Proc. Natl. Acad. Sci. USA
Vol. 83, pp. 1467–1471, March 1986
Medical Sciences

Isolation of a cDNA clone encoding the amino-terminal region of human apolipoprotein B

(lipoprotein/immunochemical cross-reactivity/gene/transcription initiation/promoter)

ANDREW A. PROTTER*†, DAVID A. HARDMAN‡, JAMES W. SCHILLING*, JUDITH MILLER*, VANESSA APPLEBY*, GERI C. CHEN‡, STEVEN W. KIRSHER*, GLENN MCENROE*, AND JOHN P. KANE†§

*California Biotechnology, Inc., 2450 Bayshore Frontage Road, Mountain View, CA 94303; and ‡Cardiovascular Research Institute and the §Department of Medicine, University of California, San Francisco, CA 94143

Communicated by Richard J. Havel, October 3, 1985

ABSTRACT **A partial cDNA clone for the B-26 region of apolipoprotein B was isolated from an adult human liver DNA library by screening with an oligonucleotide probe derived from amino-terminal protein sequence obtained from purified B-26 peptide. Antisera against a synthetic 17-residue peptide whose amino acid sequence was encoded by the clone cross-reacts with apolipoproteins B-26, B-100, and B-48, but not with B-74. The nucleotide sequence immediately upstream from the amino terminus of B-26 codes for an apparent signal sequence, implying that the B-26 moiety is in an amino-terminal locus in the B-100 protein. That this sequence represents a 5′ end region is further supported by primer extension analysis using a fragment of the cDNA clone and by S1 nuclease protection experiments using the corresponding region in a genomic clone.**

Apolipoprotein B (apoB) is a major protein constituent of low density lipoprotein (LDL) and the triglyceride-rich lipoproteins. It is believed that apoB plays a central role in the transport and metabolism of plasma cholesterol by serving as a ligand for the receptor-mediated uptake of LDL by cells (1). Attempts to characterize the apoB protein by size or other physical parameters have been hampered by its insolubility in aqueous buffers upon delipidation (2, 3), and susceptibility to cleavage by proteases (4, 5) and oxidation (6) during isolation.

Kane *et al.* (7) have described four major plasma species of apoB on the basis of their relative migration in $NaDodSO_4$/polyacrylamide gels; B-100, B-74, B-48, and B-26 with apparent molecular weights of 549,000, 407,000, 264,000, and 145,000, respectively. Recent work by Cardin *et al.* (8) and Yamamoto *et al.* (9) indicates that B-26 and B-74 may arise from B-100 by the action of plasma proteases such as kallikrein.

In an effort to elucidate the structure of apoB we describe here the isolation and characterization of a cDNA clone coding for part of the B-26 region of the protein. We have used RNA from HepG2 cells and liver tissue to characterize the 5′ end of the mRNA coding for apoB. Analysis of the cDNA clone and its corresponding genomic region shows that the clone represents the 5′ end of the mRNA for apoB.

MATERIALS AND METHODS

Materials. The 192-fold degeneration 23-base-oligonucleotide probe GARGARGARATG(CTN or TTR)GARAAYGT (R = A or G, Y = T or C, N = A, G, C, or T) was synthesized on a SAMI oligonucleotide synthesizer (Biosearch, San Rafael, CA) and purified by polyacrylamide gel electrophoresis. Radioisotopes were from Amersham and all restriction enzymes were from New England Biolabs.

Purification and Sequence Analysis of B-26. Homogeneous B-26 was isolated as described previously (7). The amino acid sequence of the purified protein was determined with an Applied Biosystems model 470A protein microsequencer, using standard programs and reagents supplied by the manufacturer. Phenylthiohydantoin amino acid derivatives were identified by HPLC on a Beckman 334 apparatus with an IBM cyano (CN) column as described by Hunkapiller and Hood (10).

Preparation of Liver RNA. Total RNA was isolated from an adult human liver (supplied by G. Assman) by extraction with guanidine isothiocyanate and sedimentation through cesium chloride (11). The RNA was enriched for poly(A)$^+$ species by two rounds of chromatography on oligo(dT)-cellulose (Collaborative Research, Waltham, MA) (12). Total RNA from HepG2 cells was supplied by D. L. Williams.

