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Isolation, Expression and Characterization of a Human Apolipoprotein B 100-Specific cDNA Clone

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Summary: The isolation and characterization of a human apolipoprotein B 100-specific cDNA clone (λgt-B1) containing a 1321 base pairs (bp) spanning insert is described. It encodes the 3′-nontranslated 281 bp long region up to the polyadenylation site and 1040 bp of the C-terminal coding region of 345 amino-acid residues of human apo B 100 and the stop codon. The λgt-B1 cDNA clone has been isolated from a human hepatoma cDNA expression library by immunoscreening using affinity-purified polyclonal anti apo B 100 antibodies. The nucleotide sequence of the apo B 100 insert has been determined. A part of the polypeptide sequence derived from this nucleotide sequence was identical with the amino-acid sequence obtained by protein sequencing of a purified cyanogen bromide fragment of apo B 100. The fusion protein consisting of β-galactosidase and the 345 amino-acid residue long C-terminus of apo B 100 had an apparent molecular mass of 148 kDa in $NaDodSO_4$ polyacrylamide gel electrophoresis. In Northern blot hybridization analysis the insert of the apo B 100-cDNA clone hybridized to a 20 to 22 kb mRNA from adult human liver.

Isolierung, Expression und Charakterisierung eines menschlichen Apolipoprotein-B-100-spezifischen cDNA-Klons

Zusammenfassung: Die Isolierung und Charakterisierung eines Apolipoprotein-B-100- (Apo-B-)-spezifischen cDNA-Klons (λgt-B1) mit einem 1321 Basenpaare (bp) großen Insert wird beschrieben. Das DNA-Insert umfaßt die gesamte 3′-nichttranslatierte Region bis zur Polyadenylierungsstelle, die 1040 bp große kodierende C-terminale Region des Apo B und das Stop-Codon. Der cDNA-Klon wurde aus einer menschlichen Hepatom-cDNA-Expressions-Bibliothek durch immunologischen Nachweis mit affinitätsgereinigten, polyklonalen, monospezifischen anti-Apo-B-100-Antikörpern isoliert. Die Nucleotidsequenz des Apo-B-100-cDNA-Klons wurde bestimmt. Das Fusionsprotein besteht aus der β-Galactosidase und den C-terminalen 345 Aminosäureresten des Apo B 100 und wurde als 148-kDa-Bande in der $NaDodSO_4$-Polyacrylamid-Gelelektrophorese nachgewiesen. Die aus der Nucleotidsequenz abgeleitete Polypeptidsequenz enthielt eine partielle Aminosäuresequenz, die durch die Proteinsequenzierungs-Daten eines Bromcyanfragments des Apo B 100 bestätigt werden konnte. Nach Übertragung auf Nitrocellulose konnte die mittels Elektrophorese aufgetrennte RNA untersucht werden; die Länge der Apo-B-100-mRNA beträgt 20 bis 22 kb.

Enzymes:
Restriction endonucleases: *Ava*II (EC 3.1.23.4), *Eco*RI (EC 3.1.23.13), *Hae*III (EC 3.1.23.17), *Hin*dIII (EC 3.1.23.21), *Hin*fI (EC 3.1.23.22).

Abbreviations:
cDNA = Complementary desoxyribonucleic acid; apo B = apolipoprotein B 100, the apolipoprotein of LDL; LDL = low density lipoprotein; bp = base pairs; kb = kilo base pairs; DMF = *N*,*N*-dimethylformamide; $NaDodSO_4$ = sodium dodecyl sulphate; pfu = plaque-forming unit; IPTG = isopropylthiogalactoside.

Key words: Apo B 100 cDNA clone, expression of apo B 100 C-terminal sequence, human apo B 100 mRNA.

