

## Analysis of the Apolipoprotein B Gene and Messenger Ribonucleic Acid in Abetalipoproteinemia

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### Abstract

The apolipoprotein B-100 (apoB-100) gene in leukocytes and the apoB-100 messenger RNA (mRNA) and translated apolipoprotein in the livers from normal and abetalipoproteinemic individuals were evaluated. Four complementary DNA probes for apoB-100 covering the 5', middle, and 3' regions of the apoB-100 mRNA were utilized and Southern blot analysis indicated that the apoB-100 gene is present in abetalipoproteinemia without major insertions or deletions. Polyadenylated hepatic apoB-100 mRNA from two abetalipoproteinemic patients was normal in size, and the concentration of apoB-100 mRNA was increased sixfold compared with control hepatic apoB-100 mRNA levels. ApoB-100 was detected in hepatocytes of abetalipoproteinemic patients by immunohistochemical techniques. These results indicate that the biochemical defect in abetalipoproteinemic patients studied is most consistent with a posttranslational defect in apoB-100 processing or secretion with an up-regulation of the apoB-100 mRNA.

### Introduction

Abetalipoproteinemia (ABL)<sup>1</sup> was first described by Bassen and Kornzweig in 1950 (1). Initially the disease was considered a form of heredoataxia accompanied by retinitis pigmentosa and erythrocyte abnormalities. The lack of detectable plasma apolipoprotein B (apoB) in affected individuals was discovered a decade later (2). The disease is transmitted as an autosomal recessive trait. Homozygous subjects are characterized by the virtually complete absence of both forms of plasma apoB, apoB-100, and apoB-48, as well as the apoB-containing lipoproteins, chylomicrons, very low density lipoproteins (VLDL), and low density lipoproteins (LDL) (3). Heterozygotes have normal to slightly increased plasma apoB levels. A similar dyslipoproteinemia, hypobetalipoproteinemia, is also characterized by a defi-

ciency of plasma apoB in the homozygous state, but distinguished by the reduced plasma apoB levels in the obligate heterozygotes (3). Recently, a third variant of apoB deficiency, normotriglyceridemic ABL, was reported, which is characterized by the presence of plasma apoB-48 but not apoB-100 (4).

The symptoms of ABL are a sequelae of the absence of apoB-containing plasma lipoproteins. Malabsorption results from the inability of the intestinal cells to secrete absorbed lipids as chylomicrons into the lymphatics leading to a lipid engorged small intestinal mucosa. Lipid malabsorption results in deficiencies of vitamins A, E, and K. Vitamin D is not affected because the luminal phase of absorption is not impaired, and vitamin D has its own carrier protein independent of chylomicron formation (5). Clotting abnormalities are present, but are usually not clinically significant (6). The neurologic syndrome present in ABL is similar to Friedreich's ataxia. However, the development and progression of the neurological symptoms may be retarded or eliminated by vitamin E supplementation. The retinal degeneration in ABL is a pigment degeneration and has also been attributed to vitamin E deficiency (7, 8).

Previous studies have reported that apoB cannot be detected in the plasma of patients with ABL (3). In addition, immunohistochemical studies on intestinal mucosa from ABL patients revealed no detectable apoB in the mucosal cells (9). These studies have been interpreted as indicating that there is a defective synthesis of both apoB-100 and apoB-48.

We have recently determined the complete complementary DNA (cDNA) and derived amino acid sequence of apoB-100. The mRNA of apoB-100 is 14.1 kilobase pair (kb), and codes for a protein of 4,536 amino acids with an amino acid molecular weight of 512,723 (10–12). The cloning of human apoB-100 has enabled us to analyze both the apoB-100 gene as well as the messenger RNA (mRNA) in ABL and compare it with the normal apoB-100 gene.

