A cross-species comparison of the apolipoprotein B domain that binds to the LDL receptor

Adam Law[1] **and James Scott**

Division of Molecular Medicine, Clinical Research Centre, Watford Road, Harrow, Middlesex HA1 3UJ, UK

Abstract Apolipoprotein (apo)-B-100 is the ligand that mediates the clearance of low density lipoprotein (LDL) from the circulation by the apoB,E (LDL) receptor pathway. Clearance is mediated by the interaction of a domain enriched in basic amino acid residues on apoB-100 with clusters of acidic residues on the apoB,E (LDL) receptor. A model has been proposed for the LDL receptor binding domain of apoB-100 based on the primary amino acid sequence (Knott, T. J., et al. 1986. *Nature.* **323:** 734–738). Two clusters of basic residues (A: 3147–3157 and B: 3359–3367) are apposed on the surface of the LDL particle by a disulfide bridge between Cys 3167 and 3297. Support for this single domain model has been obtained from the mapping of epitopes for anti-apoB monoclonal antibodies that block the binding of apoB to the LDL receptor. Here we test this model by comparing the nucleotide (from 9623 to 10,442) and amino acid sequence (from 3139 to 3411) of apoB-100 in seven species (human, pig, rabbit, rat, Syrian hamster, mouse, and chicken). Overall, this region is highly conserved. Cluster B maintains a strong net positive charge and is homologous across species in both primary and secondary structure. However, the net positive charge of region A is not conserved across these species, but the region remains strongly hydrophilic. The secondary structure of the region between clusters A and B is preserved, but the disulfide bond is unique to the human sequence. This study suggests that the basic region B is primarily involved in the binding of apoB-100 to the apoB,E (LDL) receptor. — **Law, A., and J. Scott.** A cross-species comparison of the apolipoprotein B domain that binds to the LDL receptor. *J. Lipid Res.* 1990. **31:** 1109–1120.

Supplementary key words apoB-100 • LDL receptor • apoE • receptor binding domain • LDL • VLDL

Apolipoprotein B-100 (apoB-100)- and E-containing lipoproteins are cleared from the circulation predominantly by receptor-mediated endocytosis in the liver. The portion of the apoB-100 and apoE molecule responsible for interacting with the apoB,E (LDL) receptor is termed the receptor binding domain. The ligand binding domain of the apoB,E (LDL) receptor and the receptor binding domain of apoE have been extensively characterized at the molecular level (1, 2). A cluster of basic amino acid residues within apoE (140–150) on the surface of β-VLDL interacts with clusters of acidic residues on seven 40-amino acid cysteine-rich repeats at the amino terminal of the apoB,E (LDL) receptor (1).

Identification of the apoB LDL receptor binding domain has proved to be more difficult than that of apoE. This is due to the length of apoB-100 (4536 residues, compared with 299 for apoE) and its insolubility in water. ApoB-100 in LDL is cleaved in vitro into four fragments by thrombin, which are termed T1 which is a partial digest product, T2 which corresponds to residues 3250–4536, T3 residues 1298–3249, and T4 residues 1–1297 (3). ApoB-100 is rich in basic residues; eight clusters have been identified by inspection of the primary sequence (4), seven of these have also been demonstrated by heparin affinity chromatography of tryptic digests of delipidated apoB-100 (5). The two tryptic peptides that bound to heparin agarose with the highest affinity correspond to residues 3134–3209 and residues 3356–3489 (5). The two peptides flank the T3/T2 junction (residue 3249) of the apoB-100 thrombin cleavage fragments (3) and are the only two heparin-binding sites available on the surface of native LDL. Analysis of the protein sequence in this region identifies two clusters of basic amino acids, 'A' (3147–3157) and 'B' (3359–3367), that are good candidates for the apoB LDL receptor binding domain as they have the highest charge density of the eight basic clusters described in apoB-100. There is a disulfide bond between cysteine residues 3167 and 8297 (3) (unpublished observation, R. Pease and J. Scott), which may link these two regions to form a functional single binding domain.