Identification of an apoB cDNA Clone. An oligo(dT)-primed cDNA library from adult human liver was provided by John Fiddes (California Biotechnology). The 5×10^5 member library consists of cDNA, with an average insert size of about 1 kilobase (kb), ligated into the *Eco*RI site of λgt10 (13). Then 9×10^5 plaques propagated in *Escherichia coli* C600 (HFL) cells were transferred to replica nitrocellulose filters (Schleicher & Schuell) and processed as described (14). Baked filters were washed for 2 hr in 3× NaCl/Cit (1× NaCl/Cit is 150 mM NaCl/15 mM sodium citrate, pH 7.0) containing 0.1% $NaDodSO_4$ at 65°C and then were incubated in 6× NaCl/Cit containing denatured salmon sperm DNA at 200 μg/ml, 5% Denhardt's solution (15), and 0.05% sodium pyrophosphate for 1 hr at 50°C. The oligonucleotide probes, 5′-end-labeled with T4 polynucleotide kinase and [γ-^{32}P]ATP, were then added to the filters and incubated for 14 hr at 50°C. The filters were washed twice at room temperature in 5× NaCl/Cit containing 0.1% $NaDodSO_4$ and 0.05% sodium pyrophosphate for 15 min and once at 50°C for 20 min, dried, and autoradiographed with intensifying screens. Clones giving duplicate positive signals were plaque purified and the resulting DNA inserts were subcloned in M13 phage for sequence analysis.

λ phage clones containing specific cDNA and genomic inserts were prepared by liquid lysis and isolated by sedimentation through cesium chloride step gradients (ref. 16, pp. 80–83).

Peptide Synthesis and Purification. The 17-residue peptide Bpep#1, corresponding to a region of B-26 as derived from the cDNA clone (Fig. 2), Thr-Arg-Phe-Lys-His-Leu-Arg-Lys-Tyr-Thr-Tyr-Asn-Tyr-Glu-Ala-Glu-Ser, was prepared

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Abbreviations: apoB, apolipoprotein B; LDL, low density lipoprotein; kb, kilobase(s); bp, base pair(s).
†To whom reprint requests should be addressed.

by solid-phase peptide synthesis using the Biosearch peptide synthesizer. Amino acids were coupled successively as their *N*-Boc derivatives (Peninsula Laboratories, San Carlos, CA) with standard protection strategy (17). After completion of synthesis the peptide was removed by treatment with anhydrous HF/10% (vol/vol) anisole/1.7% ethyl methyl sulfide. The crude peptide was purified by HPLC on a Vydac C-18 column (218TP1010) (Vydac, Hesperia, CA), eluted with a gradient of acetonitrile in 0.1% trifluoroacetic acid/water. The peptide homogeneity was judged to be greater than 98% by HPLC analysis of purified products.

Preparation of Peptide Antisera. Two milligrams of peptide was dissolved in 1 ml of 1 mM HCl, treated with 1 mg of 1-ethyl-3-carbodiimide (Bio-Rad) for 15 min at 4°C, and then mixed with 2 mg of keyhole limpet hemocyanin (Worthington) for 12 hr at 23°C. The mixture was then dialyzed against phosphate-buffered saline for 24 hr at 4°C. Antiserum against the mixture, emulsified with Freund's complete adjuvant, was raised in New Zealand rabbits by Berkeley Antibody (Richmond, CA). The antiserum was titered against the 17-residue peptide, using a solid-phase enzyme-linked immunoadsorption assay (18).

Immunoblotting of apoB Peptides. The apoB peptides were separated on a $NaDodSO_4$ 3–16% exponential gradient polyacrylamide gel (19) and transferred to 0.2-μm-pore nitrocellulose filters (Millipore), as described by Towbin *et al.* (20), using a trans-blot apparatus (Bio-Rad) and 5% nonfat dry milk instead of bovine serum albumin to reduce nonspecific binding. The nitrocellulose filter was incubated with antiserum (diluted 1:500) against the synthetic 17-residue peptide for 2 hr at 23°C, washed (20), incubated with 5 μCi (1 Ci = 37 GBq) of ^{125}I-labeled staphylococcal protein A (Amersham), washed, and exposed to Kodak X-Omat film.

