Nucleotide sequence encoding the carboxyl-terminal half of apolipoprotein B from spontaneously hypercholesterolemic pigs

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Abstract Previous studies from this laboratory characterized the hypercholesterolemia of pigs with a mutant allele of apolipoprotein B (apoB), designated Lpb^5. This apoB allele is associated with low density lipoprotein (LDL) particles deficient in binding to the LDL receptor. To identify potential causative mutations in Lpb^5 DNA, 10.6 kb of genomic DNA, encoding the carboxyl-terminal 58% of apoB were sequenced from the Lpb^5 allele and from an allele encoding phenotypically normal apoB. Comparison of the two DNA sequences revealed 33 polymorphisms, 13 of which resulted in amino acid polymorphisms. To determine whether any of the amino acids at the polymorphic positions in Lpb^5-encoded apoB were unique to that isoform, those positions were sequenced in four other pig apoB alleles encoding phenotypically normal apoB. None of the amino acids were by themselves uniquely encoded by the Lpb^5 allele. However, a unique haplotype consisting of Asp_{3164} in conjunction with Ala_{3447} distinguished the Lpb^5-encoded apoB from all other allelic isoforms sequenced in this region. To gain insight into changes in the tertiary structure of the mutant apoB, ^{13}C-NMR analysis of LDL reductively methylated with $[^{13}C]$-formaldehyde was performed. LDL has lysine residues that titrate at pH 10.5 and others that titrate at pH 8.9. The latter residues are thought to include those involved in the interaction of LDL with the LDL receptor. LDL from Lpb^5 pigs possessed a smaller proportion of lysine residues titrating at pH 8.9 than did LDL from non-Lpb^5 pigs, suggesting that the Lpb^5-encoded apoB is altered in a manner affecting the microenvironment of particular lysine residues.—Purtell, C., N. Maeda, D. L. Ebert, M. Kaiser, S. Lund-Katz, S. L. Sturley, V. Kodoyianni, K. Grunwald, D. N. Nevin, R. J. Aiello, and A. D. Attie. Nucleotide sequence encoding the carboxyl-terminal half of apolipoprotein B from spontaneously hypercholesterolemic pigs. *J. Lipid Res.* 1993. **34:** 1323–1335.

Supplementary key words low density lipoproteins • DNA • LDL receptor-related protein

Hypercholesterolemia is a major risk factor for coronary heart disease. Genetic factors contribute about 50% to the variance in plasma cholesterol levels in the human population (1). Approximately two-thirds of plasma cholesterol is transported on low density lipoprotein (LDL) particles. The best-characterized mutations associated with hypercholesterolemia are those affecting the function and expression of the LDL receptor, which is primarily responsible for LDL catabolism (2). However, since LDL receptor mutations account for only about 1% of individuals with hypercholesterolemia, mutations in other genes undoubtedly contribute to the high prevalence of hypercholesterolemia.

Apolipoprotein B (apoB) is the major protein component of LDL and a ligand for the LDL receptor. Immunogenetic studies in pigs first showed that variation in apoB structure might be associated with hypercholesterolemia (3). In these studies, pigs were cross-immunized with pig serum and the resulting allo-antisera were used to follow the inheritance pattern of epitopes, some of which were subsequently shown to reside on apoB (4). This approach enabled the discrimination of eight pig apoB alleles, designated Lpb^{1-8} (3).

One pig apoB allele, designated Lpb^5, occurs in pigs that have an elevation in plasma LDL (4). Cultured fibroblasts from these pigs express normal LDL receptor activity (4). The LDL elevation is due to an 85% lower affinity of this apoB variant for the LDL receptor (5) and a pronounced overproduction of a buoyant LDL subspecies (6, 7).

Abbreviations: apo, apolipoprotein; LDL, low density lipoprotein; PCR, polymerase chain reaction; CD, circular dichroism; NMR, nuclear magnetic resonance; LRP, LDL receptor-related protein; PBS, phosphate-buffered saline.

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The cross-immunization experiments that identified the allelic apoB isoforms in pigs constituted a non-prejudiced screen for polymorphic proteins in plasma. A similar cross-immunization occurs in patients undergoing multiple blood transfusions. Analysis of sera from such patients led to an immunological classification of human apoB isoforms, the *ag* system (8). Because the most antigenic protein identified in these analyses was apoB, it was inferred that apoB is an unusually polymorphic protein. More recently, studies of a highly repetitive region downstream of the human apoB gene indicate that this locus is also unusually polymorphic in non-coding regions of the gene (9).

Most of the human apoB polymorphisms do not appear to be correlated with a clinical phenotype. However, a relatively common Arg → Gln substitution at amino acid residue 3500 is associated with an apoB molecule essentially devoid of receptor binding activity (10). Since apoB truncations that delete the region surrounding Arg_{3500} do not reduce the binding affinity of apoB for the LDL receptor (11), it would appear that the Arg → Gln substitution prohibits receptor binding by altering the structure of a distant segment of apoB.

This interpretation is supported by structural analysis of intact mutant apoB. Lund-Katz et al. (12) developed an NMR technique for assessing the microenvironment of lysine residues on apoB. By labeling the exposed lysine residues via reductive methylation with [^{13}C]formaldehyde, they were able to discern two distinct lysine chemical shifts, corresponding to distinct pK_as for the ϵ-amines. Upon denaturation in 7 M urea, the lysine residues were consolidated into one chemical shift, suggesting that the more complex pattern was a reflection of the unique environments of two distinct pools of apoB lysine within the context of an intact LDL particle (12). Lund-Katz et al. (13) showed a significant decline in the proportion of lysines titrating at the lower pH in apoB possessing the Arg_{3500}→ Gln mutation.

We recently cloned and sequenced the portion of a normal pig apoB allele, *Lpb7*, encoding a carboxyl-terminal segment of apoB (14). This region is suspected to contain domains necessary for binding to the LDL receptor. In addition, we previously sequenced a short (1.1 kb) segment from the *Lpb5* allele (15). Although several sequence differences between *Lpb5* and *Lpb7* were detected, none of the individual sequence variations were found to be unique to the *Lpb5* allele when three other apoB alleles from non-hypercholesterolemic pigs were sequenced within that segment. We concluded that mutations responsible for the defective receptor recognition by the *Lpb5*-encoded apoB might lie outside the putative receptor binding domain encoded by the 1.1-kb segment (15). Alternatively, the causative alteration is more complex than a single missense mutation.

In the present study, we have sequenced 10,686 and 10,403 bp at the 3' ends of pig apoB alleles *Lpb5* and *Lpb7*, respectively (the difference in length relates to a unique 283-bp insertion in intron 28 in the *Lpb5* allele). The sequence described here encodes 58% of the apoB protein. This enabled the identification of all positions where the two alleles differ within this segment. In addition, the ^{13}C-NMR technique was applied to the comparison of normal
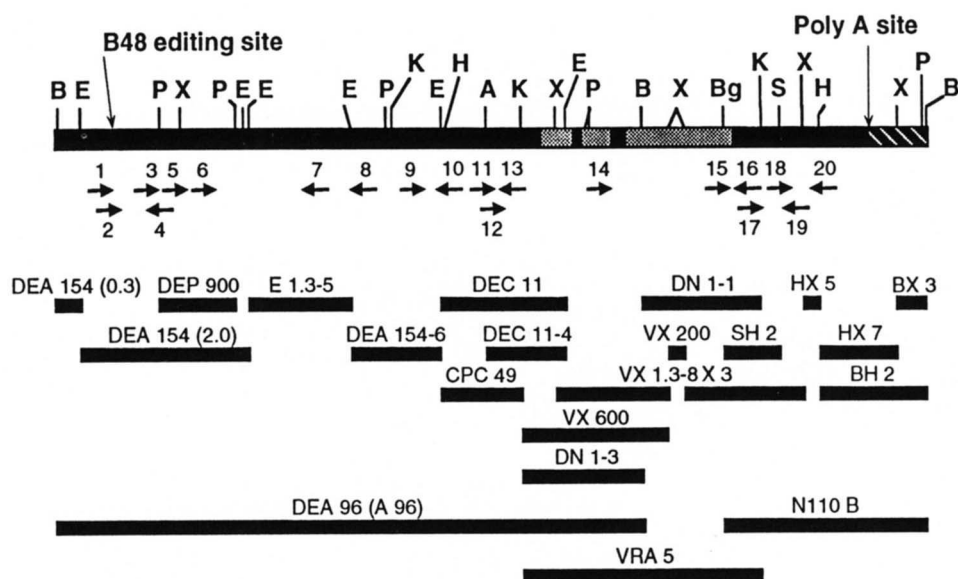