Further evidence implicates this region of apoB-100 in receptor binding. Panels of monoclonal antibodies have been raised to LDL (6–8). Epitope maps of these monoclonal antibodies have recently been constructed (9–11) and the epitopes for monoclonal antibodies that

Abbreviations: VLDL, very low density lipoprotein; LDL, low density lipoprotein.

[1]To whom reprint requests should be addressed. Present address: Department of Physiology, University of California San Francisco, San Francisco, CA 94143-0444.

completely block the binding of ^{125}I-labeled LDL to the apoB,E (LDL) receptor have been mapped to regions flanking basic clusters A and B (10, 11). Epitopes of monoclonal antibodies that do not interfere with apoB receptor binding are mapped in groups throughout the rest of the apoB-100 molecule, excluding these regions from a direct role in ligand binding. Circumstantial evidence also suggests that the apoB LDL receptor binding domain is in the carboxyl half of the apoB-100. ApoB-48, which in humans is the form of apoB synthesized in the adult intestine, is composed of the amino terminal 1–2152 residues of apoB-100 (12, 13). ApoB-48-containing lipoproteins bind to the apoB,E (LDL) receptor independently of apoB (14). Furthermore, a truncated from of apoB, apoB-37 which contains only the amino terminal 37% of the protein sequence, does not bind to the apoB,E (LDL) receptor (15).

Comparison of the amino acid sequence of apoB-100 with apoE reveals that there is homology between residues 3359–3367 of apoB-100 (i.e., region B) with residues 142 and 150 of apoE:

	3359							3367
ApoB	-Arg-	Leu-	Thr-	Arg-	Lys-	Arg-	Gly-	Leu-Lsy-
	142							150
ApoE	-Arg-	Lys-	Leu-	Arg-	Lys-	Arg-	Leu-	Leu-Arg-

This region of homology can be extended by secondary structural analysis to apoB-100 residues 3352–3371 and apoE residues 135–155 (16). The functional significance of this cluster of basic residues in apoB has been directly tested in binding studies using a synthetic oligopeptide that includes this sequence (residues 3345–3381). When this peptide was constituted into trypsinized VLDL from a type 4 hypertriglyceridemic patient it was able to suppress HMG-CoA reductase activity in cultured human fibroblasts, whereas a control synthetic oligopeptide derived from the human apoB sequence (residues 4154–4189) was not (17).

A model has been proposed for the human apoB LDL receptor binding domain (3). Basic regions 'A' (residues 3147–3157) and 'B' (residues 3359–3367) are brought into apposition on the surface of the LDL particle by a disulfide bond between Cys 3167 and Cys 3297. The protein sequence between these two basic regions is proline-rich with amphipathic β-sheet potential and is associated with lipid. We here test this model by a comparative study of this region of apoB-100 in six mammalian and one avian species.

METHODS

The following bacteriophage libraries were screened: Syrian hamster genomic DNA library—Charon 4a (18); mouse liver cDNA library—λgt10. (19); rabbit intestinal cDNA library—λgt10 (20); rat liver cDNA library—λgt11 (Clonetech); chick liver cDNA library—λgt11 (Clonetech).

These libraries were screened by standard methods (21) using cDNA probes from the human apoB LDL receptor binding domain labeled by random priming (22). DNA was prepared from phage lysates (23) and the inserts were subcloned into pUC13 for restriction mapping. Restriction fragments were subcloned into M13 vectors MP18 and MP19 and sequenced by the dideoxy chain termination method (24) using both Klenow and sequenase methods (sequenase DNA sequencing kit, United States Biochemical Corporation).

Nucleotide sequences were analyzed using the Microgenie program on an IBM AT computer, which provided alignments, translation, restriction maps, and paired homologies. Protein secondary structure (25, 26) and hydrophobicity/hydrophilicity predictions (27, 28) were made using the Wisconsin sequence analysis program on a SUN4 280/S computer.