One of the most abundant plasma apolipoproteins is apolipoprotein B 100[1]. This single polypeptide synthesized by the hepatocyte is associated with the triacylglycerol-rich very low density lipoproteins (VLDL) and the cholesterol-rich low density lipoproteins (LDL). Apo B 100 is the only protein component of LDL which remains in the particle during the catabolism of metabolized intermediate density lipoproteins (IDL) by lipoprotein lipase.

The physiological function of apo B 100 consists of the lipid transport particularly of cholesterol and its esters as LDL particle in the plasma. It is recognized by the specific LDL receptor and thereby contributes to the regulation of the cholesterol metabolism[7]. Disturbances in cholesterol metabolism with elevated serum cholesterol concentrations inevitably lead to severe hypercholesterolemia and atherosclerosis. A limited number of individuals suffering from atherosclerosis show an impaired (defective or absent) LDL receptor-mediated LDL degradation. The majority, however, might have a normal LDL receptor, but a structurally altered ligand apo B 100 or disturbed apo B 100 expression. Therefore extensive efforts in many laboratories are being made to resolve the primary structure of apo B 100. Intractabilities such as the insolubility of the delipidated apoprotein B 100 and its larger polypeptide fragments, its susceptibility to proteolytic cleavage and its high tendency to aggregate have impeded the characterization of apo B 100 by standard techniques of protein sequencing. Even the molecular size is not known exactly but estimated to be around 500 kDa[2–6].

Therefore cloning techniques are being applied in several laboratories to obtain the complete amino-acid sequence of apo B 100[8–15]. In the present report we describe the isolation of apo B 100-specific cDNA clones from a Li7 cell line cDNA expression library in λgt11[16] utilizing affinity-purified polyclonal, monospecific antibodies against human LDL. The nucleotide sequence of our longest cDNA clone of 1321 bp (λgt-B1) includes the 3′-noncoding region up to the polyadenylation recognition site (281 bp) and the deduced amino-acid sequence of the 345 carboxyterminal residues followed by the stop codon. The λgt-B1 clone produced a 148 kDa fusion protein, as shown in $NaDodSO_4$-polyacrylamide gel electrophoresis. Several polypeptide sequences from cyanogen bromide fragments of apo B 100 have been determined. One of them is represented within the λgt-B1 nucleotide sequence-deduced polypeptide structure. Northern blot hybridization utilizing the insert of the present clone as hybridization probe revealed a 20–22 kb long apo B 100-specific mRNA in human liver mRNA.

The results reported here might contribute to the elucidation of the primary apo B 100 structure, to the isolation of the gene and to the facilitation of the studies on the regulation of apo B 100 expression.

Materials and Methods

Monospecific, polyclonal antibodies against human apo B 100

Human LDL obtained by desorption from LDL-apheresis columns[17] was passed over an antialbumin column and contained apo B 100 as its only apoprotein constituent, which was used for the immunization of sheep and for the construction of an apo B 100-Sepharose CL-4B column. The γ-globulin fraction of the LDL antiserum was purified by chromatography over the LDL-Sepharose and an *E. coli* Y1089/λgt11-Sepharose CL-4B column.

Immunoscreening of the cDNA expression library in λgt11

The cDNA expression library from cell line Li7[16] was a generous gift of *Dr. J. S. O'Brien*, La Jolla. cDNA was ligated with *Eco* RI-linkers and cloned into the *Eco* RI site of λgt11. Phages were plated on *E. coli* Y1090 at a density of 1.5×10^4 pfu per 150 mm plate and isopropylthiogalactoside (IPTG)-induced plaques were transfered to nitrocellulose filters[18–20]. Filters were rinsed in Tris/NaCl/Tween (100mM Tris/HCl, pH 7.5, 170mM NaCl, 0.05% Tween 20) and incubated in Tris/NaCl containing 3% gelatine for 1 h. After a short wash in Tris/NaCl the filters were transfered to Tris/NaCl/gelatine containing affinity-purified anti-LDL IgG (50 μg/ml) and incubated for 16 h with gentle agitation. Then the filters were washed in NaCl/Tris/Tween three times (10 min each time) and afterwards incubated for at least two hours with alkaline phosphatase coupled to rabbit anti-sheep IgG (Bio-Rad, dilution 1:1000) dissolved in Tris/NaCl/3% gelatine, followed by three washing steps in Tris/NaCl for 5 min each time and finally incubated for 2 min in 0.1M $NaHCO_3$, 1mM $MgCl_2$, pH 9.8. The filters were transfered into the phosphatase substrate solution: 100 ml 0.1M $NaHCO_3$, 1mM $MgCl_2$, pH 9.8, 1 ml of a solution of 5-bromo-4-chloro-3-indolyl phosphate in DMF (15 mg/ml) and 1 ml of a solution of nitro blue tetrazolium in 70% DMF (30 mg/ml). After about 15 min the colour had developed. The filters were washed with bidistilled water and allowed to dry.