Fig. 1. DNA sequencing strategy. The shaded areas indicate introns. Letters indicate restriction endonuclease sites used to generate subclones: B, *Bam*H1; S, *Sac* I; E, *Eco* R1; P, *Pst* I; X, *Xba* I; K, *Kpn* I; Bg, *Bgl* I, H, *Hind* III. Heavy bars indicate segments subcloned into plasmids. Numbers indicate the locations and orientations of the synthetic oligonucleotide primers.

versus mutant pig LDL particles to assess the relative conformations of lysine residues in particles with varying affinities for the LDL receptor.

MATERIALS AND METHODS

Subcloning of genomic DNA fragments

Genomic clones (7.0–10.4 kb) from non-hypercholesterolemic (*Lpb7*) and hypercholesterolemic (*Lpb5*) pigs (14) were digested with appropriate restriction enzymes; fragments were separated by agarose gel electrophoresis, and isolated from agarose gel slices (16). The gel-purified fragments were subcloned into the Bluescript M13 +/– plasmid (Stratagene).

DNA sequencing (Fig. 1.)

Templates were sequenced by the chain termination sequencing method (17) using the modified DNA polymerase, Sequenase® (U.S. Biochemical) according to the protocol supplied by the manufacturer. Polymerization reactions were primed with the T7 or SK primers (Stratagene) or internally primed with synthetic oligonucleotide primers, which were synthesized on an Applied Biosystems 430A oligonucleotide synthesizer.

In regions where there was band compression, *Taq* polymerase (Taquence® kit; U.S. Biochemical) was used in place of Sequenase®; annealing was at 37°C, incubation with [35S]dATP, and extension reactions were at 65°C.

Sequencing of polymerase chain reaction amplification products

Pig genomic DNA was prepared by the method of Poncz et al. (18). A 2.5 kb sequence (nucleotides 6781–9338)[2] encoding a region containing a 10-amino acid insertion unique to pig apoB was amplified by the polymerase chain reaction (PCR) using the primers and conditions recently described (19). The PCR products from three 100-μl reactions were purified from agarose gels and precipitated with ethanol. Two μg of purified PCR product was annealed with 60 ng of the nested primer: 5'-AGAGCCAGGAACTACACTGGAGATGAGCTCT-3' (position 8852–8882 bp of porcine apoB). Sequencing was performed following a modified Sequenase® procedure (20). Reaction products were subjected to electrophoresis on a 7 M urea, 5% LongRanger® (AT Biochem) gel for 1.5 h at 1200 V.

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[2]The nucleotide sequence presented in this paper has been deposited in the GenBank database and has been assigned accession number L11235.

Sequence analysis

More than 90% of the reported sequence, including all exon sequence, was obtained three or more times. The remaining 10% was performed twice. In more than 50% of the reported sequence, both strands were sequenced. Autoradiograms were read at least twice by different people. The sequence was entered by typing or via digitizer into the University of Wisconsin Genetics Computer Group (UWGCG) program "SeqEd." Subsequent analysis was done employing other GCG programs (21) and DNA Strider (22). Homology searches in the Genbank database were carried out using the FASTA program (23) accessed through the EMBL Network file server (24, 25).

Lipoprotein preparation

Blood was collected aseptically from animals fasted overnight into a phosphate-buffered saline (PBS)/1 mM EDTA cocktail as described previously (6). LDL was subfractionated and the fractions were pooled into two density ranges: d 1.019–1.038 g/ml (buoyant LDL) and d 1.038–1.063 g/ml (dense LDL) (7). The density cutoff at 1.038 g/ml was used for all animal LDL separations and represents the most common density at which there was a trough in the mutant LDL density profile (6).

NMR spectroscopy

Nuclear magnetic resonance (NMR) spectroscopy was used to characterize the exposed lysine residues of apoB in the mutant and control pig LDL subspecies. The reductive methylation procedure of Jentoft and Dearborn (26) was used to introduce ^{13}C-labeled methyl groups into apoB lysine residues. The degree of methylation was determined by measuring the incorporation of [^{14}C]formaldehyde into LDL using scintillation counting and by measuring the incorporation of [^{13}C]formaldehyde into LDL using NMR spectroscopy. For a given sample, these values agreed to ± 0.1%. The number of visualized lysines was derived by integration of the $(^{13}CH_3)_2$ lysine resonances (δ = 43.1 and 42.7 ppm), as described previously (12). The integrated intensities from $(^{13}CH_3)_2$ Lys resonances were obtained by using a gated proton decoupler routine to eliminate the nuclear Overhauser effect. In these experiments, the irradiation for proton decoupling was switched off for a period at least 5 times the longest T_1 of the resonances compared. The integrated areas from expanded printouts of the $[^{13}CH_3]_2$Lys resonances were determined (13).

Circular dichroism (CD) measurements were carried out as described previously (12) on control and mutant LDLs before and after reductive methylation. The α-helix content was within ± 5% of the unlabeled particle (data not shown). Thus, no apparent changes in apoB secondary structure were detected in the LDL particles as a result of methylation.

RESULTS

DNA sequence analysis

The nucleotide sequence analysis of the *Lpb⁵* allele presented here identified several new restriction fragment length polymorphisms (**Table 1, Fig. 2**). In total there are 33 nucleotide differences between the *Lpb⁵* and *Lpb⁷* alleles, of which 13 result in changes at the amino acid level. The sequence of both alleles indicates the absence in pig apoB of the 'hypervariable' non-coding region located at the extreme 3' end of the human apoB gene (9).

The intron–exon arrangement of the *Lpb⁵* allele is similar to that of the *Lpb⁷* allele and thus, human apoB. The *Lpb⁵* allele has a unique 283-bp insertion within intron 28 (nucleotides 7078–7360) which is not predicted to affect intron splicing (14). A homology search through Genbank revealed 87% identity within a 260-bp overlap with pig inhibin β subunit cDNA, 76% identity within a 274-bp overlap with pig MHC class I PD6 glycoprotein cDNA, 76% identity within a 277-bp overlap with pig DNA for interferon-γ, 72% identity within a 247-bp overlap with pig relaxin DNA, and 72% identity within a 283-bp over-

TABLE 1.   Nucleotide (nt) and amino acid differences between *Lpb⁵* and *Lpb⁷* pig apoB alleles