RESULTS

cDNA and genomic DNA clones encoding the LDL receptor binding domain were isolated from five species (rabbit, rat, hamster, mouse, and chicken) and the primary protein sequence was inferred from the nucleotide sequence. The human apoB-100 sequence (3, 17, 29–32) and the pig sequence have been previously characterized (33). The nucleotide sequences for the seven species, corresponding to nucleotides 9623–10,442 of the human sequence, are aligned in **Fig. 1** and the amino acid sequences, corresponding to residues 3139–3411 of the human sequence, are aligned in **Fig. 2**. This region is highly conserved between all the species and this homology is greater at the nucleotide than at the amino acid level (**Table 1**). There are a number of insertions and deletions found in individual species, which leave the amino acid sequence in frame. The human sequence has deleted three nucleotides at residue 10,063, which are present in all the other species (TAT in the pig, TCT in rabbit, rat, hamster, and mouse, and CCT in the chicken). There is a Leu residue at this position in all species, except the pig where an Ile codon is present. The hamster has a deletion of 15 nucleotides corresponding to nucleotides 9819 to 9833 of the human amino acid sequence. This results in the loss of the consensus amino acid sequence Asp-Lys-Tyr-Lys-Thr found in the other species. The chick has an insertion of three nucleotides, AAT, at the equivalent position of nucleotides 10332–4 of the human sequence, which is not seen in the other species and results in an extra Asn residue.

The similarity between the seven amino acid sequences is demonstrated in **Fig. 3**. The similarity score was deter-

mined using the Venn diagram scoring method based upon Dayhoff's mutation matrix (34–36). The two boxes represent the basic regions A (3147–3157) and B (3359–3367) thought to interact with the apoB,E (LDL) receptor in the human sequences (1). Both basic regions A and B have a high similarity score. Comparison of the primary sequence of these two basic regions shows a significant difference in the type of residues conserved. The basic region B has highly conserved, positively charged residues. The only nonconserved charged residue is the first Arg which is Ser in the pig, rabbit, and chicken. There is, therefore, a net positive charge of 4 or 5 across all species examined. In contrast, the basic region A is less well preserved across species. The net positive charge is human 7, pig, rabbit, rat, and hamster 5, mouse 4, and chicken 1. The amino acid substitutions are hydrophilic and in several instances acidic residues are substituted for basic ones (Fig. 2).

There are several areas of low homology between the sequences compared (Fig. 3). The troughs at amino acid positions 29 and 160 correspond to the region of the two Cys residues at positions 3167 and 3297 of the human sequence. Inspection of the amino acids at these two positions in Fig. 2 confirms that these Cys residues are not conserved between species and are in regions that appear to have the greatest divergence of amino acid residues. The proline-rich area between the two basic regions is highly conserved. The Lys residue at position 3249, which in the human sequence is the thrombin cleavage site at the T3/T2 junction, is conserved in all species except the mouse which has a Thr residue at this position.

Different procedures for predicting protein secondary structure from primary amino acid sequence have been developed. Each of these methods is based upon different assumptions and empirically determined x-ray crystallographic data of predominantly globular proteins. Thus, different algorithms give rise to heterogeneous predictions of protein secondary structure from the same primary sequence. These secondary structure prediction algorithms must be interpreted with some caution with lipoproteins, as the protein x-ray crystal structure data upon which they are based does not include proteins that interact extensively with lipid. In order to extract the maximum amount of information from a group of cross-species sequence data, a simple graphic method of comparing the secondary structure predictions has been devised (36). The results of such an analysis are shown in **Fig. 4**. The top panel represents the aligned primary sequences of the seven compared species; above each line the boxes represent the Chou and Fasman secondary structure prediction, and below the line the Garnier, Osguthorpe, and Robson prediction as derived by the PeptideStructure and PlotStructure programs of the Wisconsin sequence analysis package (25, 26). Black boxes represent β-sheet, speckled boxes α-helix, and empty areas random coil or β-turn. The second panel is a composite histogram of the information from the first panel, taking 5 amino acid residue cuts throughout the length of the aligned sequences. Hence the black bars on the histogram represent the probability of β-sheet. The third panel represents the composite secondary structure prediction derived from the histogram data, and the two boxes underneath correspond to basic regions A and B. The basic region B is predicted to be in a region of α-helix, whereas region A may be either α-helix or β-sheet. The region between the two basic regions is largely predicted to be β-sheet.