Characterization of fusion proteins

Lysogenized *E. coli* Y1089, grown and induced as described by Young and Davis[18,19], were recovered by centrifugation and resuspended in 50mM Tris/HCl pH 7.5 with 5% $NaDodSO_4$. After sonification and centrifugation the supernatant was analysed by $NaDodSO_4$-polyacrylamide gel electrophoresis (7.5%). Staining was performed with Coomassie Brilliant Blue.

Isolation of human liver poly $(A)^+$ RNA

Total RNA of adult human liver was isolated using established procedures[21]. Poly$(A)^+$ RNA was enriched from total RNA by repeated chromatography over oligo$(dT)_{12-18}$ cellulose (Type 3, Collaborative Research)[22].

Northern blot hybridization analysis

10–12 µg aliquots of poly$(A)^+$ RNA samples were treated with formaldehyde and separated by electrophoresis in 0.75% agarose slab gels (2.2M formaldehyde and 0.2M morpholinopropane sulfonic acid pH 7.0)[23]. RNA was blotted to a gene screen membrane (New England Nuclear) and hybridized with the nick-translated 1 321 bp λgt-B1 insert according to the manufacturer's instructions.

DNA sequence analysis

The cDNA insert from the λgt-B1 clone was isolated from the *Eco*RI restriction digest by preparative agarose electrophoresis[24] and cloned into M13mp19 and pUC13. The nucleotide sequence was determined by the chain-termination method[25] either in M13 or partially under denaturing conditions in pUC13 by double strand sequencing[26]. Our sequencing strategy consisted of the use of short synthetic oligonucleotides deduced from our sequence data as primers in the chain-termination method. The oligonucleotides were synthesized with an automated Applied Biosystems DNA synthesizer model 380 A by the solid phase phosphoramidite method[27].

Results

Isolation and characterization of apo B 100 clones

The initial screening of 5×10^5 recombinants of a human liver cDNA λgt11 expression library yielded 2 clones, which reacted with the polyclonal monospecific anti human apo B 100 IgG. These immunoglobulin fractions serve as antibody for LDL-apheresis in familial hypercholesterolemic patients[17]. They have been further purified by affinity chromatography over LDL-Sepharose CL-4B-column and freed from antibacterial antibodies by passing over an affinity column Sepharose CL-4B cross linked with *E. coli* lysate[16]. Fig. 1 A–C shows developed filters of two purified positive apo B 100 clones (λgt-B1, λgt-B2) in comparison to a randomly picked λgt11 recombinant as a negative control after incubation with polyclonal IgG. One of these clones, λgt-B1, was used to lysogenize *E. coli* Y1089. Total bacterial proteins were separated and the fusion protein band could be detected in the range of 148 kDa (as shown in Fig. 2). The restriction digest of the λgt-B1 DNA with *Eco*RI released a fragment approximately 1 300 bp long (Fig. 3), which has been isolated by preparative agarose gel elec-