Nucleotide substitutions causing amino acid substitutions

Position	Pig *Lpb⁵* nt	Pig *Lpb⁵* Amino Acid	Pig *Lpb⁷* nt	Pig *Lpb⁷* Amino Acid	Human Equivalent Amino Acid #	Human Equivalent Amino Acid	Exon	RFLPs *Lpb⁷*→ *Lpb⁵*, Sites Lost (−) or Gained (+)
2041	T	Ser	G	Ala	2601	Ser	26	− *NsB* II
3590	T	Val	C	Ala	3117	Thr	26	− *Alu* I
3936	A	Glu	C	Asp	3232	Glu	26	− *Mae* II, + *Afl* III
4582	G	Ala	A	Thr	3447	Lys	26	+ *Fnu*4H I
4660	G	Val	A	Ile	3473	Val	26	− *Aha* II, + *Aat* II, + *Mae* II
5208	G	Met	A	Ile	3655	Lys	26	− *Hind* III, + *Nla* III
5321	C	Ser	A	Tyr	3693	Ser	26	(no RFLP)
5432	G	Arg	A	Gln	3730	Gln	26	− *Eco*57 I
6917	C	Leu	G	Val	3974	Ala	28	− *Dde* I, + *Mae* I
6929	G	Ala	T	Ser	3978	Ala	28	− *Fok* I
8801	G	Val	A	Ile	4163	Ile	29	+ *Hinc* II
9009	G	Arg	T	Leu	*a*		29	(no RFLP)
9665	C	Gln	G	Glu	4441	Glu	29	(no RFLP)

Nucleotide substitutions not causing amino acid substitutions and nucleotide substitutions within introns

Position	*Lpb⁵* nt	*Lpb⁷* nt	Exon/intron	RFLP's *Lpb⁷*→ *Lpb⁵* Sites Lost (−) or Gained (+)
1461	T	C	exon 26	(no RFLP)
1512	C	T	exon 26	(no RFLP)
2022	G	A	exon 26	(no RFLP)
2625	A	G	exon 26	− *Nsp*B II, + *Mae* I
3243	A	G	exon 26	(no RFLP)
3297	T	C	exon 26	− *Sau* 3A,*Mbo* I, *Dpn* I
3624	G	A	exon 26	(no RFLP)
4020	T	C	exon 26	− *Bsp* 1286 I, *Ban* II, *Hph* I
4224	C	T	exon 26	(no RFLP)
4422	T	C	exon 26	− *Mae* II
4596	T	C	exon 26	− *Hinc* II, + *Sau*3A I, *Mbo* I, *Dpn* I, *Bcl* I
4956	C	T	exon 26	− *Alu* I, + *Mnl* I, *Eco*N I
5664	G	C	exon 26	(no RFLP)
6673	G	A	intron 27	− *Mae* III
7058	G	A	intron 28	(no RFLP)
7562	T	G	intron 28	(no RFLP)
7994	G	A	intron 28	− *Dpn* I, *Mbo* I, *Sau*3A I, + *Hpa* II, *Msp* I, *Bsm*A I
10060	A	G	3' non-coding	(no RFLP)
10079	G	A	3' non-coding	+ *Fok* I
10218	A	G	3' non-coding	(no RFLP)

*a*Within 10-amino acid insertion at site corresponding to Val$_{4223}$↓Ile$_{4224}$ in human apoB.

Exon: 26 27 28 29

1921 3500 3903 3941 4002 4536

Lpb5 - S V E A V M S R L A ■ V R Q → 10-AA insertion

Lpb7 - A A D T I I Y Q V S I L E → 10-AA insertion
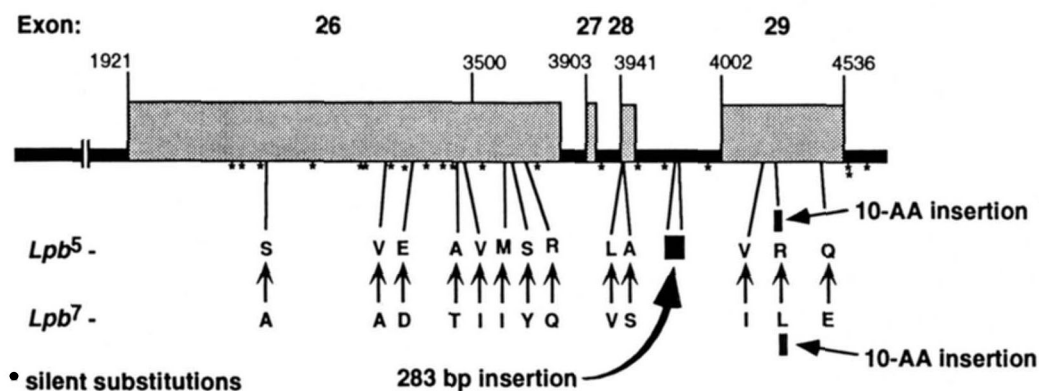
• silent substitutions 283 bp insertion →

Fig. 2. Locations of sites where the Lpb^5 and Lpb^7 alleles have sequence differences. Sequence differences not resulting in amino acid changes or located within introns are indicated with asterisks.

lap with the pig heart calpastatin gene. This insertion has facilitated the routine identification of pigs carrying the Lpb^5 allele using the polymerase chain reaction (19).

ApoB mRNA has the unique property of undergoing post-transcriptional editing to produce a modified transcript encoding a prematurely truncated protein, termed apoB48 (27–29). The editing mechanism is sequence-specific to a region of 26 bp flanking the editing site at nucleotide 6666 (27, 30). The pig apoB sequence in this region is identical to that of human apoB, implying conservation of this mechanism.

Protein sequence analysis

The ligand binding domain of the LDL receptor is comprised of seven imperfect repeats enriched in acidic amino acids (31). Charge modification of arginine (32) or lysine (33) residues on apoB abolishes binding to the LDL receptor. Thus, it has been inferred that the receptor–ligand interaction involves an electrostatic interaction between basic amino acids in the ligand and acidic amino acids in the receptor. Several segments with clusters of basic amino acids have been identified in the apoB sequence. This has led to the proposal of a region between amino acids 3100 and 3500 as the LDL receptor binding

domain (34–36). The Lpb^5 allele encodes an alanine residue while the Lpb^7 encodes a threonine residue at a position corresponding to Lys_{3447} in human apoB. Three conservative substitutions were found, $Ala_{3117} \rightarrow Val$, $Asp_{3232} \rightarrow Glu$, and $Ile_{3473} \rightarrow Val$. In addition, nine amino acid substitutions were discovered outside of the 3100–3500 region (Table 1, Fig. 2).

To determine whether any of the amino acid substitutions were unique to the Lpb^5 allele, several other alleles were sequenced at each of the polymorphic sites. In every position where the deduced amino acid corresponding to the Lpb^5 and Lpb^7 alleles differed from one another, other alleles (which encode phenotypically normal apoB) were found that encoded the same amino acid at that position as did the Lpb^5 allele (**Table 2**).

We next considered whether multiple alterations to the Lpb^5 allele might confer unique properties to LDL. The combination of Asp at residue 3164 in conjunction with Ala at 3447 is unique to the Lpb^5 encoded protein. The other alleles sequenced in this region encode either Gly_{3164}/Ala_{3447} (Lpb^2) or Asp_{3164}/Thr_{3447} (Lpb^3/Lpb^7).