Primer Extension Analysis. Twenty-five micrograms of pB25-1 plasmid DNA was digested with *Nar* I (see Fig. 2) and the 577-base-pair (bp) fragment containing the 5′ region was isolated on a 1.2% agarose gel. The purified fragment was treated with bacterial alkaline phosphatase (Amersham) and then 5′ end labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase. The DNA was digested with *Pvu* II and the labeled 79-bp fragment (Fig. 2) was recovered from a polyacrylamide gel. One-fifth of the eluted fragment was co-precipitated with 2 μg of either adult human liver poly(A)$^+$ RNA or 20 μg of Hep G2 total RNA, resuspended in 80% (vol/vol) formamide/0.4 M NaCl/0.04 M 1,4-piperazinediethanesulfonic acid (Pipes), pH 6.4/0.001 M EDTA (FNPE buffer), and heated to 68°C for 10 min and then 55°C for 3 hr (21). After precipitation with ethanol the annealed primer-template complex was incubated with unlabeled deoxynucleoside triphosphates and reverse transcriptase (21), and the size of the primer extension products was measured by electrophoresis on a 5% polyacrylamide/8 M urea gel.

Genomic Screening. A human genomic library (22) was screened as described previously (14) with the cDNA insert of LB25-1 labeled with [^{32}P]dCTP by nick-translation (ref. 16, pp. 109–112). Fifteen hybridizing plaques were purified and the DNA from three representative clones was subcloned in plasmid puC8 or plasmid pBR322 and then further subcloned in phage M13 for sequence analysis.

S1 Nuclease Analysis. The *Sma* I genomic fragment was isolated, digested with *Pvu* II, treated with bacterial alkaline phosphatase, and then 5′-phosphorylated with [^{32}P]ATP and T4 polynucleotide kinase. The 204-base-pair *Sma* I–*Pvu* II fragment was purified by polyacrylamide gel electrophoresis. The eluted radiolabeled fragment was incubated with 2.5 μg of human liver poly(A)$^+$ RNA or 20 μg of total Hep G2 RNA in FNPE buffer for 5 min at 70°C and then for 14 hr at 65°C or 55°C. After hybridization the products were diluted to 0.5 ml in 0.3 M NaCl/0.03 M sodium acetate, pH 4.5/0.003 M $ZnCl_2$, and then incubated with 500 units of S1 nuclease (Bethesda Research Laboratories) for 30 min at 37°C. The S1 nuclease-resistant products were analyzed on a denaturing 8% polyacrylamide gel.

Sequencing of cDNA and Genomic Fragments. Restriction enzyme fragments were inserted into the appropriate sites in M13/mp8 and M13/mp9 phage and the sequences of both strands were determined by the dideoxy method (23). Regions of sequence ambiguity were analyzed by using the Maxam and Gilbert technique (24).

RESULTS

Isolation of a cDNA Clone for apoB. The human liver cDNA library was screened with a degenerate 23-base oligonucleotide corresponding to the first eight amino acids of B-26 peptide (see Fig. 1). One positive plaque (LB25-1) was purified and the DNA insert was subcloned in both orientations in M13/mp8 for sequence analysis. The nucleotide sequence of the 970-bp insert is shown in Fig. 1. The deduced amino acid sequence of one reading frame agrees completely with the amino-terminal sequence determined from the B-26 peptide. The sequence of LB25-1 contains one open reading frame extending 800 nucleotides downstream, coding for 30,000 daltons of protein. Further analysis of the predicted protein sequence directly upstream from the region coding for the amino terminus of B-26 reveals a highly hydrophobic region characteristic of a signal sequence (25) and preceded by a methionine residue.

The presence of an apparent signal sequence immediately upstream from the amino terminus of B-26 supports the idea that the cDNA clone LB25-1 includes the 5′ coding region of apoB as well as 86 nucleotides of 5′ untranslated sequence. The sequence TGGCGATGG found in the cDNA clone around the putative methionine initiation codon conforms reasonably well with the consensus sequence CC(A or G)CCATGG described by Kozak (26).

Primer Extension Analysis of apoB mRNA. The human hepatoma cell line HepG2 has been shown to synthesize and secrete a variety of serum proteins, including apoB (27, 28). We have used total RNA from HepG2 cells and poly(A)$^+$ RNA from liver tissue to characterize the 5′ end of the mRNA coding for apoB. To determine the proximity of LB25-1 to the 5′ end of the apoB mRNA a restriction fragment was isolated from the clone and used to prime cDNA synthesis from total HEPG2 RNA (Fig. 2*A*, lane 1). The resulting major product indicates that there are approximately 41 additional bases upstream from the 5′ end of LB25-1. Similar products were obtained when the primer extension reaction was performed with adult human liver poly(A)$^+$ RNA (data not shown).