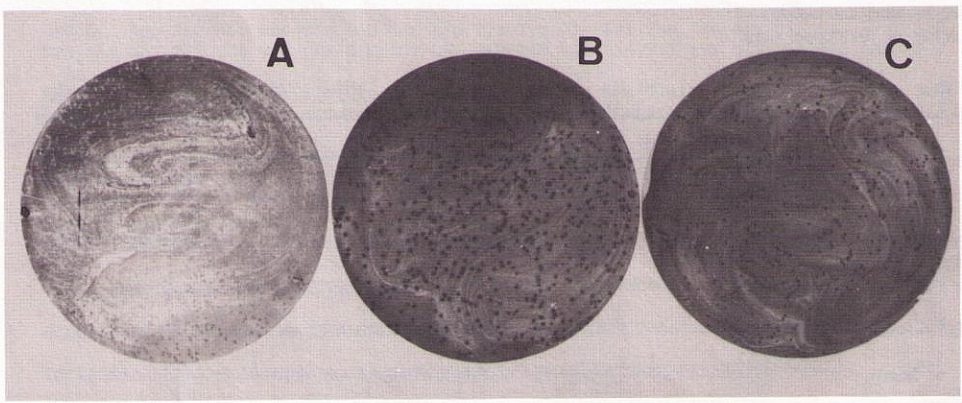


Fig. 1. Screening of λgt11-cDNA library with anti apo B 100 antibodies.

λgt-B1 clone and λgt-B2 after plaque purification reacted with polyclonal affinity purified anti apo B 100 IgG. A) λgt11 recombinant, negative control; B) clone λgt-B1; C) clone λgt-B2. (Signal to noise ratio could be increased by a shorter incubation with the colour developing reagent.)

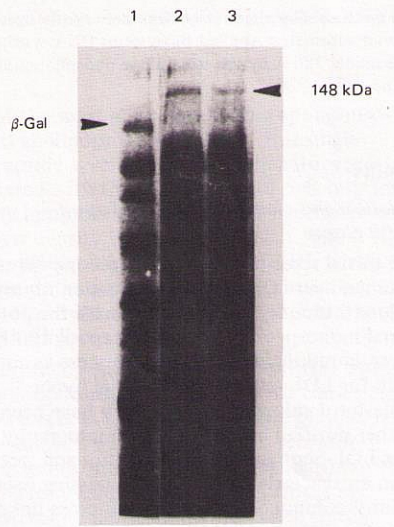


Fig. 2. $NaDodSO_4$-Polyacrylamide gelelectrophoresis (7.5%) of total protein present in the *E. coli* Y1089 lysate lysogenized with λgt-B1 clone.

Arrows indicate electrophoretic position of β-galactosidase (116 kDa) and of the fusion protein (148 kDa). Lane 1: wild type λgt11; lane 2: λgt-B1; lane 3: λgt-B1 (half the concentration of 2).

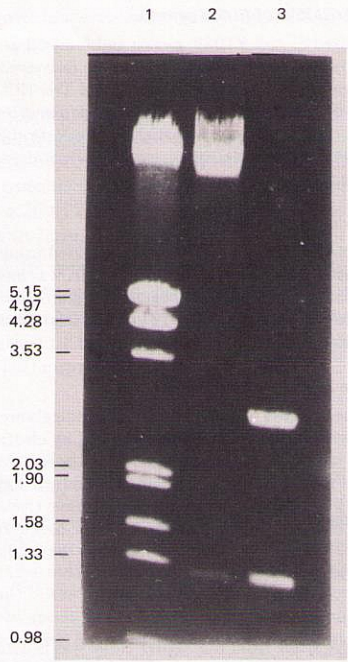


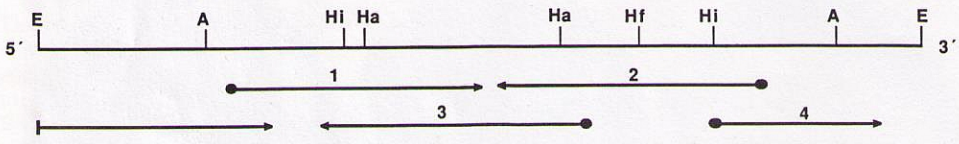
Fig. 3. *Eco*RI restriction digest of λgt-B1.