A 10-amino acid segment in pig apoB was identified that, relative to the human apoB sequence, represents an insertion between Ile_{4224} and Val_{4223} (**Fig. 3**). Five of the

TABLE 2. Amino acid residues at polymorphic* sites in pig apoB

Allele	2601	3117	3232	3447	3473	3655	3693	3730	3974	3978	4163	•	4441
Lpb^2			Glu	Ala	Val	Met						Arg	
Lpb^3	Ser	Val	Glu	Thr	Val	Ile						Leu	
Lpb^4	Ser	Val					Ser	Arg	Val	Ala		Leu	Glu
Lpb^5	Ser	Val	Glu	Ala	Val	Met	Ser	Arg	Leu	Ala	Val	Arg	Gln
Lpb^7	Ala	Ala	Asp	Thr	Ile	Ile	Tyr	Gln	Val	Ser	Ile	Leu	Glu
Lpb^8									Leu	Ala	Val	Arg	Gln

*Sites where Lpb^5 and Lpb^7 differ.

```
      GSTSHHLLSRRSMNTALDHKVSALLTLAEQTGTWKLKTQLNQNEYSQNFDAYNTKDKVGVELNGRGLADLTVLDSTITVPLLLSEPINVIDALEMRDTVD
      ||||||:||:|:..||:||||||| ||||||||||||||:|.|||||:|.||||||:|||||:|||.||.|||||:|||.|.||||||||: |||||||.|:
 1921 GSTSHHLVSRKSISAALEHKVSALLTPAEQTGTWKLKTQFNNNEYSQDLDAYNTKDKIGVELTGRTLADLTLLDSPIKVPLLLSEPININDALEMRDAVE

      QPQEFTVVASVKYDKNQNHHTINLPFFNILPEYFEKTRLVGIVALEAIQRELKHININQPMRKYGAALDKFPQQVNDYLSTFSWERQVLSAKKKLSDFME
      .|||||:|| ||||||||: |.||||||:.|.||||:.| . ||.||..:||:||||||:| :||| |||:|:|||.||||..|.||||| ||.||..: .
 2021 KPQEFTIVAFVKYDKNQDVHSINLPFFETLQEYFERNRQTIIVVLENVQRNLKHINIDQFVRKYRAALGKLPQQANDYLNSFNWERQVSHAKEKLTALTK

      DYRITENDVRIALDNAKINLNEKLTQLQTYVIQFDQYIKDNYDLHDFKTAIARIIDEIIATLKILDERYHIHVNLVKKIHDLHLFIEKIGFNKTRSSTAS
      .|||||||:.||||:||||:||||.|||||||||||||||||||:|.|||.||||||.|| |||:||.:|||||.||||||||||.|:|||. |||||
 2121 KYRITENDIQIALDDAKINFNEKLSQLQTYMIQFDQYIKDSYDLHDLKIAIANIIDEIIEKLKSLDEHYHTRVNLVKTIHDLHLFIENIDFNKSGSSTAS

      WFQNVNINYQIRLWIQEKLQQLKAQIQNIDIQHLAEKLKQQVEDIDARVLLDQLRTTIPFQEIKEVIEHVKYCVKSLFEDFEVTKEINAFRAMVHELIKM
      |:|||:..|||| ||||||||| :|||||||||:|||||::|.||.||||||| |||.|: |.:|:||||. | .|.::||||...|||||.|||||..
 2221 WIQNVDTKYQIRIQIQEKLQQLKRHIQNIDIQHLAGKLKQHIEAIDVRVLLDQLGTTISFERINDVLEHVKHFVINLIGDFEVAEKINAFRAKVHELIER

      YKIDEHIQVLMDNSVHLAHQYKLQETVQKLSSSILQQVKIQDLLETLTWFIDDTLKQLEALSFQQLIEEVNKFLDMLVKRLRSFDYHHFLDETNKNLHEVT
      |.:|::|||||| |.||||||||.||:||||:|||||:||||.|.:|.|. ||||:|.|.|||. :||:|||||||:|:|:|||||:|:||||||:|||..::|||
 2321 YEVDQQIQVLMDKLVELAHQYKLKETIQKLSNVLQQVKIKDYFEKLVGFIDDAVKKLNELSFKTFIEDVNKFLDMLIKKLKSFDYHQFVDETNDKIREVT

      QRINGKIQALELPQKFTALKLLSEDIKALISVHLENLKATKITLFFDWLQGTLSSTPIVHMKAKFHETLEDVRDRMYQMDVQQEIQRYLFLVGQVYSTLV
      ||:||.||||||||| .|||| ||:. ||.||| :.||.:||.:| :.||||||||:|||||||||||||:.||||||||||||:|||:||||:| |||||||||||
 2421 QRLNGEIQALELPQKAEALKLFLEETKATVAVYLESLQDTKITLIINWLQEALSSASLAHMKAKFRETLEDTRDRMYQMDIQQELQRYLSLVGQVYSTLV

      TYISDWWTLAAKNLTDFAEQYSIQDWAKNLKALVEQGFTVPEIQTTFGTLPAFEVSLRALQEATYQTPDFIVPLTDLKIPSVQINFKKLRDIEIPSRFST
      ||||||||||||||||||||||||||||||.:||||||||||||.|.:||:|||||||.|||.||:||||||||||||||:|||||||||||||.|::|.|||||||
 2521 TYISDWWTLAAKNLTDFAEQYSIQDWAKRMKALVEQGFTVPEIKTILGTMPAFEVSLQALQKATFQTPDFIVPLTDLRIPSVQINFKDLKNIKIPSRFST

      PEFTVLNTFHVPSFTIDLVEIKAKIIRTIDQMLNSELQWPVPEVYLRDLNGVETMLARITLPDLYLPEIRIPEFVIPNLNLTDFQVSDLHIPEFQLPHIP
      |||||.|||||.|||||||.||||||||:.|.||||||||||||||::||||.. :. |||||||:.|||| ||||:||.|||.|||||||||:||||||||||||.
 2621 PEFTILNTFHIPSFTIDFVEMKVKIIRTIDQMLNSELQWPVPDIYLRDLKVEDIPLARITLPDFRLPEIAIPEFIIPTLNLNDFQVPDLHIPEFQLPHIS

      HTVEVPAFGKLHGILKIQSPLLILEANANIQNITTSENTAEMEASFTAKGESKFEVLNFDFQANAQLSDPEINPLVLKESVRFSSKYLKTEHGSEVLFVG
      ||:|||.|||.:|||||||||:.|:|||||:|.|:|:.|. |||.|.|::.||:||||||:||||||||||:|.||||.|||||:||||||:|:|||||||:.|.|
 2721 HTIEVPTFGKLYSILKIQSPLFTLDANADIGNGTTSANEAGIAASITAKGESKLEVLNFDFQANAQLSNPKINPLALKESVKFSSKYLRTEHGSEMLFFG

      NSIEGKSNTVAGLYTGKNTLKLSNDVLLKVNNQLTLNSNTKYFHELNIPKLDFSSQADLRSEIKSLLEASHIAWTSSGKGSWKLACHRFSDEGTHESQIS
      |.|||||||||.|:|.|:||||.|||:|:::|.:||||||||||||||||||||||||||||.|||.||.|:||||||||||||||:||.|||||||||||||||
 2821 NAIEGKSNTVASLHTEKNTLELSNGVIVKINNQLTLDSNTKYFHKLNIPKLDFSSQADLRNEIKTLLKAGHIAWTSSGKGSWKWACPRFSDEGTHESQIS

      FTVEGPITSFGLSNEIRSKHLRVNQSMVYESAFLKFSKFEIHSQAECQYVGRSVLTAKGMVLPRERKAEITGNHDAHLNGKVIGTLKNSLFFSAQPLEIT
      ||:|||:||||||||.|.:|||||.:||:|||| |.|||:.:|..::.||.||||||||||.|.| | |||:.|.|||||||||||||||||||||||||||||:|||
 2921 FTIEGPLTSFGLSNKINSKHLRVNQNLVYESGSLNFSKLEIQSQVDSQHVGHSVLTAKGMALFGEGKAEFTGRHDAHLNGKVIGTLKNSLFFSAQPFEIT

      RSTINEGNLRVSFPLKLTGKIDFLNNYALLLSPSAQQASWQASARFNQYKYNQNFSAVNNERSTEAHIGINGEANLDFLNIPLTIPEMTLPYTRLTVPQV
      || ||||:|.|||:||||||||||||||||.|||||:|||||||||||||||||.|||. |||:|||||||||||||||||||| |||| :|.|.:
 3021 ASTNNEGNLKVRFPLRLTGKIDFLNNYALFLSPSAQQASWQVSARFNQYKYNQNFSAGNNENIMEAHVGINGEANLDFLNIPLTIPEMRLPYTIITTPPL

      KDISLWEKTGLKEFLKTTKQSFDLSVKAQYKKNKDKHSIPNPLDVFYAFINRNINSFNRHFETVRDKALDFFTESYNEIKITFDKYKVEKPLDQQPRTFQ
      ||:|||||||||||||||||||||||K̲A̲Q̲Y̲K̲K̲N̲K̲H̲R̲H̲SITNPLAVLCEFISQSIKSFDRHFEKNRNNALDFVTKSYNETKIKFDKYKAEKSHDELPRTFQ