### Methods

**RNA preparation.** Liver tissue was obtained from an ABL patient post-mortem and by needle biopsy from a second patient, as well as from normal subjects, at the time of organ donation. The tissue was stored frozen at  $-70^{\circ}\text{C}$  until further used. RNA was isolated by the guanidinium thiocyanate procedure (13). Briefly, liver tissue was ground to a fine powder on dry ice, and homogenized in the presence of 4 M guanidinium thiocyanate. The homogenate was clarified by centrifugation at 10,000 g for 10 min. The supernatant was layered on a 5.7 M CsCl cushion and centrifuged in a Beckman SW 50.1 rotor at 36,000 rpm for 18 h. The RNA pellet was resuspended in  $\text{H}_2\text{O}$ , ethanol precipitated (two times), and reconstituted at 5 mg/ml in  $\text{H}_2\text{O}$ . Poly(A)<sup>+</sup> RNA was prepared by affinity chromatography over oligo(dT)-cellulose (14).

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1. *Abbreviations used in this paper:* ABL, abetalipoproteinemia; apo, apolipoprotein; apoB, apoB-48, and apoB-100, apolipoproteins B, B-48, and B-100, respectively; kb, kilobase pair; SSC, 150 mM NaCl, 15 mM Na citrate, pH 7.2; TBS, Tris-buffered saline.

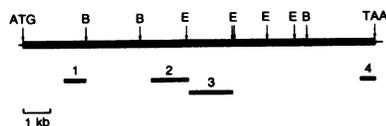
**DNA preparation.** DNA from leukocytes was prepared as previously described (15). To obtain DNA from liver, frozen liver tissue was ground and lysed in a buffer containing 0.5 M EDTA and 0.5% Sarcosyl. Proteinase K was added to achieve a final concentration of 200  $\mu\text{g}/\text{ml}$ , and the mixture incubated at 50°C for 3 h. The lysate was extracted three times with phenol, and dialyzed extensively against 50 mM Tris-HCl (pH 8.0), 10 mM EDTA, and 10 mM NaCl. RNaseA was added to a final concentration of 100  $\mu\text{g}/\text{ml}$  and the sample incubated at 37°C for 3 h. After one extraction each with phenol (1 vol), 0.5 vol phenol and 0.5 vol chloroform/isoamylalcohol (24:1), and finally 1 vol chloroform/isoamylalcohol, the DNA was precipitated with ethanol and resuspended at  $\sim 0.5$  mg/ml in 10 mM Tris-HCl pH (8.0) and 1 mM EDTA.

**cDNA probes.** Double-stranded-cDNA probes were prepared from four separate apoB-100 cDNA clones as previously described (10). The apoB-100 clones were designated as  $\lambda\text{MDB-1}$  (probe 1),  $\lambda\text{MDB-3}$  (probe 2),  $\lambda\text{MDB-4}$  (probe 3), and pMDB-6 (probe 4) (Fig. 1). Probe  $\lambda\text{MDB-1}$  is 1.9 kb from the 5' end of the apoB-100 mRNA. Probes  $\lambda\text{MDB-3}$  and  $\lambda\text{MDB-4}$  are 5 and 6.4 kb from the 5' end of the apoB-100 mRNA. Probe pMDB-6 is located at the 3' end of the apoB-100 mRNA (Fig. 1). The probes were labeled by nick translation with [ $\alpha$ - $^{32}\text{P}$ ]dCTP to a specific activity of  $\sim 3 \times 10^8$  dpm/ $\mu\text{g}$ .