Immunological Characterization of apoB. To confirm the identity of the clone, antiserum raised against a synthetic 17-residue peptide (Bpep#1) whose amino acid sequence was derived from the nucleotide sequence of the clone was tested for its ability to cross-react with apoB peptides purified from plasma (Fig. 3). The sequence corresponding to Bpep#1 is located 15 amino acids downstream from the amino terminus of the secreted protein (see Fig. 1). Total apoproteins from LDL, B-100, B-74, and B-26 were electrophoresed through a $NaDodSO_4$/3–16% gradient polyacrylamide gel, transferred to nitrocellulose, incubated with antisera against Bpep#1, and then incubated with ^{125}I-labeled protein A (20). The resulting autoradiograph (Fig. 3) shows that the antiserum reacts specifically with B-26 (lane a), B-100 (lane c), and B-48 (lanes e and f) but not with B-74 (lane b) or bovine serum albumin (lane g). Antisera directed against B-100 reacted with B-100, B-74, and B-26 (data not shown). Similar results were obtained when the antisera against Bpep#1 was tested against the purified apoB peptides by using a solid-phase enzyme-linked immunoadsorption assay (data not shown).

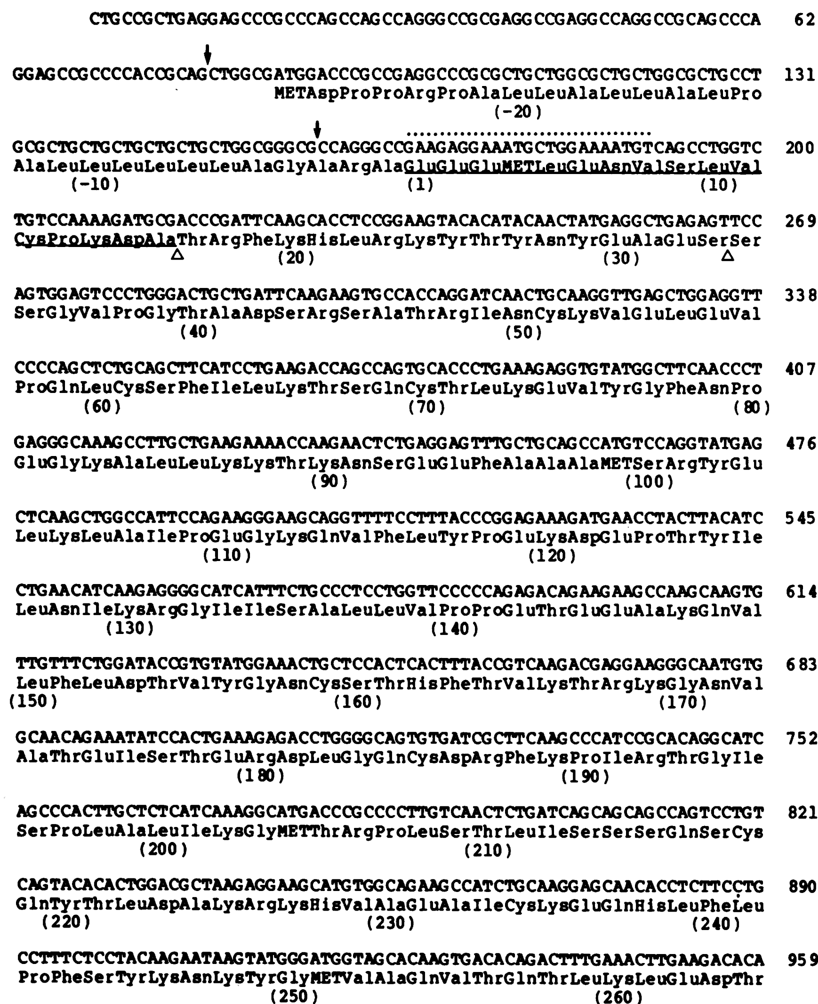

FIG. 1. Nucleotide sequence and inferred amino acid sequence of clone LB25-1. The dotted line above the sequence indicates the region corresponding to the oligonucleotide probe. The portion determined from sequence analysis of apoB-26 is underlined. The numbers in parentheses below the sequence refer to amino acids, with (1) being defined as the amino terminus. The amino acid sequence of the synthetic 17-residue peptide Bpep#1 is indicated between △s. The ends of the *Pvu* II–*Nar* I fragment used for primer extension are indicated by arrows.