 3121 KDFSLWEKTGLKEFLKTTKQSFDLSV

      IPGYTIPVINIEVSPFTVKMLTFGYVIPKEISTPNITILGSGISVPSYTLGLQFLELPALDVPRNILQISLPEL.AVSGTSNKILIPAMGNITYDFSFKS
      |||||:||:|:||||||||||.||||:||.|:| |..:.::.|||||| |. ||||.|.||| |.:|||:: .:::..|: |:||||||||||||||||||
 3221 IPGYTVPVVNVEVSPFTIEMSAFGYVFPKAVSMPSFSIIGSDVRVPSYTLILPSLELPVLHVPRN.LKLSLPDFKELCTISH.IFIPAMGNITYDFSFKS

      SVITLNTNVGLYNQSNIVAHFLTFSSSVIDALQYKLEGTSSLMRKRGLKLATALSLSNKFMEGNHDSTISFTKKNVDASLTTTARVQLPILRMNFKQELK
      ||||||||.:|:|||:||||.|. ||||||||||||||||||||..| ||||||||||||||||:|.:|:|:|:.||::.|.:...|.:.::|||||||||||.
 3319 SVITLNTNAELFNQSDIVAHLLSSSSSVIDALQYKLEGTTR̲L̲T̲R̲K̲R̲G̲L̲K̲LATALSLSNKFVEGSHNSTVSLTTKNMEVSVAKTTKPEIPILRMNFKQELN

      GNTKSKPTISSSIELKYDFNSPKLHSTAAGAVDHKLSLESLTSYFSIEMSTKGDVKGSILSRVYSGSLASEASTYVNSKGTRSSVKLQGASKVDDIWNLE
      |||||||:|||:|:||||||:.||..|.|||||||||||||||||||||||||||| ||'||||||:|||| |||.:||||.||:|||:|||||||||||||.||:|||||||
 3419 GNTKSKPTVSSSMEFKYDFNSSMLYSTAKGAVDHKLSLESLTSYFSIESSTKGDVKGSVLSREYSGTIASEANTYLNSKSTRSSVKLQGTSKIDDIWNLE
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Fig. 3. Alignment of the pig *Lpb*[5] (top row) and human apoB (bottom row) deduced protein sequences. The numbering is based upon the human apoB sequence. The horizontal bars indicate two regions of high positive charge density and the ten-amino acid insertion in pig apoB.

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     VKENFAGEATLLRIYTIWEHHTKNHLQLEGLILTSGEHTSKATLELSPWNIAALVQVHTRQPSSLLNIHYLSQEVSLNANTENQKISWKSQVQVHSGSLQ
     ||||||||||| |||.:|||  ||||||||||||::|.||||||||||||||.:.||||||..||||: ::. |:|||.|||||.||||.||.:|.:||||:|
3519 VKENFAGEATLQRIYSLWEHSTKNHLQLEGLFFTNGEHTSKATLELSPWQMSALVQVHASQPSSFHDFPDLGQEVALNANTKNQKIRWKNEVRIHSGSFQ

     NNLQLSNDKEEARFDISGSVEGYLSFLKYTVLPVYDMSLWDLLKLDVTTSINRKQYLRASTALVYTKNPNAYPVSIPVQELTDKFIIPGLKLNNLKSVYV
     ..::||||.|.|::||.||:||.|.||| .:|||||.|||||:||||||||||.|:|.||.|||:|||||||:|..||||. |.||||||||||||:|.||.|
3619 SQVELSNDQEKAHLDIAGSLEGHLRFLKNIILPVYDKSLWDFLKLDVTTSIGRRQHLRVSTAFVYTKNPNGYSFSIPVKVLADKFIIPGLKLNDLNSVLV

     TPAFQVPFTDLRFPSYTLDFSEIKIYKKLSTSSFSLNIPTLPKVKFPKVDVLTQYSNPETSSVPFFEVTVPAYQLTVSPFTLPKSISVGSAVLDLDVVAS
     |.|:|:||||||.||:.|||.||.|||||.||||.||||:.||||.||||.|||||.||.||.:|||||:|||.|||||.||||||||:|  | |.|||:.||.
3719 MPTFHVPFTDLQVPSCKLDFREIQIYKKLRTSSFALTLPTLPEVKFPEVDVLTKYSQPEDSLIPFFEITVPESQLTVSQFTLPKSVSDGIAALDLNAVAN

     YIADFEVPTVTMPEQTIEIPSIKFSVPAGISLPSFGTLTARFGVASPLYNATWSAGLKNKEDRVETFLDSTCSSTIQFLEYDLNVVGTHKIEDGMLVCST
     |||||:||.:||||||||||||||||||||  :|||.:|||||:|.||.||||:||||.| |||.||||||||:|||.||||:|||:|||||||||||| |.:.|
3819 KIADFELPTIIVPEQTIEIPSIKFSVPAGIVIPSFQALTARFEVDSPVYNATWSASLKNKADYVETVLDSTCSSTVQFLEYELNVLGTHKIEDGTLASKT

     KGSFAHRDLSAEYEENGKYHGL.YWDGKIILNITSPTLTDVYLRYQQSKNHLFSSLASPAIGTVALEQENINTSLKSNLYYRPQSSPDKKLNIFKIEKRD
     ||.:||||:||||||||:|||.|| |:|| |||.||.:||:.||||..|. : .| ||||:|||::: :: :. |.|:||.||||||||||.||||.| |
3919 KGTLAHRDFSAEYEEDGKYEGLQEWEGKAHLNIKSPAFTDLHLRYQKDKKGISTSAASPAVGTVGMDMDEDDDFSKWNFYYSPQSSPDKKLTIFKTELRV

     LESDEDVQIKANWEEEAVSELLRSLKDNVPKATGAFYDYVNKYHQEYTGLDLRDASLKLRKGLQNSADQAYQGAVRQIDEVDRGLRKIARGTTKSYQQWK
     ||||:.|||.|||||||.|:|||  |||||||||.|||||||||.|.|||.||:.| |||.|.|:|||||.||:|  ||.||||:::| :.|  |.||| .||:||
4019 RESDEETQIKVNWEEEAASGLLTSLKDNVPKATGVLYDYVNKYHWEHTGLTLREVSSKLRRNLQNNAEWVYQGAIRQIDDIDVRFQKAASGTTGTYQEWK

     DRAQSLYQKLLAQEDQGDFQGLRNKVFDSVIGVTQEYNVTVKRAVDSLIKSLKFTRFQLPGRARNYTGDELCNMVMTEVGEVLSQIYSKIHSGLEILLSY
     |:||.||||.||.||:|:.||||::.|||||::.  |||.::::.||.:::.||: :||||. |.|.|||:||:: ||  :|||.|.| |||.||||:|||:|
4119 DKAQNLYQELLTQEGQASFQGLKDNVFDGLVRVTQKFHMKVKKLIDSLIDFLNFPRFQFPGKPGIYTREELCTMFMREVGTVLSQVYSKVHNGSEILFSY

   _____.
     FQDLMEKSKLNKARKIKFTFDSVKYQLTDVVSEYGEQLKSLSQDVQKALSDLHSINITEILSELQIFLEGIFQEIEEELTRLKEKKLADFINDIQQNINT
     ||||:         |.:.|:   |..|.||:| | | ||.||.:.|..:..::|:.||:|.:|| :|: ||| ||:::..|||.|:. :|| ||::|||
4219 FQDLV..........ITLPFELRKHKLIDVISMYRELLKDLSKEAQEVFKAIQSLKTTEVLRNLQDLLQFIFQLIEDNIKQLKEMKFTYLINYIQDEINT

     TFNIYAPLGFRLLKENLDSPFGMLNEFIQNTLWEASQELQQLHQYIKALRKEYFDPSMVGWTVKYYELEEKVINLIKNLVDVLKDFHSKYTVSATDFASQ
     .||  | |..|:|||||   :  .:||||||.| ||||||||:||||.|||.||||||:|||||||||||::.|||||: .||||||.|.|||.:.|.|||.||
4309 IFNDYIPYVFKLLKENLCLNLHKFNEFIQNELQEASQELQQIHQYIMALREEYFDPSIVGWTVKYYELEEKIVSLIKNLLVALKDFHSEYIVSASNFTSQ

     LSSQVEQFVQEDIQEYLSILADADGKGKEKIAQLSSRAQEIIKSWAVTMKEIISDYHQQFIYKLQDFSDQLSDYYEKFIAETERLIDLSIQKYHMFLRYI
     ||||||||:::  :|||||||.|:||||||||||:||.||:||.  |||||| ||:.  |.|||||||| ||||||||||||||:|||||||||||..||||||||.||  || ||
4409 LSSQVEQFLHRNIQEYLSILTDPDGKGKEKIAELSATAQEIIKSQAIATKKIISDYHQQFRYKLQDFSDQLSDYYEKFIAESKRLIDLSIQNYHTFLIYI

     MKLLKELQSDTVNDMRPYIKVAPGEFTITF*
     .|||.||||.|| |.||:|:||||:||.:|
4509 TELLKKLQSTTV..MNPYMKLAPGELTIIL* 4536
```