Lane 1: λDNA, *Eco*RI/*Hin*dIII; lane 2: λgt-B1, *Eco*RI; lane 3: pUC13-B1, *Eco*RI.

4a)

Restriction map of Apo B cDNA and sequencing strategy

Length: 1321 nucleotides
Number of restriction sites: 9



Enzymes:

A = AvaII Hi = HindIII

Ha = HaeIII Hf = HinfI

E = EcoRI

Primer types:

Synthetic oligonucleotide

M13 primer

Primer sequences:

	5′	3′
1	CAG CTG AAA GAG ATG AA	
2	AGT TCA ATT GGA AAA AG	
3	GCT GGT GGT AAT CAG	
4	GAG AAC TTA CTA TCA TCC TC	

4b)

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                                                                ...aattcc CT    2

Met Phe Ile Arg Glu Val Gly Thr Val Leu Ser Gln Val Tyr Ser Lys Val His Asn Gly   20
ATG TTC ATA AGG GAG GTA GGG ACG GTA CTG TCC CAG GTA TAT TCG AAA GTC CAT AAT GGT   62

Ser Glu Ile Leu Phe Ser Tyr Phe Gln Asp Leu Val Ile Thr Leu Pro Phe Glu Leu Arg   40
TCA GAA ATA CTG TTT TCC TAT TTC CAA GAC CTA GTG ATT ACA CTT CCT TTC GAG TTA AGG  122

Lys His Lys Leu Ile Asp Val Ile Ser Met Tyr Arg Glu Leu Leu Lys Asp Leu Ser Lys   60
AAA CAT AAA CTA ATA GAT GTA ATC TCG ATG TAT AGG GAA CTG TTG AAA GAT TTA TCA AAA  182

Glu Ala Gln Glu Val Phe Lys Ala Ile Gln Ser Leu Lys Thr Thr Glu Val Leu Arg Asn   80
GAA GCC CAA GAG GTA TTT AAA GCC ATT CAG TCT CTC AAG ACC ACA GAG GTG CTA CGT AAT  242

Leu Gln Asp Leu Leu Gln Phe Ile Phe Gln Leu Ile Glu Asp Asn Ile Lys Gln Leu Lys  100
CTT CAG GAC CTT TTA CAA TTC ATT TTC CAA CTA ATA GAA GAT AAC ATT AAA CAG CTG AAA  302

Glu Met Lys Phe Thr Tyr Leu Ile Asn Tyr Ile Gln Asp Glu Ile Asn Thr Ile Phe Ser  120
GAG ATG AAA TTT ACT TAT CTT ATT AAT TAT ATC CAA GAT GAG ATC AAC ACA ATC TTC AGT  362
                                                                        ▲

Asp Tyr Ile Pro Tyr Val Phe Lys Leu Leu Lys Glu Asn Leu Cys Leu Asn Leu His Lys  140
GAT TAT ATC CCA TAT GTT TTT AAA TTG TTG AAA GAA AAC CTA TGC CTT AAT CTT CAT AAG  422

Phe Asn Glu Phe Ile Gln Asn Glu Leu Gln Glu Ala Ser Gln Glu Leu Gln Gln Ile His  160
TTC AAT GAA TTT ATT CAA AAC GAG CTT CAG GAA GCT TCT CAA GAG TTA CAG CAG ATC CAT  482

Gln Tyr Ile Met Ala Leu Arg Glu Glu Tyr Phe Asp Pro Ser Ile Val Gly Trp Thr Val  180
CAA TAC ATT ATG GCC CTT CGT GAA GAA TAT TTT GAT CCA AGT ATA GTT GGC TGG ACA GTG  542