Isolation of Genomic Clones for apoB. The insert of the cDNA clone LB25-1 was subcloned in pBR322 and used as a probe to isolate genomic clones from a human genomic library in λ Charon 4A. Southern analysis of three representative clones and total genomic DNA gave identical patterns with a variety of restriction enzymes when the probe was the radiolabeled insert of LB25-1 (data not shown). Exon-containing restriction fragments of DNA isolated from one clone, LBgl5, were identified by hybridization with the cDNA probe. Sequence analysis of three exon regions shows complete identity with the cDNA clone LB25-1 (unpublished data).

Sequence analysis of a genomic fragment of LBgl5, corresponding to the 5′ end of the cDNA clone LB25-1, is shown in Fig. 4. Sixty-three nucleotides upstream from the nucleotide corresponding to the 5′ end of the cDNA clone is a region characteristic of a promoter that includes a "CAAT box" (29) and a Goldberg–Hogness ("TATA") sequence (20). The primer extension product maps to a region 15 bases downstream from the TATA sequence.

To define accurately the 5′ end of the mRNA, S1 nuclease analysis was performed with a 204-bp *Sma* I–*Pvu* II genomic fragment extending from nucleotide −211 to −7 relative to the adenosine residue of the translation initiation codon (see Fig. 4). The fragment was 5′ end labeled at the *Pvu* II site and incubated with total HepG2 RNA or poly(A)$^+$ liver RNA at 55°C or 60°C, and the S1 nuclease-resistant products were analyzed by gel electrophoresis (Fig. 2*B*). The resulting 118-bp protected fragment maps the site of transcription initiation to 22 base pairs downstream from the TATA sequence (see Fig. 4). This confirms the results obtained by primer extension analysis described above.

DISCUSSION

We have described the isolation and characterization of a cDNA clone coding for part of the B-26 region of B-100. The amino acid sequence inferred from nucleotide sequence analysis of the cDNA clone matches that obtained from the amino terminus of the B-26 peptide. The identity of the clone was confirmed with the demonstration that antiserum raised against synthetic 17-residue peptide whose amino acid sequence was derived from the sequence of the clone, cross-reacts with B-100 and B-26 and not with B-74.

The predicted amino acid sequence directly upstream from the region of the clone coding for the amino terminus of B-26 is highly characteristic of a signal sequence (25). This includes a charged residue (aspartic acid) near the amino terminus, a central hydrophobic core, a helix-breaking residue (glycine) four residues from the cleavage site, and an amino acid with a small aliphatic side chain (alanine) at the putative cleavage site (25). In addition, a region homologous to the consensus sequence for eukaryotic translation initiation (26) is located proximal to the signal sequence. Primer