10 amino acids encoded by the *Lpb⁵* allele are positively charged, making it the region of highest positive charge density in the 2625 amino acids we have deduced. A Genbank homology search revealed that 7 out of 10 amino acids in the insertion are identical with a segment in the pig rotavirus NS26 protein, which is encoded by genome segment 11 (37, 38). The three amino acids that are not identical to those in the rotaviral protein are in positions that are highly variable in other rotavirus strains (38). Antibodies to NS26 (kindly provided by Dr. M. Estes) failed to block the binding of pig LDL to its receptor (S. L. Sturley and A. D. Attie, unpublished observations). In addition, recombinant NS26 protein (kindly provided by Dr. M. Estes) failed to compete with pig LDL for binding to the LDL receptor (S. L. Sturley and A. D. Attie, unpublished observations).

We previously showed that pig LDL has a higher affinity for the LDL receptor than does human LDL (15).

It therefore seems plausible that the aforementioned basic segment may represent an LDL receptor binding domain and may account for the higher affinity of pig LDL for the LDL receptor. Interestingly, within this basic segment, a leucine residue in *Lpb⁷* is replaced by arginine in the *Lpb⁵* apoB allelic product. This raised the possibility that the receptor binding defect associated with the *Lpb⁵*-encoded apoB may be the result of the Leu → Arg substitution. To test this hypothesis, genomic DNA from four different pig apoB alleles, *Lpb²*, *Lpb³*, *Lpb⁴*, and *Lpb⁸*, was PCR-amplified and sequenced within this segment. All four of these alleles are associated with normal LDL receptor binding and normal in vivo LDL catabolism (M. Kaiser and A. D. Attie, unpublished observations). Nevertheless, two of the alleles encode leucine and two encode arginine at the polymorphic site, suggesting that the polymorphism is not, by itself, responsible for the receptor binding defect (Table 2). Since one of these apoB variants (*Lpb⁸*)

also possesses the *Lpb*[5]-encoded allotypic antigen (3), the insertion is unlikely to mediate expression of this epitope.

Human apoB contains seven cysteine residues in the segment corresponding to the pig apoB region analyzed here. Five of the seven cysteines in the human apoB segment have no counterpart in pig apoB. Four of the six cysteine residues in the pig apoB segment do not appear to have a counterpart in human apoB (Fig. 3 and **Fig. 4**). Thus, only cysteines at positions 2906 and 3890 are conserved between the two species.

Within the segment sequenced in this study, there were 16 potential N-linked glycosylation sites. Human apoB has 13 in this region, but only 6 were in precisely the same position as the pig glycosylation sites (Figs. 3 and 4). Furthermore, there were no differences between alleles at the predicted N-linked glycosylation sites.

Glycosaminoglycans are linked to protein via a xyloside residue. The xylose residue is typically attached to serine. Target serine residues have been characterized previously and all conform to a consensus pattern Ser-Gly-X-Gly (39, 40). In addition, the consensus requires two acidic amino acids 2–4 residues upstream from serine. Both human and pig apoB have one serine residue within the appropriate context for xyloside attachment (Ser$_{2897}$).

Spectroscopic analyses (Fig. 5)

The mutant pigs were first identified through a screen involving allo-antisera specific for the Lpb5 epitope. Thus, both the impaired receptor binding properties and the altered epitope expression are consistent with significant changes in the tertiary structure of the mutant apoB.

Alterations in tertiary structure were therefore probed by assessing the microenvironment of lysine residues on apoB. It had previously been suggested that certain lysines on the surface of human LDL, which titrate with a pKa of 8.9 ("active" lysines), as opposed to "normal" lysines, which titrate with a pKa of 10.5, may be involved in the interaction between apoB and the LDL receptor (12). The properties of the exposed lysine residues in apoB were therefore investigated by NMR spectroscopy.

Pig LDL was subfractionated into buoyant (d 1.019–1.038 g/ml) and dense (d 1.038–1.063 g/ml) LDL subspecies. Prior studies showed that the receptor binding defect was primarily demonstrable within the dense LDL subspecies (5). When dense LDL from normal and mutant pigs were compared, there was a substantially lower proportion of "active" lysines in the samples from mutant pigs. In addition, in the mutant pigs the dense LDL had a lower proportion of "active" lysines than did buoyant LDL. In control pigs, there was no difference in the proportion of "active" lysines in buoyant and dense LDL (**Table 3**).

DISCUSSION

The complexity of apoB has made it difficult to accurately map the domains required for lipoprotein assembly and for interaction with the LDL receptor. Comparisons of apoB across various species as well as the analysis of apoB alleles associated with well-defined phenotypes provide valuable information about functional domains of apoB. Such information will establish the basis for clinical screens of selected genomic DNA segments from patient populations and also will guide the selection of apoB segments for in vitro expression to further delineate their functions.

Despite the extensive polymorphism of apoB and intensive investigation, mutations that correlate with hypercholesterolemia have only rarely been detected. Several methodological problems are responsible for this difficulty. First, the apoB gene is 42 kb in length, hence thorough characterization of each new allele is not practical with current technology. Second, since most mutations
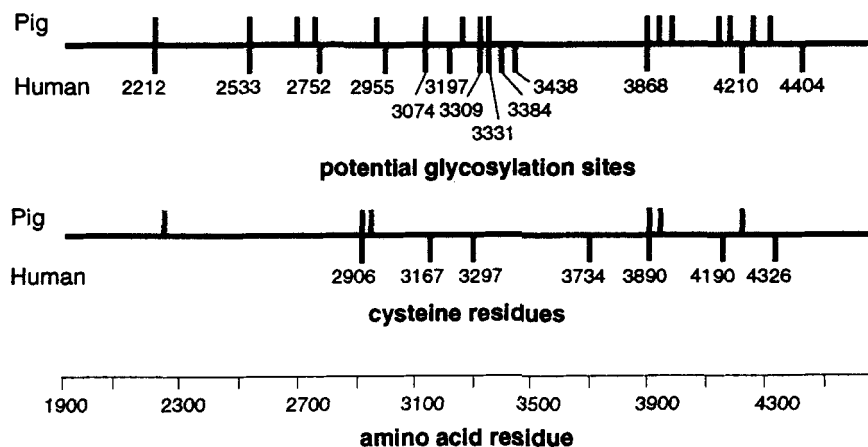

