,22) contained both more extensive 5′- and 3′-sequences than p158 meant that we could not draw conclusions regarding whether the intestinal element was located upstream or downstream from the structural gene. To address this issue, we subcloned the two *Not*I fragments from BAC(70,22) (Figs. 1 and 10*A*) into a P1 bacteriophage vector. P1–70 contained 70 kb of 5′-flanking sequences; P1–66 contained 22 kb of 3′-flanking sequences. Additional lines of transgenic mice were generated by co-microinjecting fertilized mouse eggs with p158 and either P1–70 or P1–66. Transgenic lines generated by co-microinjecting p158 with P1–70 expressed the human apoB gene at high levels in both the liver and the intestine (Fig. 10*B*). Southern blot analysis indicated that both DNA fragments had integrated into the mouse genome (Fig. 10*C*). In contrast to these results, human apoB transgenic mice generated by co-microinjecting p158 and P1–66 lacked apoB gene expression in the intestine (Fig. 11).

DISCUSSION

Soon after the apoB cDNA and gene were cloned (20–22), the function of the proximal promoter sequences of the apoB gene

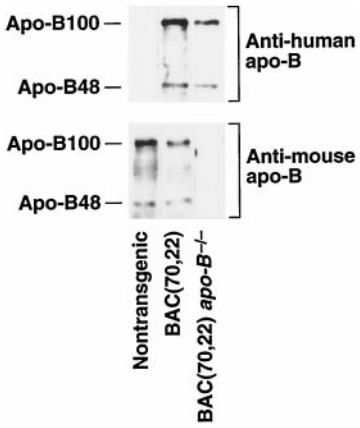

FIG. 7. **Western blot of the plasma from a BAC(70,22) transgenic mouse that was homozygous for a knockout mutation in the mouse apoB gene (BAC(70,22)*apoB*$^{-/-}$) using mouse and human apoB-specific antibodies.**

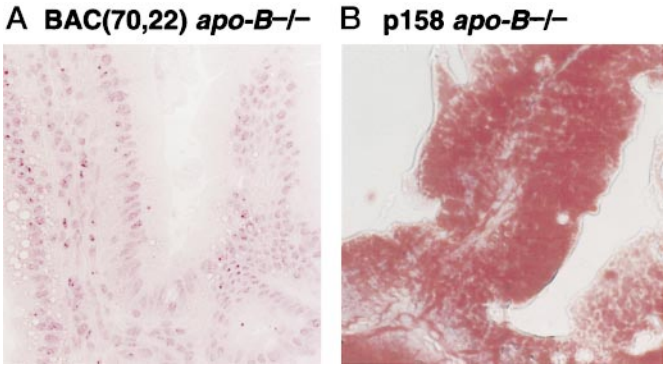

FIG. 8. **Oil red O-stained duodenal sections from human apoB transgenic mice that were homozygous for a knockout mutation in the mouse apoB gene.**

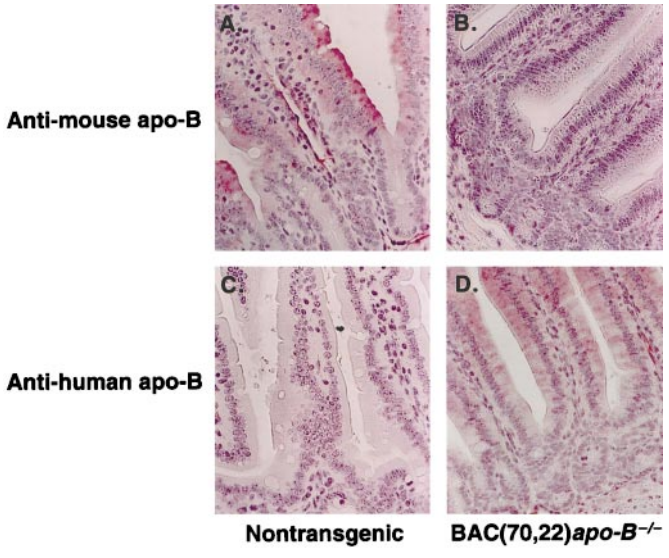

FIG. 9. **ApoB expression in the intestines of mice that express human apoB, but not mouse apoB.** Immunohistochemical staining is shown of apoB in sections of duodenum from a BAC(70,22) transgenic mouse that was homozygous for a knockout mutation in the endogenous apoB gene (*B* and *D*) or from a nontransgenic mouse (*A* and *C*). Sections were stained with a rabbit antiserum to mouse apoB (*A* and *B*) or with a rabbit antiserum to human apoB (*C* and *D*).

