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

EVIDENCE THAT A DISTANT 5'-ELEMENT CONFER APPROPRIATE TRANSGENE EXPRESSION IN THE INTESTINE

(Received for publication, May 23, 1997, and in revised form, August 22, 1997)

Lars B. Nielsen‡§, Sally P. A. McCormick‡§, Vincenzo Pierotti‡, Carmen Tam§, Michael D. Gunn§, Hiromu Shizuya**, and Stephen G. Young‡§

From the *Gladstone Institute of Cardiovascular Disease, the ‡Cardiovascular Research Institute, and the §Department of Medicine, University of California, San Francisco, California 94141-9100 and the **Division of Biology, California Institute of Technology, Pasadena, California 91125

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 coinjecting 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 (1), 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 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, apoB 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
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 70 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 NotI fragments of BAC(70,22) (a 70-kb fragment spanning from the 5′-polylinker NotI fragment in the BAC to the NotI site within intron 1 of the apoB gene and a 66-kb fragment spanning from the NotI site within intron 1 to the 3′-polylinker NotI site in the BAC) were purified from NotI-cleaved BAC(70,22) DNA and ligated into a NotI-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—The microinjected DNA transgene was cleaved with NruI or BshHI (see Fig. 1), and P1 bacteriophage DNA was cleaved with NruI or MluI. 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 largest insert was excised and amplified in Escherichia coli (Life 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 (3). Transgenic lines were established by mating founder animals with C57BL/6J mice. To generate transgenic mice lacking expression of the 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 32P-labeled 1857-bp NruI fragment from the 5′-polylinker NotI fragment in the BAC to the NotI site within intron 1 of the human apoB gene and a 66-kb fragment spanning from the NotI site within intron 1 to the 3′-polylinker NotI site in the BAC was used to screen transgenic C57BL/6J × SJL mice (15). To investigate whether sequences within BAC(120,35) could be amplified from transgenic mouse tail DNA (Fig. 2), the transgene could be amplified from transgenic mouse tail DNA (Fig. 2). To investigate whether sequences within BAC(120,35) could be amplified from transgenic mouse tail DNA (Fig. 2), the transgene could be amplified from transgenic mouse tail DNA (Fig. 2). To investigate whether sequences within BAC(120,35) could be amplified from transgenic mouse tail DNA (Fig. 2), the transgene could be amplified from transgenic mouse tail DNA (Fig. 2). To investigate whether sequences within BAC(120,35) could be amplified from transgenic mouse tail DNA (Fig. 2), the transgene could be amplified from transgenic mouse tail DNA (Fig. 2). To investigate whether sequences within BAC(120,35) could be amplified from transgenic mouse tail DNA (Fig. 2), the transgene could be amplified from transgenic mouse tail DNA (Fig. 2). 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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 NotI and size-fractionated on a pulsed-field agarose gel also indicated that the
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. 5A). This expression pattern was identical to that of the endogenous mouse apoB gene (Fig. 5B). As judged by a quantitative analysis of RNase protection assays with a PhosphorImager, the amounts of human apoB mRNA in the duodenum were 55 and 61% of that in the liver, respectively, whereas the amounts of endogenous mouse apoB mRNA in the duodenum 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

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2 NotI cleaves BAC(70,22) in the vector sequences at the 5’- and 3’-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 32P-labeled 1.1-kb HindIII-StuI fragment from the apoB gene promoter region (i.e. 5’ to the intron 1 NotI site) (48) and a 32P-labeled 2.7-kb HindIII fragment from exon 26 of the human apoB gene (i.e. 3’ to the intron 1 NotI site) (5) to confirm the presence of the two NotI fragments in genomic DNA of BAC(70,22) transgenic mice (data not shown).
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 knock-out mice and ultimately generated transgenic mice lacking mouse apoB gene expression (BAC(70,22)apoB22/2). The absence of 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 p158apoB22/2 mice, which lacked all apoB expression (mouse or human) in the intestine. The p158apoB22/2 mice had growth retardation and manifested a massive accumulation of fat within the villus enterocytes (Fig. 8). In contrast to the p158apoB22/2 mice, the BAC(70,22)apoB22/2 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)apoB22/2 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 NotI fragments from BAC(70,22) (Figs. 1 and 10A) 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. 10B). Southern blot analysis indicated that both DNA fragments had integrated into the mouse genome (Fig. 10C). 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
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 expression.
expression. 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 e-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.
be very useful for achieving this goal and for further localizing the sequences controlling intestinal apoB gene expression.

**Acknowledgments**—We thank J. Ng for help with mouse breeding, L. Prentice for preparing tissue sections for *in situ* hybridization, L. Flynn for the rabbit antiserum to mouse apoB, J. Carroll and A. Corder for graphics, and S. Ordway and G. Howard for editorial assistance.

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