Fig. 4. Locations of potential asparagine-linked glycosylation sites and cysteine residues in pig and human apoB.
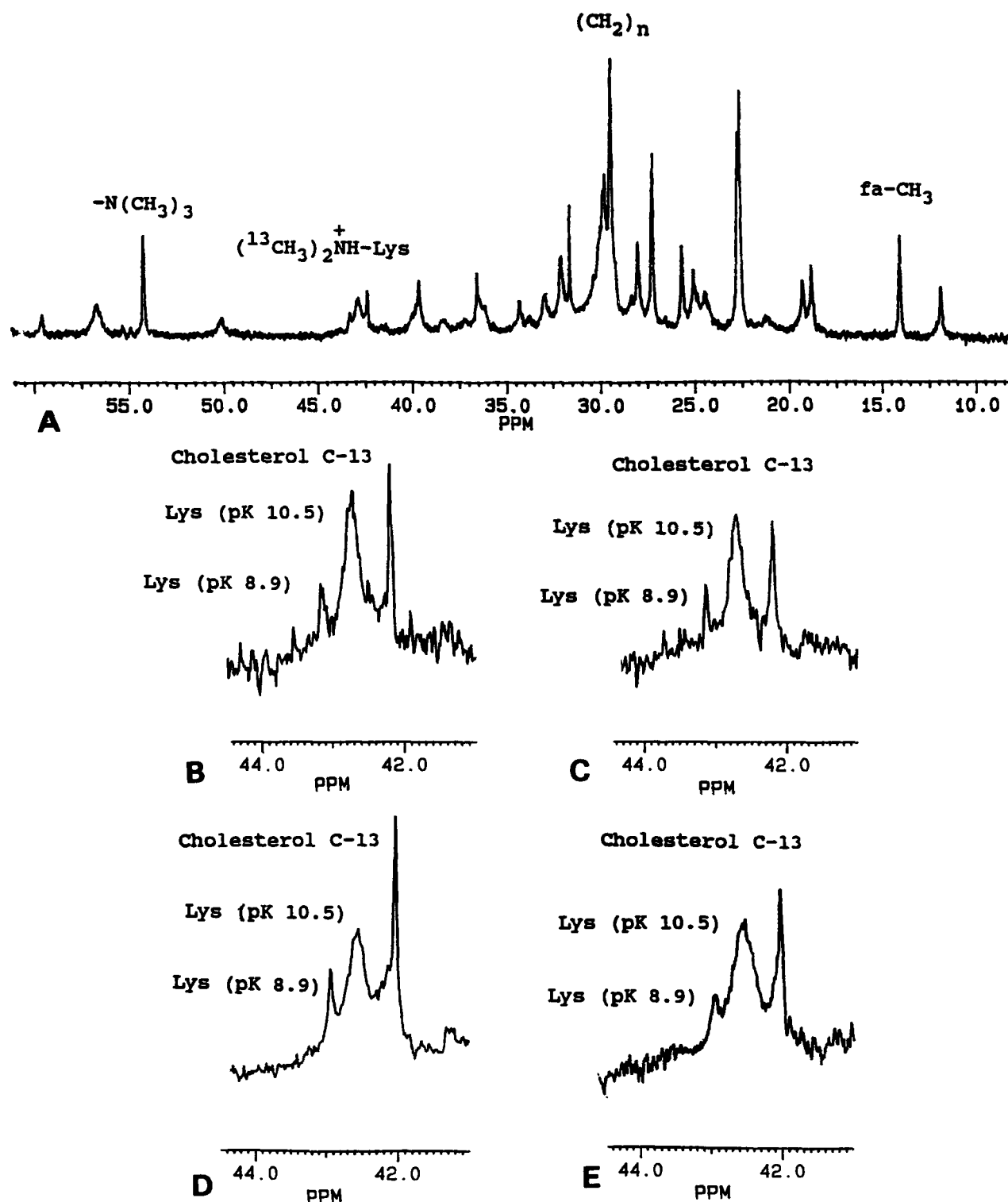
Fig. 5. ^{13}C NMR spectra of pig LDLs. Proton-decoupled ^{13}C NMR spectra (126 MHz) of pig LDL from normal buoyant (A, B), normal dense (C), and mutant buoyant (D) and mutant dense LDL (E) in which lysine residues have been converted to $[^{13}CH_3]_2$Lys by reductive methylation. The spectra were obtained at 37°C on a Bruber AM500 spectrometer and were accumulated using 90° pulses of 8 µsec duration and a recycling time of 1.08 sec. Chemical shifts were measured to external aqueous 1,4-dioxane (66.55 ppm). Spectra were processed with 2.0 Hz exponential filtering. A) The upfield region of the spectrum (56,002 acquisitions) of LDL from normal buoyant pig (3.2 mg of protein in 1.5 ml of 0.15 M NaCl, 1 mM EDTA, 0.02% w/v NaN$_3$, pH 7.6) in which 15.4% of the lysine residues were methylated. B) Expansion of spectrum in panel A showing $[^{13}CH_3]_2$Lys resonances. C) $[^{13}CH_3]_2$Lys resonances of normal dense pig LDL in which 17.8% of the lysine residues were methylated (2.9 mg of protein in 1.5 ml saline solution; 40,731 acquisitions). D) $[^{13}CH_3]_2$Lys resonances of LDL from mutant buoyant pig LDL in which 15.2% of the lysine residues were methylated (10.9 mg of protein in 1.5 ml saline solution; 33,801 acquisitions). E) $[^{13}CH_3]_2$ Lys resonances of LDL from mutant dense pig LDL in which 9.5% of the lysine residues were methylated (9.1 mg of protein in 1.5 ml of saline solution; 30,108 acquisitions). The $[^{13}CH_3]_2$Lys resonances are referred to as Lys (pK 8.9) and Lys (pK 10.5) by inference to normal human LDL (12). Chemical shifts of $[^{13}CH_3]_2$ Lys resonances from all pig samples were identical to those obtained from human LDL at the same pH.

TABLE 3. Content of normal and active apoB lysines in control and mutant pig LDL

LDL	% Lysines Modified	No. of ^{13}C-Labeled Lysine Residues[a]		
		(Active)	(Normal)	(Active/Normal)
Dense LDL				
Control 1 (Lpb$^{4/4}$)	12.6	17	28	0.61
Control 2 (Lpb$^{4/4}$)	17.8	21	43	0.49
Control 3 (Lpb$^{2/2}$)	11.4	14	27	0.52
Control 4 (Lpb$^{4/4}$)	10.4	12	25	0.48
Control 5 (Lpb$^{2/8}$)	9.3	10	23	0.43
Mutant 1 (Lpb$^{5/5}$)	9.5	9	25	0.36
Mutant 2 (Lpb$^{5/5}$)	9.5	7	27	0.26
Buoyant LDL				
Control 1 (Lpb$^{4/4}$)	10.5	14	24	0.58
Control 2 (Lpb$^{4/4}$)	15.4	19	36	0.53
Control 3 (Lpb$^{2/2}$)	13.8	16	33	0.49
Control 4 (Lpb$^{4/4}$)	13.5	15	33	0.45
Control 5 (Lpb$^{2/8}$)	9.6	11	23	0.48
Mutant 1 (Lpb$^{5/5}$)	15.2	18	37	0.49
Mutant 2 (Lpb$^{5/5}$)	10.2	13	23	0.57

[a]Per mole of apoB; derived by integration ($\pm$ 10%) of [^{13}CH$_3$]$_2$-lysine resonances in NMR spectra of the type shown in Fig. 2. Controls 1 and 2 are separate LDL preparations from the same pig.

are likely to abrogate the receptor binding activity of LDL only partially, and will most frequently occur in heterozygotes, a screen involving LDL binding to its receptor must be exquisitely sensitive in order to detect a partially deleterious mutation in an LDL preparation consisting of a mixture of normal and defective particles. One way to overcome some of these methodological problems is to identify the sites on the apoB molecule where mutations are most likely to alter binding of LDL to its receptor. This information can then be used to screen selected apoB genomic DNA segments from hypercholesterolemic individuals.