The basic region B can be modelled as an amphipathic α-helix using an Edmundson wheel diagram (37). **Fig. 5** demonstrates that the amphipathic α-helix is conserved for region B; the asterisks designate the basic residues. These lie along the face of approximately 60% of the circumference of the helix, whereas hydrophobic residues lie in the remaining 40%. Region A is neither a classic amphipathic α-helix nor a β-sheet (data not shown).

Fig. 6 shows the aligned plots of the Kyte and Doolittle and the Goldman, Engelman, and Steitz hydrophobicity scale for the seven apoB LDL receptor binding domain sequences (27, 28). Hydrophobic areas are shown above the horizontal axis and hydrophilic areas below the horizontal axis. The two scales show close agreement and there is a great similarity between the profiles of the species compared. The two boxes represent the basic regions A and B, which are markedly hydrophilic despite the many amino acid substitutions between the human and chick sequence in region A. The proline-rich area and the region predicted to have a β-sheet secondary structure are predominantly amphipathic.

DISCUSSION

In this study we have tested a model for the apoB,E (LDL) receptor binding domain of apoB-100 (3) by comparing the sequence in seven species—human, pig, rabbit, rat, mouse, and chicken. The main features of this model are as follows. First, the region around the T3/T2 junction (residue 3249) is involved in binding to the apoB,E (LDL) receptor. Second, two clusters of basic residues A (residues 3147–3157) and B (residues 3359–3367) are found on the surface of LDL and interact with the acidic ligand binding domain of the apoB,E (LDL) receptor. Third, the two basic regions are brought into juxtaposition by a disulfide bond between residues 3167 and 3297 and function as a single receptor binding domain in humans. Fourth, the sequence between regions A and B is comprised of amphipathic β-sheet and is rich in proline residues.

Evidence for the importance of the region surrounding the T3/T2 junction of apoB-100 in ligand binding includes analysis of the primary sequence data, heparin