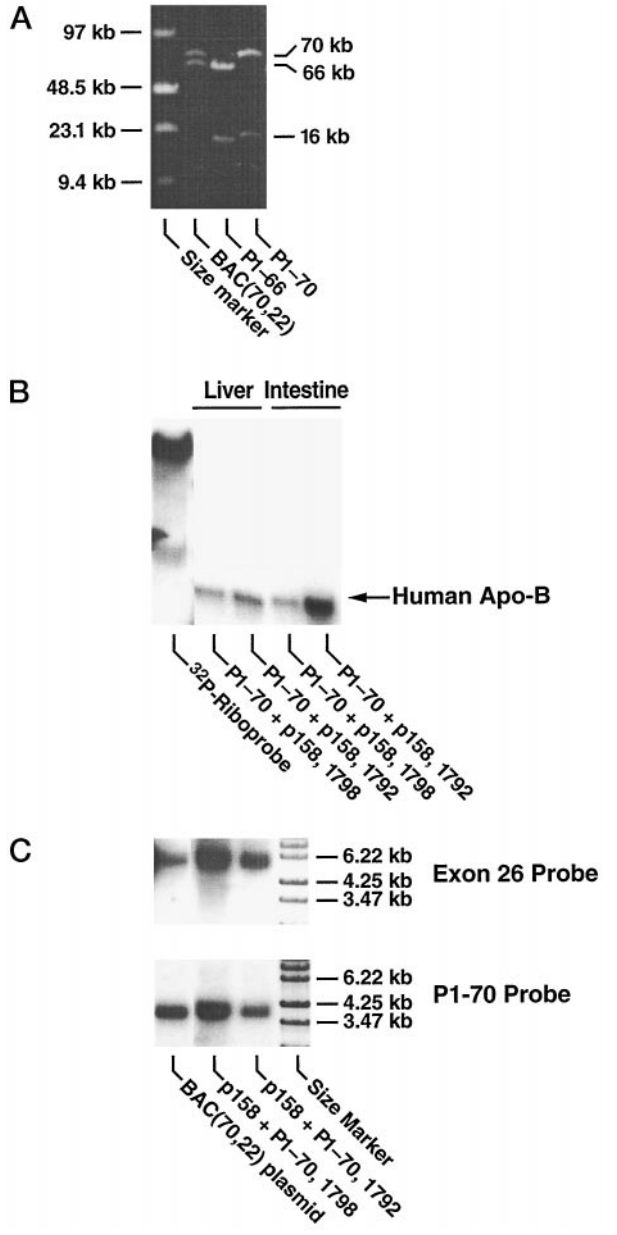

FIG. 10. **DNA sequences 5′ to the apoB gene direct intestinal expression in transgenic mice.** *A*, ethidium bromide-stained pulsed-field agarose gel of *Not*I-cleaved BAC(70,22), P1–70, and P1–66 DNAs; *B*, RNase protection assay with a 121-bp human apoB riboprobe demonstrating expression of human apoB in the liver (5 μg of RNA) and the intestine (25 μg of RNA) in two transgenic mouse lines (1792 and 1798) generated by co-microinjection of p158 and P1–70; *C*, Southern blot analysis of *Eco*RI-digested transgenic mouse tail DNA and BAC(70,22) plasmid DNA. Transgenic lines 1792 and 1798 were generated by co-microinjecting fertilized mouse eggs with p158 and P1–70. The Southern blot was probed with either a ^{32}P-labeled 1857-bp *Bam*HI-*Eco*RI fragment from exon 26 of the human apoB gene or with a ^{32}P-labeled ~4-kb *Eco*RI fragment located ~58 kb 5′ to the human apoB gene. The exon 26 probe hybridizes selectively to p158, whereas the P1–70 probe hybridizes selectively to P1–70. Both probes hybridize to BAC(70,22) plasmid DNA.

was evaluated by transient transfection of reporter gene constructs into liver and intestinal cell lines (HepG2 and CaCo2 cells) (8, 23). The results of these experiments suggested that 260 bp of upstream sequences were sufficient to direct the expression of the apoB gene in both the liver and the intestine. The conclusion that proximal promoter sequences might control expression in both tissues seemed plausible. The physiologic role of apoB is essentially the same in both tissues, and there are convincing precedents that proximal promoter sequences are sufficient to direct both liver and intestinal gene