**Northern blot and dot blot analysis of RNA.** Total and poly(A)<sup>+</sup> RNA were size fractionated by 1% agarose gel electrophoresis in the presence of 6% formaldehyde. Transfer of RNA to nitrocellulose (Schleicher & Schuell, Inc., Keene, NH) was performed as described (16, 17). For dot blots, total RNA was spotted in serial dilutions on nitrocellulose paper using a Schleicher & Schuell manifold. After baking at 80°C for 2 h, filters were prehybridized in 5  $\times$  SSC, 5  $\times$  Denhardt's solution (0.04% bovine serum albumin, 0.04% Ficoll 400, 0.04% polyvinylpyrrolidone), 0.1% sodium dodecyl sulfate (SDS), 20 mM Na<sub>2</sub>PO<sub>4</sub> (pH 7.0), 100  $\mu\text{g}/\text{ml}$  salmon sperm DNA, and 50% formamide for 4 h at 42°C. Hybridization was performed in the same solution with 10<sup>7</sup> dpm/filter of the cDNA probe added for 48 h at 42°C. Subsequently, filters were washed twice in 3  $\times$  SSC at room temperature for 30 min, followed by one wash each at 65°C for 30 min in 1  $\times$  SSC and 0.1% SDS, followed by 0.5  $\times$  SSC and 0.1% SDS. For dot blots, another 30-min wash in 0.2  $\times$  SSC and 0.1% SDS at 65°C was added to decrease background radioactivity. Filters were autoradiographed; dot blots were scanned by laser densitometry, the dots cut, and the radioactivity quantitated in a scintillation counter.

**Southern blot analysis of DNA.** 10  $\mu\text{g}$  of DNA were cleaved with restriction enzymes (Bethesda Research Laboratories, Gaithersburg, MD, Boehringer Mannheim Biochemicals, Indianapolis, IN, and New England Biolabs, Beverly, MA) at 5 U/ $\mu\text{g}$  for 3 h under the conditions specified by the suppliers. The DNA fragments were separated by 0.7% agarose gel electrophoresis at 25 V for 16 h. Southern transfer to nitrocellulose paper was performed as described (15). Prehybridization was performed in 6  $\times$  SSC, 5  $\times$  Denhardt's, 0.1% SDS, and 100  $\mu\text{g}/\text{ml}$  salmon sperm DNA for 4 h at 65°C. Hybridization was carried out in the same solution with 10<sup>7</sup> dpm/filter of the labeled cDNA probe. The filters were rinsed briefly in 2  $\times$  SSC and by one wash each in 2  $\times$  SSC and 0.1% SDS followed by 1  $\times$  SSC and 0.1% SDS at 65°C for 30 min. Subsequently the filters were autoradiographed for 3–5 d at  $-70^\circ\text{C}$  with intensifying screens.

**Immunohistochemistry.** Liver tissues from normal controls and ABL patients were fixed in Bouin's fixative for 4 h, washed overnight in running tap water, and embedded in paraffin. 4  $\mu\text{m}$ , paraffin-embedded sections



**Figure 1.** Schematic map of the apoB-100 mRNA, and the four cDNA probes utilized in the analysis of the apoB-100 gene in ABL. Probes 1–4 are  $\lambda\text{MDB-1}$ ,  $\lambda\text{MDB-3}$ ,  $\lambda\text{MDB-4}$ , and pMDB-6, respectively. B, Bam HI; E, Eco RI.

were dried and deparaffinized in fresh, completely paraffin-free alcohols and xylene. Endogenous peroxidase activity was eliminated by incubation in 3% hydrogen peroxide in absolute methanol for 3 min at 30°C. Sections were subsequently brought to distilled water through graded alcohols, treated with 10% egg albumin (to block nonspecific antibody binding), rinsed in Tris-buffered saline (TBS), and incubated in the primary antiserum that contained a mix of four mouse monoclonal apoB antibodies (1:1:1:1, ABB1, ABB2, ABB3, and ABB5, Canadian Bioclinical, Ltd., Scarborough, Ontario, Canada).

After overnight incubation at 4°C, the primary antibody was rinsed from the slide with TBS containing 1% albumin, and incubated with a peroxidase coupled rabbit anti-mouse IgG antibody (Cappel Laboratories, Malvern, PA) for 30 min at 20°C, and rinsed with TBS. Sections were then covered with freshly prepared diaminobenzidine colorimetric reagent (0.3 mg/ml in TBS and 0.18% H<sub>2</sub>O<sub>2</sub>) for 10 min (18), rinsed with deionized water, and counterstained with hematoxylin. Sections were then dehydrated through graded alcohols, coverslipped, and photographed.