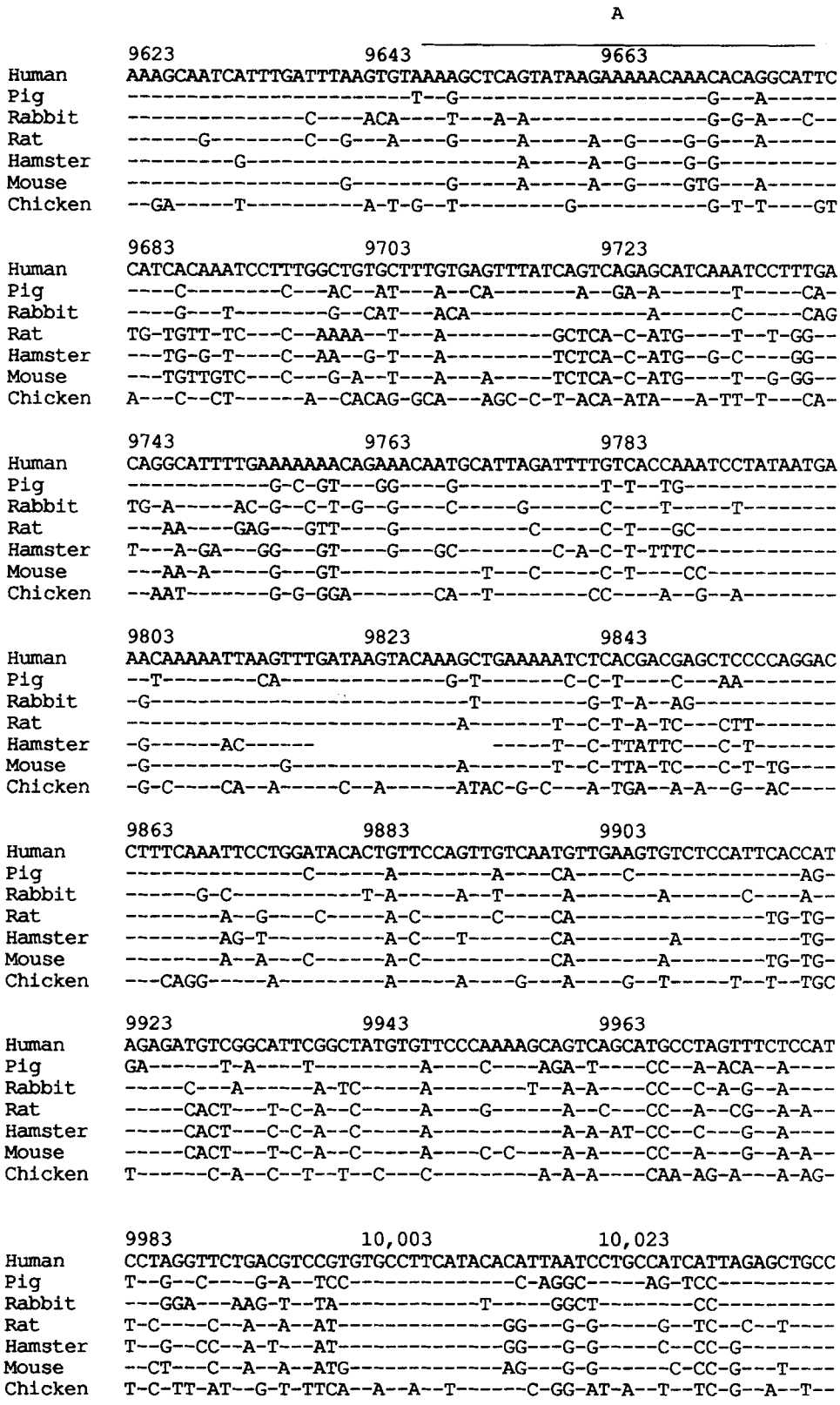