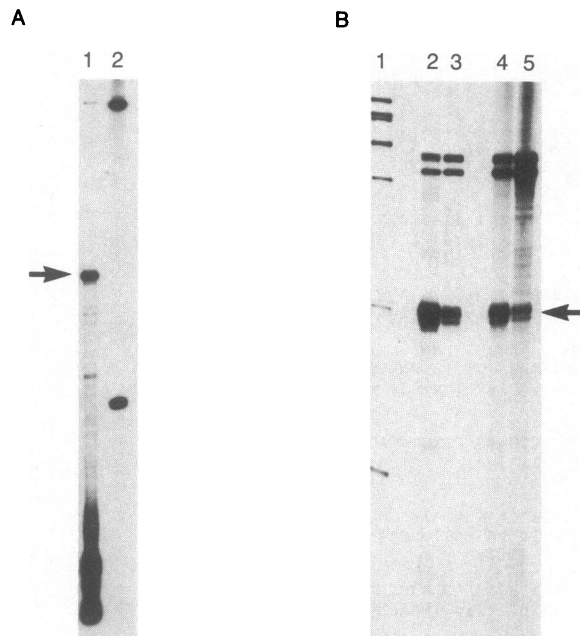

FIG. 2. Characterization of the 5′ end of the apoB mRNA. (*A*) Primer extension analysis. The *Nar* I–*Pvu* II restriction fragment, 5′ end labeled at the *Nar* I site, was incubated with total HepG2 RNA and then extended with reverse transcriptase. The resulting products were analyzed on a denaturing 5% polyacrylamide gel (lane 1). An arrow indicates the major extension product. Radiolabeled *Hin*dIII-cut λ DNA standards (lane 2) are 130 and 470 nucleotides long. (*B*) S1 nuclease mapping. The *Sma* I–*Pvu* II restriction fragment, 5′ end labeled at the *Pvu* II site, was incubated with total HepG2 RNA (lanes 2 and 3) or poly(A)$^+$ liver RNA (lanes 4 and 5) at 60°C (lanes 2 and 4) or 55°C (lanes 3 and 5) for 20 hr. The products were treated with S1 nuclease and the resistant products were analyzed by electrophoresis on a denaturing 8% polyacrylamide gel. The arrow indicates the protected fragment. Radiolabeled *Hae* III-cut phage ϕX174 DNA standards (lane 1) are 72, 118, 194, and 234 nucleotides long.

extension analysis of apoB-specific mRNA using a restriction enzyme fragment of the clone confirms the close proximity of LB25-1 to the 5′ end.

The exact size of the apoB protein is unknown. As discussed recently by Elovson *et al.* (31), published estimates of molecular weight based on migration in $NaDodSO_4$/polyacrylamide gels range from 335,000 to 550,000. Denaturing sedimentation equilibrium analysis (31) yields a molecular weight of 387,000. An mRNA with sufficient coding capacity would be 9.0 to 14.9 kb long. Blot hybridization analysis of liver RNA using the DNA insert of clone LB25-1 as a probe has failed to detect a discrete RNA species but does reveal the presence of an abundant broad size class (1–15 kb) of material capable of hybridizing to the probe under very stringent wash conditions (data not shown). Such a result would be expected if the apoB mRNA was 15 kb or greater and the RNA preparations used for the analysis were partially degraded.

While this manuscript was in preparation, Lusis *et al.* (32) and Deeb *et al.* (33) reported the isolation of partial cDNA clones for rat and human apoB, respectively. The exact regions of the apoB protein coded for by these cDNA clones is unknown, but the published nucleotide sequences show no homology with LB25-1.

The partial apoB cDNA clone LB25-1 allows us to predict the sequence of 30,000 daltons of protein, not including the amino-terminal signal sequence. The protein shows no homology with any sequence previously obtained from apoB (34, 35). Furthermore, there is no apparent homology to any

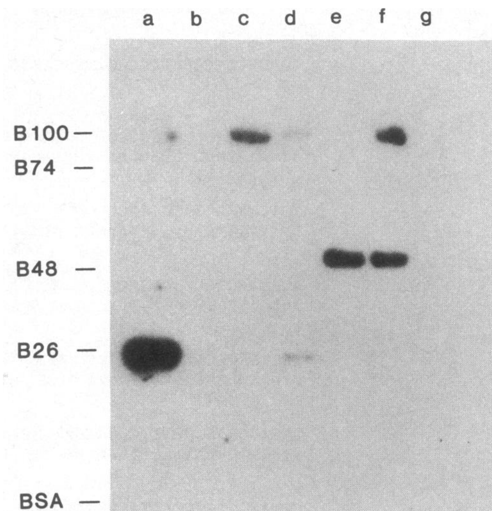

FIG. 3. Immunoblotting of apoB protein. Samples (10 μg) of B-26 (lane a), B-74 (lane b), B-100 (lane c), total LDL apoprotein (lane d), B-48 (lane e), total chylomicron apoprotein (lane f), and bovine serum albumin (BSA; lane g) were separated by polyacrylamide gel electrophoresis and processed. B-100, B-74, and B-26 were purified from LDL and B-48 from chylomicrons, as in ref. 7. The position of each protein was determined by Coomassie blue staining of a duplicate gel.

other plasma apolipoprotein such as apolipoproteins E, A-I, and A-IV (36).