## Results

The four ABL patients analyzed in this report had no detectable plasma apoB by radioimmunoassay. These patients presented with characteristic neurological and ophthalmological signs and symptoms of ABL including spinocerebellar ataxia and retinal pigment degeneration with decreased visual acuity as well as acanthocytosis. The four patients studied were from three unrelated kindreds, and their parents, obligate heterozygotes, did not have any detectable lipoprotein abnormalities. The two ABL patients whose RNAs were analyzed have been described in detail previously (19, 20).

Southern blots with the four cDNA probes revealed no major differences in the DNA from the ABL patients and normolipidemic subjects (Figs. 2 and 3). The restriction fragments analyzed cover  $\sim 30$  kb. Probe  $\lambda\text{MDB-1}$  hybridizes to more than one band with the four enzymes used to cleave the DNA. None of these enzymes have sites within the probe, which suggests that the probe contains sequences from at least two exons. The same is true for  $\lambda\text{MDB-4}$  (Figs. 2 and 3), whereas  $\lambda\text{MDB-3}$  (Fig. 3) as well as pMDB-6 (data not shown) hybridize to only one genomic DNA fragment in each digest.

Northern blot analysis of liver poly(A)<sup>+</sup> RNA was performed on two ABL patients and a normal control. Both ABL patients gave similar results, and a representative Northern blot of one of the ABL patients and a control is illustrated in Fig. 4. These results established that ABL hepatocytes contain an apoB-100 mRNA which is similar in size to the apoB-100 mRNA of normal controls.

To obtain a more precise estimate of the levels of hepatic apoB-100 mRNA which are not affected by partial degradation or variability of RNA transfer, we analyzed normal and ABL total hepatic RNA by dot blot hybridization. Varying quantities of normal and ABL total RNA were applied directly to nitrocellulose paper. Fig. 5 depicts dot blots of total RNA from the livers of two ABL patients and three normal livers utilizing the  $\lambda\text{MDB-1}$  cDNA probe. Subsequent to autoradiography, the dot blots were quantitated by laser densitometry. In normal liver the apoB-100 mRNA level was  $17 \pm 1.3$  U (mean of triplicate samples  $\pm$  SD, arbitrary absorbancy units). The apoB-100 mRNA in the ABL liver was 98 units. Thus, the liver from the ABL patients contained approximately six times the quantity of apoB-100 mRNA as normal liver. These results not only confirmed the Northern blot data that the liver from ABL patients contains apoB-100 mRNA, they also indicated that the apoB-100 mRNA is present in increased concentration.