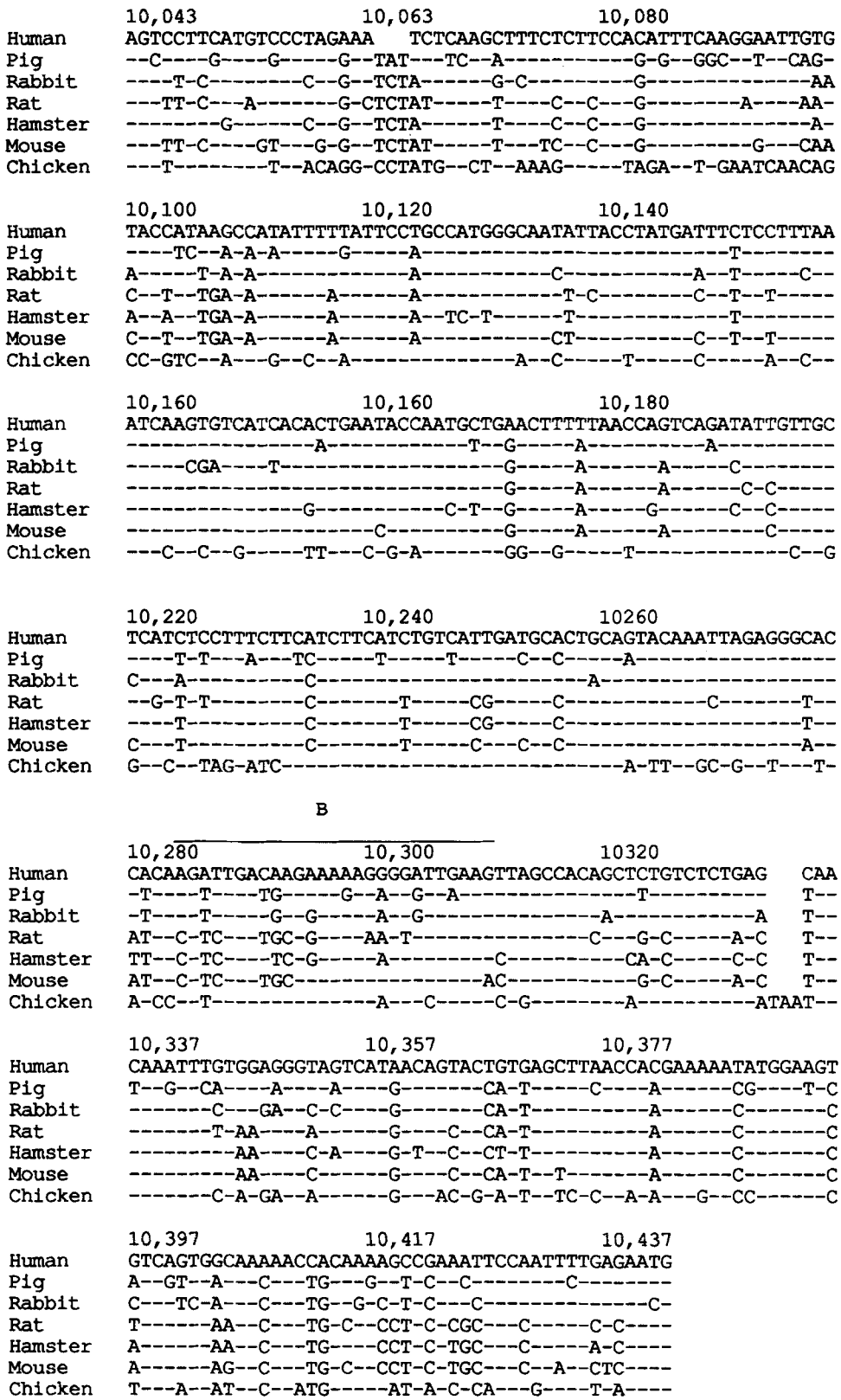