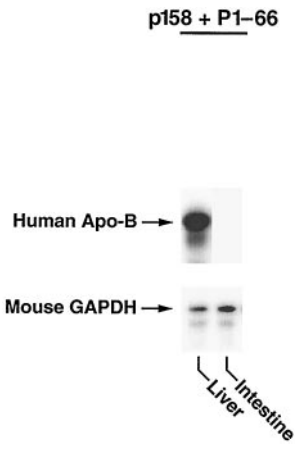

FIG. 11. **ApoB gene expression in a transgenic mouse generated by co-microinjection of p158 and P1–66.** Total liver RNA (5 μg) and total intestinal RNA (25 μg) were used in a RNase protection assay with a 121-bp human apoB riboprobe. To assess GAPDH expression, a total of 2 μg of RNA were used for both tissues in an RNase protection assay with a 316-bp mouse GAPDH riboprobe.

expression.[3] More recently, however, transgenic mouse expression studies with P1 bacteriophage clones have strongly suggested the possibility of a very different scenario for apoB: that the DNA sequence elements governing apoB gene expression in the intestine and the liver might be entirely distinct, with intestinal expression requiring a distant enhancer element, perhaps located >33 kb from the transcription initiation site of the apoB gene (3, 5, 6, 9, 17, 24, 25). In this study, we put that hypothesis to the test by generating human apoB transgenic mice from BACs containing more extensive 5′- and 3′-flanking sequences. The initial set of experiments demonstrated that a 207-kb BAC directed apoB expression in both the liver and the intestine, and subsequent experiments revealed that a much smaller (145-kb) BAC was sufficient for intestinal apoB gene expression.

To further localize the DNA sequences that direct intestinal expression of apoB, we co-microinjected p158 (which alone does not confer intestinal apoB expression) with DNA fragments containing either the 5′- or 3′-portion of BAC(70,22). When two DNA fragments are co-microinjected into mouse embryos, they typically integrate into the same site within the genome and co-segregate in subsequent breeding experiments (26). We chose the co-microinjection strategy because it had been used previously to examine a distant enhancer element for the immunoglobulin λ gene; co-microinjection of a cosmid containing the immunoglobulin λ gene coding region and a cosmid harboring a distant 3′-enhancer element yielded high-level, tissue-specific expression of the λ transgene in B lymphocytes of transgenic mice (27). In our studies, we found that transgenic mice generated by co-microinjection of p158 and P1–70 (containing 70 kb of 5′-flanking sequences) manifested robust human apoB expression in the intestine. When considered in combination with our previous P1 bacteriophage experiments, the current studies indicate that intestinal expression of the apoB gene requires a distant DNA sequence element located between 33 and 70 kb 5′ to the apoB gene (Fig. 12).

Gordon and co-workers (28–33) have provided important documentation that gene expression patterns in the intestine are regulated genetically on multiple levels: temporally during development and spatially along both the cephalocaudal axis and the crypt-to-villus axis. Moreover, the DNA sequences responsible for different spatial patterns of gene expression in the intestine can be entirely distinct and can involve both positive and negative elements. For example, Simon *et al.* (28), using reporter gene expression studies in transgenic mice, identified seven distinguishable *cis*-acting elements within the ~4 kb of sequences upstream from the *Fabpl*-coding sequences that affected the spatial pattern of *Fabpl* gene expression in the intestine. Remarkably, a reporter gene construct containing the entire 4 kb of upstream sequences directed inappropriate expression of the transgene in enterocytes of the colon, implying that additional sequence elements are important for the correct pattern of *Fabpl* gene expression. Also, transgenic mouse experiments with apoA-I genomic clones have demonstrated that an intestinal enhancer element, located ~9 kb downstream from the apoA-I gene (34, 35), is required for intestinal expression of the apoA-I gene. Unexpectedly, however, this enhancer element yielded transgene expression in the crypt cells and in neuroendocrine cells (sites where the apoA-I gene is normally silent) (34), implying that other, as yet unidentified regulatory elements were necessary for a fully appropriate pattern of apoA-I gene expression. In light of these studies, we considered it essential to determine whether the BAC clones contained sufficient sequences to yield *appropriate* patterns of apoB gene expression in the intestine. Our analysis of the BAC(70,22) mice in this study revealed that the intestinal expression pattern of the transgene was identical to that of the endogenous apoB gene, with high levels of expression in the villus enterocytes of the duodenum and the jejunum and no expression in the colon or the crypt cells. Moreover, the BAC clones yielded physiologically appropriate apoB expression, as judged by the fact that the transgenic mice lacking synthesis of endogenous *mouse* apoB grew normally and had no fat accumulation in intestinal enterocytes.