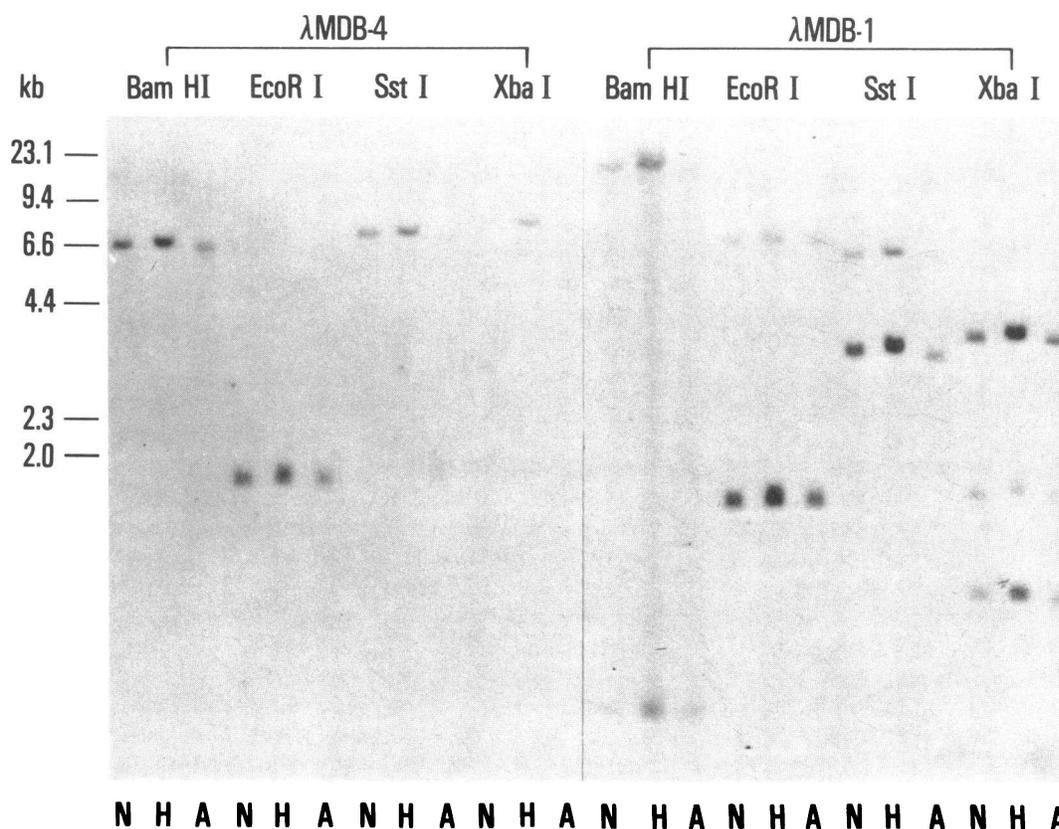


Figure 2. Southern blot of normal and ABL DNA. Genomic DNAs of a normal subject (N), ABL patient (A), and an obligate heterozygote (H) were cleaved with Bam HI, Eco RI, Sst I, and Xba I. ApoB-100

probes λMDB-1 and λMDB-4 were used for hybridization. There is no significant difference between the restriction enzyme fragments obtained from normal and the ABL subjects.

Immunohistochemistry was performed on liver tissue from two ABL subjects utilizing either a polyclonal or a mixture of four monoclonal apoB-100 antibodies. In normal liver apoB is readily detected and the apoB immunoreactivity is present exclusively in hepatocytes. The portal triads do not contain any apoB. The same pattern is seen in liver tissue from ABL patients, however, the reaction product appears to be more intense. When preimmune serum or mouse ascitic fluid is applied, no reaction product is detectable. The specificity of the monoclonal antibodies has been extensively tested (21). Fig. 6 illustrates the presence of apoB-100 in the hepatocytes of one ABL patient analyzed with the monoclonal antibodies. The results with the polyclonals are identical. The polyclonal antibodies are specific for apoB-100 and apoB-48, and show no reactivity with any other human apolipoprotein (11).

### Discussion

We have shown by Southern blot analysis that the gene for apoB-100 is present in ABL. The gene does not contain any abnormalities that could be detected by the Southern blot technique. We interpreted these results as indicating that there is no major insertion or deletion within this region in the apoB-100 gene of ABL patients. Analysis of the restriction fragments revealed that our probes covered ~30 kb of the apoB-100 gene. The probes are located at the 5', middle, and 3' region of the gene.

Further evidence for the absence of a major defect in the apoB-100 gene was obtained by Northern blot analysis of liver mRNA, which revealed the presence of an apoB-100 mRNA in

ABL liver of similar size as normal apoB-100 mRNA. The somewhat lower amount of the full length transcript (14.1 kb) is biased due to the use of poly(A)<sup>+</sup> mRNA. Partially degraded mRNA will not be analyzed in these samples. When total RNA from the livers of two ABL patients was quantitated by dot blot hybridization, a sixfold elevation of apoB mRNA was detected in both samples (Fig. 5).

The presence of an apoB-100 message of normal size indicates that there are no major deletions or insertions within the exon sequences, and that normal splicing takes place after transcription of the gene. Dot blot hybridization revealed that the apoB-100 mRNA is present in much higher concentrations in the liver of ABL patients than in normal liver. The increase in apoB-100 mRNA level is ~5-6-fold. These data provide evidence that there is an increased concentration of the apoB-100 mRNA, presumably in response to the lack of apoB-100 in the circulation. These results indicate that ABL is not due to a promoter or enhancer defect in the apoB-100 gene. Finally, the presence of apoB-100 in the ABL hepatocytes establishes that the apoB-100 mRNA is translated after it has been spliced and exported from the nucleus to the cytoplasm.