Lys Tyr Tyr Glu Leu Glu Glu Lys Ile Val Ser Leu Ile Lys Asn Leu Leu Val Ala Leu  200
AAA TAT TAT GAA CTT GAA GAA AAG ATA GTC AGT CTG ATC AAG AAC CTG TTA GTT GCT CTT  602

Lys Asp Phe His Ser Glu Tyr Ile Val Ser Ala Ser Asn Phe Thr Ser Gln Leu Ser Ser  220
AAG GAC TTC CAT TCT GAA TAT ATT GTC AGT GCC TCT AAC TTT ACT TCC CAA CTC TCA AGT  662

Gln Val Glu Gln Phe Leu His Arg Asn Ile Gln Glu Tyr Leu Ser Ile Leu Thr Asp Pro  240
CAA GTT GAG CAA TTT CTG CAC AGA AAT ATT CAG GAA TAT CTT AGC ATC CTT ACC GAT CCA  722

Asp Gly Lys Gly Lys Glu Lys Ile Ala Glu Leu Ser Ala Thr Ala Gln Glu Ile Ile Lys  260
GAT GGA AAA GGG AAA GAG AAG ATT GCA GAG CTT TCT GCC ACT GCT CAG GAA ATA ATT AAA  782

Ser Gln Ala Ile Ala Thr Lys Lys Ile Ile Ser Asp Tyr His Gln Gln Phe Arg Tyr Lys  280
AGC CAG GCC ATT GCG ACG AAG AAA ATA ATT TCT GAT TAC CAC CAG CAG TTT AGA TAT AAA  842

Leu Gln Asp Phe Ser Asp Gln Leu Ser Asp Tyr Tyr Glu Lys Phe Ile Ala Glu Ser Lys  300
CTG CAA GAT TTT TCA GAC CAA CTC TCT GAT TAC TAT GAA AAA TTT ATT GCT GAA TCC AAA  902

Arg Leu Ile Asp Leu Ser Ile Gln Asn Tyr His Thr Phe Leu Ile Tyr Ile Thr Glu Leu  320
AGA TTG ATT GAC CTG TCC ATT CAA AAC TAC CAC ACA TTT CTG ATA TAC ATC ACG GAG TTA  962

Leu Lys Lys Leu Gln Ser Thr Thr Val Met Asn Pro Tyr Met Lys Leu Ala Pro Gly Glu  340
CTG AAA AAG CTG CAA TCA ACC ACA GTC ATG AAC CCC TAC ATG AAG CTT GCT CCA GGA GAA 1022

Leu Thr Ile Ile Leu ***
CTT ACT ATC ATC CTC TAA TTTTTTAAAAGAAATCTTCATTTATTCTTCTTTTCCAATTGAACTTTCACATAGC 1095

ACAGAAAAAATTCAAACTGCCTATATTGATAAAACCATACAGTGAGCCAGCCTTGCAGTAGGCAGTAGACTATAAGCAG 1174
          ▲                ▲

AAGCACATATGAACTGGACCTGCACCAAAGCTGGCACCAGGGCTCGGAAGGTCTCTGAACTCAGAAGGATGGCATTTTT 1253

TGCAAGTTAAAGAAAATCAGGATCTGAGTTATTTTGCTAAACTTGGGGGAGGAGGAACAAATAAATGG gg...      1321
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△

◁ Fig. 4. Restriction map and sequencing strategy of cDNA insert of apo B 100 clone (a), and nucleotide sequence of λgt-B1 and derived amino-acid sequence (b).