Intramolecular disulfide bonds, present when apoB is in its native environment bound to LDL, are important for the maintenance of its secondary structure (3, 37). The 30,000 daltons of apoB protein deduced from clone LB25-1 includes eight cysteine residues. Comparison of this value with published amino acid composition values [1.1–1.8 cysteine residues per 30,000 daltons of apoB (8)] indicates that cysteine residues are overrepresented in this amino-terminal region. The significance of this region of the protein to the overall structure and function of apoB and LDL is unknown at this time.

Antibodies raised against a synthetic peptide corresponding to amino acids 17–33 have been demonstrated to cross-react with B-48, the intestinal form of apoB. Marcel *et al.* (38) and Curtiss *et al.* (39) have described monoclonal antibodies against LDL that cross-react with B-48. While the exact structural relationship between B-100 and B-48 is unknown, they appear to share antigenic determinants contained within the amino-terminal region. Intestine cDNA clones that have been selected by using the insert of LB25-1 as a probe may provide information about B-48.

In this report we also describe the isolation and preliminary characterization of genomic clones for apoB. Nucleotide sequence analysis of a genomic fragment containing the 5′ end of the cDNA clone shows the existence of a CAAT box and a TATA box, which are highly characteristic of eukaryotic promoter regions (30). The site of transcription initiation was mapped by S1 nuclease protection experiments to an adenosine residue that is 22 nucleotides downstream from the TATA box and is contained within a region homologous to the transcription initiation consensus sequence described by Corden *et al.* (30). The cDNA clone B25-1 contains 86 of the 127 nucleotides of the untranslated region.

The absence of a translation stop signal in the correct reading frame of the cDNA clone LB25-1 indicates that additional coding sequence occurs downstream. Liver cDNA clones that have been selected by using the insert of LB25-1 as a probe may allow us to find the stop signal.

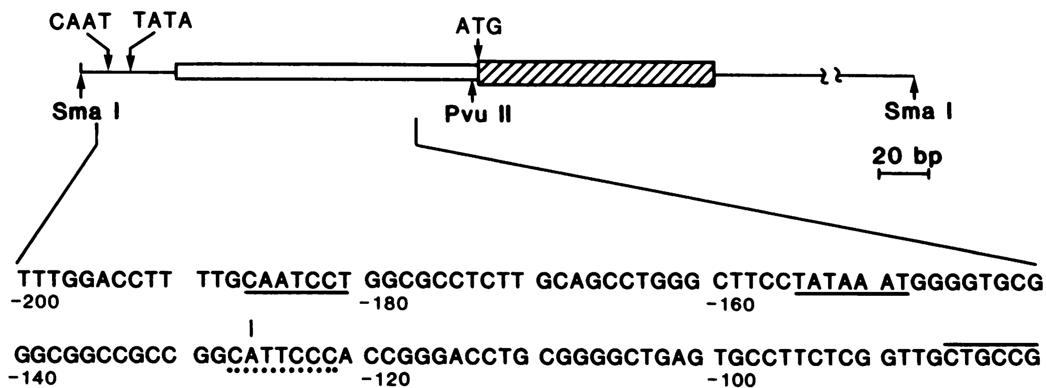

FIG. 4. Characterization of the *Sma* I genomic fragment containing the 5′ end of the gene for apoB. The schematic diagram shows the positions of the promoter region and first exon of the apoB gene. The 5′ untranslated region is indicated by an open box and the 5′ coding region is indicated by a hatched box. The *Sma* I and *Pvu* II sites below the diagram delineate the fragment used for S1 nuclease protection analysis. The nucleotide sequence shown below is derived from the region of the genomic fragment indicated. The CAAT box and TATA box regions are underlined. The consensus sequence for transcription initiation is underlined with a dotted line and the site of transcription initiation mapped by S1 nuclease protection analysis is indicated by a vertical line. The sequence corresponding to the cDNA clone LB25-1 is overlined and the numbers below the sequence refer to the distance from the adenosine residue of the translation initiation codon.

We are grateful to D. Schenk and J. Fiddes for technical advice, J. Iwassa for preparation of oligonucleotides, and all our colleagues at California Biotechnology, Inc., for their encouragement and help in critically reading this manuscript. This work was supported by Biotechnology Research Partners, Ltd. and by Arteriosclerosis Specialized Center of Research Grant HL 14237 from the U.S. Public Health Service.

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