Based on these results we now have evidence that the apoB-100 gene is present and transcribed in ABL. Neither Southern nor Northern blots reveal any insertions or deletions within the gene, nor do they provide any evidence for a splicing defect. Furthermore, the mRNA for apoB-100 is considerably increased in ABL as compared with normal. Finally, the apoB-100 mRNA is translated, and a protein is synthesized that reacts with monoclonal antibodies against apoB-100. This protein is found only

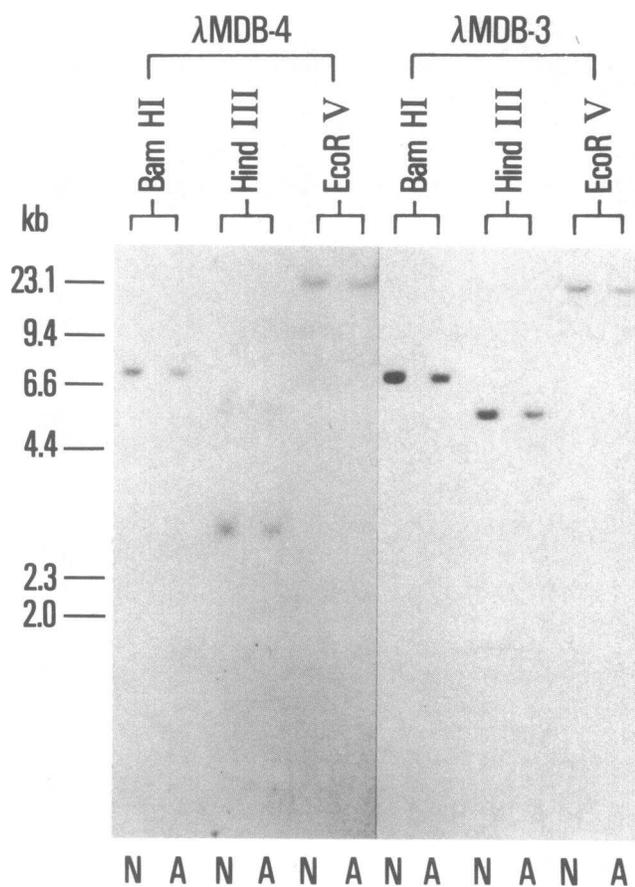


Figure 3. Southern blot of normal and ABL DNA. Genomic DNA of a normal subject (N) and an ABL patient (A) were cleaves with Bam HI, Hind III, and Eco RV. Probes λMDB-3 and λMDB-4 were used for hybridization.

within the hepatocytes, and does not appear to be secreted into the circulation in appreciable amounts.

The two most important findings of this study are the massive increase of apoB-100 mRNA in the ABL hepatocytes, and the synthesis of apoB, or an apoB-like protein, which is not secreted by these cells. The first result suggests that the apoB-100 gene may be quite considerably up-regulated, and an abnormally low plasma LDL concentration may be an appropriate stimulus for this regulation. There is also evidence that ABL is not due to a promoter or enhancer defect, because in that case an abnormally low mRNA level would be anticipated. The second result strongly suggests a defect in the secretory process of apoB-100. It is also proof that the mRNA reaches the cytoplasm and is translated. There are several potential explanations for the defective secretory pathway of apoB-100. The protein might be structurally abnormal, and not able to be processed properly for secretion. It is unlikely that a single amino acid substitution would impair lipid binding so profoundly that apoB could no longer be incorporated into VLDL particles (22). However, amino acid substitutions in apoB may result in defective posttranslational modification. Since apoB has been previously reported to contain a carbohydrate moiety (23, 24), a defect in carbohydrate addition may be a potential explanation for defective secretion. Alternatively, there could be premature termination of apoB-100 due to a single base substitution. A subtle insertion, deletion, or small splicing defect could also cause a frame-shift leading to premature termination. On the other hand, apoB-100 may undergo normal