While this study provides definitive evidence that a distant element controls intestinal expression of the apoB gene, it offers no insight into why the intestinal regulatory sequences of apoB are located so far from the coding sequences or how this complex pattern of regulation evolved. There are, of course, precedents for control of gene expression by distant *cis*-acting regulatory elements (27, 35–42). The best characterized example is within the β-globin locus, where a locus control region located 6–22 kb 5′ to the human ε-globin gene controls the temporal and spatial expression of the β-globin family of genes (39, 43–47). Tissue-specific expression of growth hormone in the pituitary is controlled by interacting DNA sequences 15 and 30 kb 5′ to the five-member growth hormone gene cluster (37). A locus control region located 15 kb downstream from the apoE gene controls the hepatic expression of several genes in the apoE/apoC-I/apoC-II/apoC-IV locus (41, 42), and the aforementioned intestinal enhancer element for the apoA-I gene probably controls intestinal expression of more than one gene within the apoA-I/apoC-III/apoA-IV locus (34, 35). In each of these cases, the distant regulatory element occurred in the setting of a family of related genes that arose by ancient gene duplication events, and it is not difficult to imagine how these duplication events might place regulatory elements at a distance from the genes they control. In contrast, the apoB gene is not known to have any neighboring family members, and although it is possible that a functionally related gene might be present in the upstream or downstream sequences, no such gene has yet been identified. An obvious future goal is to determine whether related genes are present in the adjacent sequences and then to determine if those genes are expressed in the intestine and share regulatory sequences with the apoB gene. The BAC and P1 bacteriophage clones reported here will

[3] For example, an analysis of the promoter for the liver fatty acid-binding protein gene (*Fabpl*) in transgenic mice (28) revealed that as few as 153 bp of proximal promoter sequences were sufficient to confer expression in both the liver and the intestine.

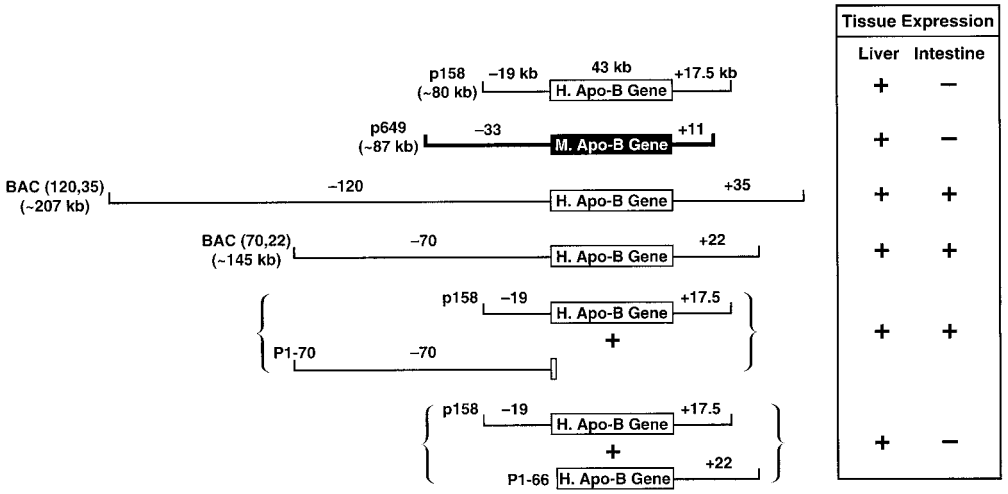

FIG. 12. **Summary of apoB gene expression patterns in transgenic mice generated with large genomic clones.** Shown is the expression of apoB in the livers and intestines of transgenic mice generated with ~80-kb P1 clones and 207- and 145-kb BAC clones and co-microinjection of two different P1 clones (p158 plus either P1–70 or P1–66). *H*, human; *M*, mouse.

be very useful for achieving this goal and for further localizing the sequences controlling intestinal apoB gene expression.

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