Underlined is the polypeptide sequence of an apo B 100-derived cyanogen bromide fragment and the polyadenylation recognition signal. Base exchanges are indicated by arrows. Sequence data belonging to the *Eco*RI-linkers are given in small letters.

trophoresis (1% in Tris-borate EDTA buffer) by electrophoretic stacking to DEAE-filters as described[24]. The fragment has been cloned into the *Eco*RI-site of dephosphorylated M13mp19 and pUC13 for nucleotide sequence determination by the chain-termination method[24,25]. The restriction map of the insert in clone λgt-B1 together with the nucleotide sequence of the 1321 bp long insert is given in Figs. 4a and 4b. The nucleotide-derived amino-acid sequence is given above the respective codons. The complete *Eco*RI restriction fragment cloned into M13mp19 was sequenced using suitable M13 primers and synthetic oligonucleotides homologous to the unambigous 3′-end of the established sequence. Northern blot analysis of human poly(A)$^+$ RNA showed a distinct band in the range of 20–22 kb after hybridization with the nick-labelled cDNA insert (Fig. 5). The signals below the apo B band are caused by partially degraded apo B-specific RNA's.

Fig. 5. Northern blot analysis of poly(A)$^+$ mRNA from adult human liver RNA.

The electrophoretically separated mRNA was probed after blotting with the nick-labelled apo B 100 insert of clone λgt-B1.

Discussion

We describe in this report a human apolipoprotein B 100-specific λgt11 clone which carries a 1321 bp long insert. This clone has been isolated from a human cDNA expression library cloned into the *Eco*RI site of λgt11. The apo B 100-specific protein sequence encoded in this DNA sequence was recognized by polyclonal, monospecific anti human apolipoprotein B 100 IgG. This IgG fraction serves in our laboratory for the preparation of immunoaffinity columns applied to patients with familial hypercholesterolemia for LDL-apheresis. The antibodies purified by affinity chromatography over a LDL-Sepharose column are free of antibodies cross-reacting with other plasma components, especially apolipoproteins. The apo B 100 specificity is further confirmed by our finding of the partial amino-acid sequence of a cyanogen bromide fragment isolated from CNBr-cleaved total apo B 100 and thrombin fragment T_2*. This CNBr-fragment is recognized not only by the polyclonal IgG fraction but also by one monoclonal antibody out of several against human apo B 100.

The 1321 bp long nucleotide sequence encodes the 345 amino-acid residues of the C-terminus and the 3′-noncoding region up to the polyadenylation site which is 273 bp downstream from the apo B 100 stop codon. Apo B 100 has an estimated molecular mass of approximately 500 kDa. It is not expected that a full length cDNA transcript can be obtained, particularly not if one considers the results of Northern blot hybridization analyses described in this paper and reported recently by others[8–15,28]. The insert of our λgt-B1 clone hybridized to an apo B 100-specific poly(A)$^+$ mRNA of 20 to 22 kb. The coding region of our cDNA clone corresponds to the C-terminal part of the sequence published by Knott et al.[10] and Wei et al.[15] with the exception of only one amino-acid exchange (Asn→Ser^{120}). With regard to the noncoding region our sequence confirms the sequence analysis given by Wei et al.[15] except the exchanges of two bases (1112 and 1126). This is caused by a one-base exchange (A→G) at the second position of the Asn-codon and thus this sequence polymorphism could be the result of a point mutation. Our finding is not surprising, because the highly polymorphous character of this protein is known for a long time[29]. Recently the N-terminal amino-acid sequence of apo B 100 has been published[30], but there are other partial cDNA sequences which do not yet overlap with N-

* Stoffel, W. & Tunggal, L. (1986), in preparation.

terminal or C-terminal sequences[8,9,11]. Their allocation within the complete apo B 100 sequence awaits clarification.

The synthesis of suitable oligonucleotide probes for the screening of randomly primed cDNA-libraries additional peptide sequences of apo B 100 are required. We have analysed a number of cyanogen bromide and tryptophan fragments of apolipoprotein B 100 which are not present in the apo B 100 sequences reported so far from cDNA data. They will be presented in a subsequent communication.

These data may help to facilitate the current work going on in several laboratories towards the elucidation of the complete primary structure of human apo B 100. By means of this cDNA clone as hybridization probe we were able to identify and characterize the C-terminal part of the human apo B gene*.

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