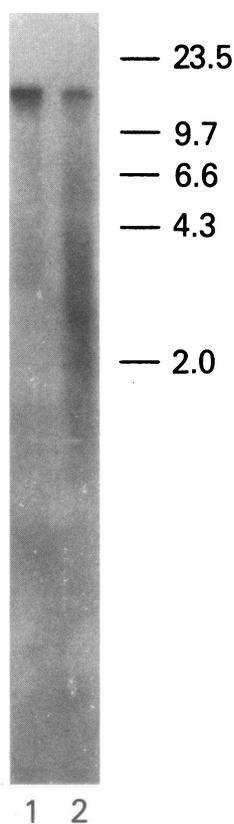


Figure 4. Northern blot analysis of poly(A)<sup>+</sup> RNA isolated from the livers of a normal subject (lane 1) and a patient with ABL (lane 2). The mRNA in both the normal and ABL subjects is ~14 kb.

posttranslational processing, and the defect is located in the chain of events leading to secretion of new VLDL particles; e.g., in intracellular receptor interaction or assembly of lipoprotein particles.

Thus, we conclude from the results of our analysis of the apoB gene, its mRNA, and the apoB content of the hepatocytes

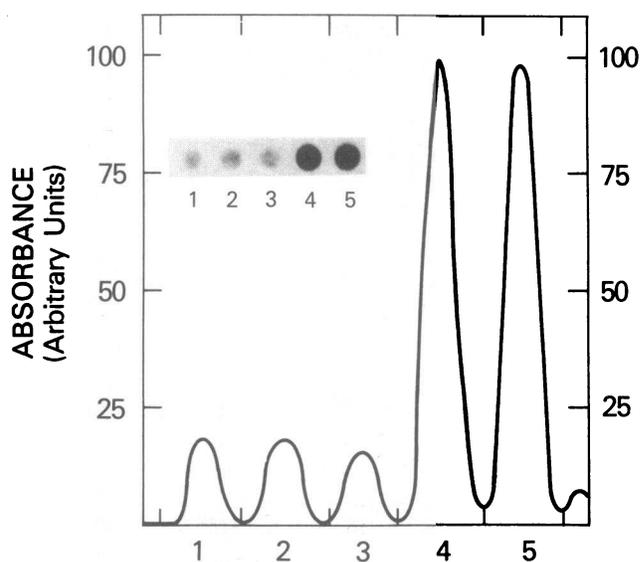
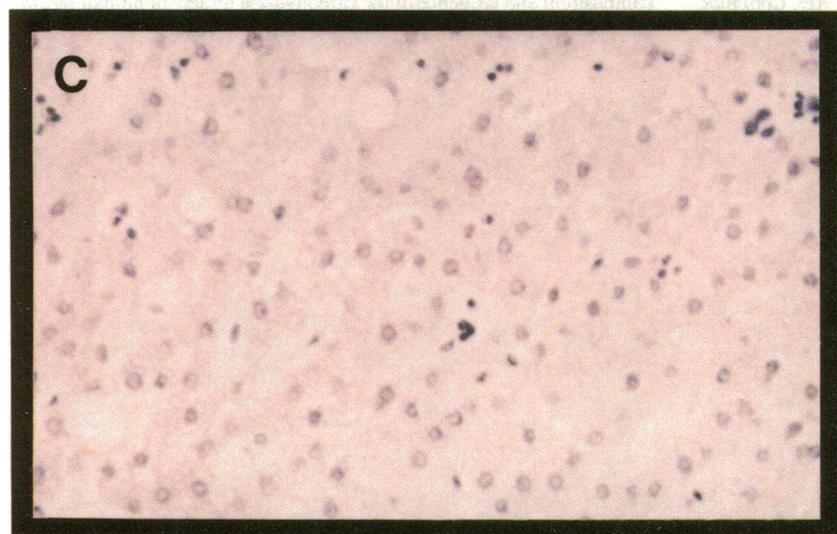
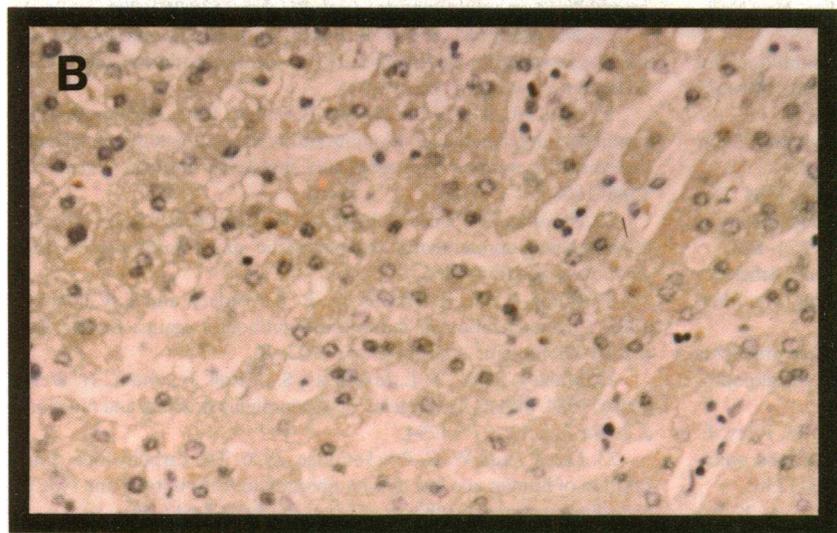
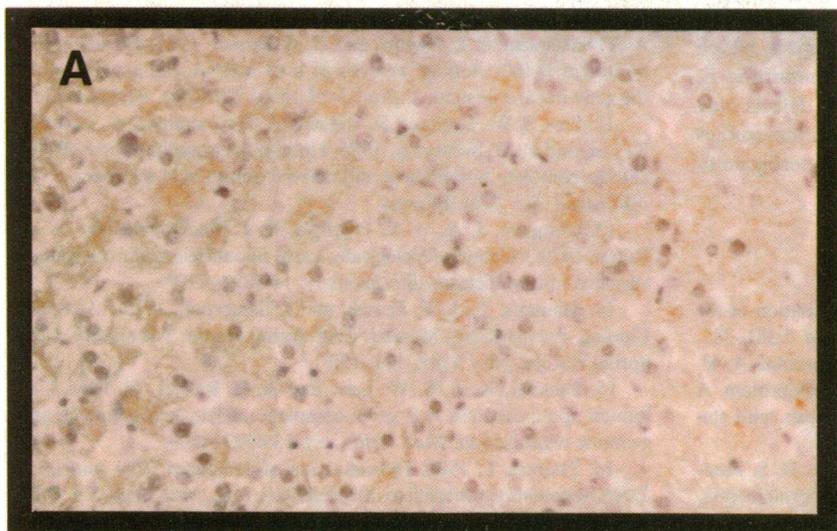


Figure 5. Dot blots and the densitometric scans of the dot blots of normal and ABL total hepatic mRNA of three normals (lanes 1, 2, and 3) and two patients with ABL (lanes 4 and 5). RNA samples were hybridized to the apoB probe λMDB-1; the absorbancy is presented in arbitrary units.



*Figure 6.* Immunohistochemical analysis of normal and ABL liver using a mixture of four monoclonal apoB-100 antibodies. The *top panel* contains normal liver, the *middle panel* ABL liver obtained by biopsy, and the *bottom panel* demonstrates ABL liver with nonimmune mouse ascitic fluid substituted for the monoclonal apoB-100 antibodies. The brownish reaction product present in normal and ABL hepatocytes indicates the presence of apoB.

in ABL patients, that the most likely biochemical defect in ABL is in posttranslational processing and secretion of apoB, which ultimately leads to defective secretion of apoB-containing lipoproteins from the cell. Several different molecular defects may ultimately result in this biochemical defect, and additional studies are underway to elucidate the potential genetic heterogeneity of ABL.

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