Several domains have been proposed to be directly involved in the binding of apoB to the LDL receptor. Since the ligand binding domain of the receptor is comprised of seven imperfect repeats of highly acidic domains, it has been proposed that there is an electrostatic interaction between these domains and positively charged domains in the ligand. Two apoB segments enriched in basic amino acids, amino acids 3147–3157 and 3352–3369, are potential candidates. The latter region is homologous to the well-characterized receptor-binding domain of apoE (41). A synthetic peptide corresponding to amino acids 3345–3381, when adsorbed to trypsin-treated hypertriglyceridemic VLDL, suppressed HMG-CoA reductase activity in cultured fibroblasts, presumably indicating that the peptide bound to the LDL receptor (36). Monoclonal antibodies that interfere with receptor binding have epitopes that map to the carboxyl-terminal half of apoB (42, 43).

When this study was initiated, the expectation was that there would be a single mutation that would appear as a strong candidate for causing the receptor binding defect associated with the mutant apoB gene product. Having

access to additional pig apoB alleles enabled us to rigorously test the hypothesis that one of the 13 polymorphisms we discovered was uniquely associated with the Lpb^5 apoB allele. Surprisingly, none of the polymorphisms was unique to the Lpb^5 allele. There are several possible explanations. The causative mutation may lie outside of the carboxyl-terminal 58% of the apoB sequence that we have determined in these studies. A more likely possibility is that the difference in primary structure responsible for the functional defect is caused by several amino acid differences. Indeed, a unique amino acid combination is encoded by the Lpb^5 allele. The presence of Asp$_{3164}$/Ala$_{3447}$ as opposed to Gly$_{3164}$/Ala$_{3447}$ or Asp$_{3164}$/Thr$_{3447}$ clearly distinguishes the Lpb^5 allele from the other pig apoB allelic variants. It is interesting to note that residue 3447 is within a putative epitope for the monoclonal antibody MB47 (44). MB47 inhibits LDL binding to its receptor and thus defines a crucial region of apoB for interaction with the receptor.

A human apoB mutation involving an Arg$_{3500}$→ Gln substitution has been identified in patients with hypercholesterolemia (10). The apoB gene product carrying this mutation is almost completely devoid of LDL receptor binding activity (45). A monoclonal antibody that blocks LDL binding to its receptor and that binds to a region close to Arg$_{3500}$ binds with higher affinity to the mutant apoB protein (46). Thus, it has been suggested that the Arg$_{3500}$→ Gln substitution is responsible for the receptor binding defect. The region surrounding Arg$_{3500}$ is well-conserved in pig apoB. Nevertheless, no mutations were observed within this region of the Lpb^5 allele.

It is noteworthy that despite intensive screening of hypercholesterolemic patients, no other single apoB mu-

tations have been identified that correlate with hypercholesterolemia. In an analysis of the 3130–3630 region of apoB from eight individuals carrying LDL that binds with reduced affinity to the LDL receptor, Dunning et al. (47) found no sequence differences, suggesting that in these individuals, the mutations responsible for reduced receptor binding lie outside this region.

Several immunochemical studies suggest that regions distant from the 3100–3600 region in apoB are involved in receptor recognition or binding. For example, Kinoshita, Krul, and Schonfeld (48) altered the core lipid composition of LDL to produce a triglyceride-enriched particle with reduced affinity for the LDL receptor. The accessibility of various epitopes to well-characterized monoclonal antibodies was measured. Surprisingly, the exposure of the epitopes corresponding to amino acids 3214–3506 or 3506–3635 was not affected. Rather, the exposure of the epitopes incorporating amino acids 690–797 and 4082–4348 was selectively affected. The latter region encompasses the position where pig apoB has the 10-amino acid insertion. In another study, Milne et al. (43) showed that epitopes spanning apoB amino acids 2980–3084 and domains in the proximity of Arg_{3500} became inaccessible to monoclonal antibodies after LDL was bound to the LDL receptor. In addition, the accessibility of the epitope encompassing amino acids 4027–4081 was also reduced. Another indication that the latter segment might be involved in receptor recognition came from the studies of Chapman and co-workers (49), who showed that a peptide corresponding to amino acids 4007–4019 could partially compete with LDL for binding to the pig adrenal LDL receptor. Numerous mutations producing truncated variants of apoB are associated with reduced plasma LDL levels (50). In some instances, this may be the result of reduced LDL production (51, 52) while in others, the truncations are associated with LDL particles with a higher affinity for the LDL receptor (11). The latter result would tend to suggest that the carboxyl-terminal segment interacts with the receptor binding region of apoB.

Pig LDL (including Lpb^5 pig LDL) has a higher affinity for the LDL receptor (pig or human receptor) than does human LDL (15). It is possible that the 10-amino acid insertion in pig apoB constitutes an extra docking domain by virtue of its high positive charge density. Alternatively, the insertion may mediate binding to another receptor, such as the LDL receptor-related protein (LRP). This would be consistent with the observation that pig LDL binding to cells fits to a two-receptor site model better than to a one-receptor site model (5). The Leu → Arg mutation within this segment in Lpb^5 apoB is contrary to what one would predict if charge density were the sole determinant of receptor binding affinity. By analyzing four additional apoB alleles, we ruled out the possibility that this mutation, by itself, causes the receptor-binding defect, but did not rule out the possibility that it comprises an additional receptor-binding domain.

The NMR analysis of the mutant pig LDLs showed a significant change in the environments of apoB lysine residues in LDLs with reduced affinity for the LDL receptor. As in the patients with the $Arg_{3500} \rightarrow$ Gln mutation, the pigs do not have mutations in the charged clusters thought to interact with the LDL receptor (15). Since the mutant pig apoB gene also does not encode Gln_{3500}, additional regions of apoB are sensitive to amino acid substitutions that alter the conformation of the domains interacting with the LDL receptor.

Only within the dense LDL subspecies were we able to observe a change in the proportion of "active" lysine residues. This correlates with the observation that within this LDL subspecies we were able to detect a difference in binding affinity for the LDL receptor. This is consistent with the proposal of Lund-Katz et al. (12) that the "active" lysines participate in receptor recognition.

When pig LDL lysine residues are chemically modified to abolish LDL receptor binding, there is still a difference in turnover rate between dense and buoyant LDL (R. J. Aiello and A. D. Attie, unpublished observations). These data are consistent with the conclusion that the faster clearance of the dense compared to the buoyant LDL subspecies involves a pathway that is not dependent on lysine interactions with the LDL receptor.

These studies provide several important insights into the analysis of apoB polymorphism and its relation to hypercholesterolemia. First, the functional domains of apoB are not sufficiently well understood to predict with certainty whether a particular mutation will have an effect on LDL function. Second, without information that would indicate the order of appearance of each new haplotype, it is difficult to interpret the significance of individual sequence differences. Third, species comparisons are extremely useful for interpreting particular sequence motifs and special amino acids, such as cysteine. Most importantly, it is clear that more information is needed regarding the functional domains of apoB. As an adjunct to sequence and haplotype analysis, a better understanding of apoB domains will necessitate tests for the functional activity of candidate protein domains in suitable biological systems. ▮

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