Fig. 1. Aligned nucleotide sequences of apoB LDL receptor binding domain in seven species. The numbering corresponds to the published human apoB-100 sequence (30). The complete human sequence is provided on the top line, and nucleotide differences from the human sequences in the other six species compared are aligned beneath. Hyphens represent nucleotides identical to the human sequence and blanks indicate deletions. The nucleotides corresponding to basic regions A and B are indicated by horizontal lines above the sequence. The pig sequence is from Maeda et al. (33).



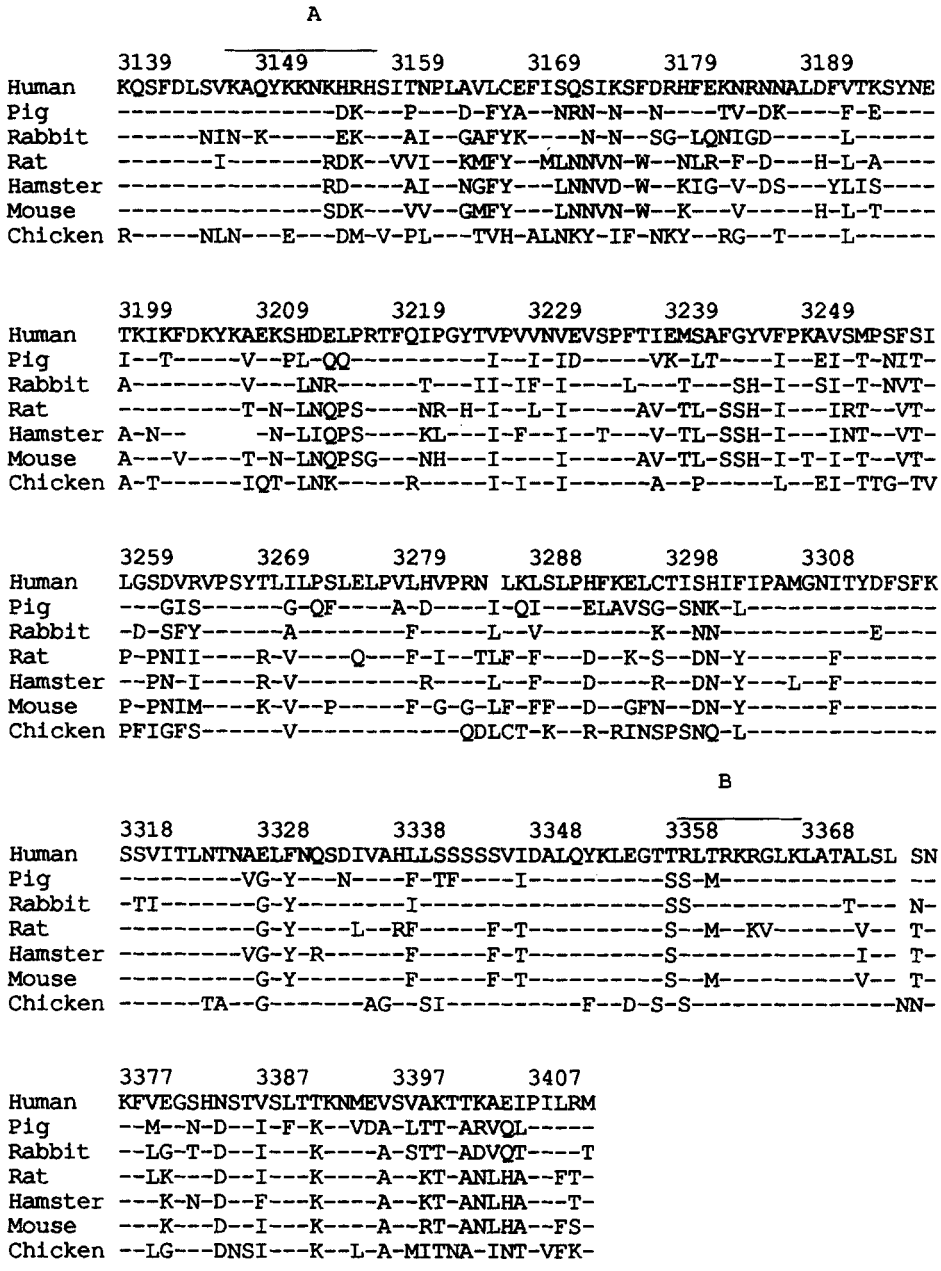


Fig. 2. Aligned amino acid sequences of the apoB LDL receptor binding domain in seven species. The numbering corresponds to the published human apoB-100 sequence (3). The complete human amino acid sequence is provided on the top line, and amino acid residue differences are indicated below. Hyphens represent amino acid residues identical to the human sequence and blanks indicate deletions. The single letter code for amino acids is employed. The basic regions A and B are indicated by horizontal lines above the sequence. The pig sequence is from Maeda et al. (33).

binding studies (5), epitope mapping, and competitive binding studies of monoclonal antibodies raised against LDL (9–11), sequence comparison with the apoE receptor binding domain (3, 16, 29, 31), and binding studies using a synthetic oligopeptide encompassing the basic region of apoB homologous to the apoE receptor binding domain (17). The region as a whole shows extensive homology between species (Table 1), with the chicken nucleotide and amino acid sequence being 68% and 62% homologous to the human sequence, respectively. This is in contrast to the C-terminal 500 amino acids of the chicken apoB nucleotide sequence, which is only 31% homologous to

TABLE 1. Homology among the apoB LDL receptor binding domains of seven species

	Human	Pig	Rabbit	Hamster	Rat	Mouse	Chick
Human	—	67.5	69.3	66.2	64.0	66.4	62.4
Pig	79.9	—	68.0	63.5	62.0	65.0	59.8
Rabbit	81.4	78.6	—	67.3	68.0	67.9	62.0
Hamster	75.4	73.6	76.5	—	79.6	79.9	55.4
Rat	75.5	74.9	76.4	84.5	—	86.1	57.0
Mouse	75.7	74.1	76.9	84.3	90.2	—	59.5
Chick	67.6	66.9	67.6	61.8	64.5	65.0	—

The comparisons are expressed as percentage homology between pairs of sequences. The figures to the left of the hyphens are the nucleotide comparisons, and to the right of the hyphens are the amino acid comparisons. The homologies were calculated using the Microgenie Compare/Align program.

the human sequence (38). The rat and mouse nucleotide and amino acid sequences are more divergent than the others, but show a high degree of homology to one another (Table 1). This results in a greater overall homology between the chicken and human sequences than between the chicken and the rat and mouse sequences. Thus, the amino acid homology between chicken and rat sequences is 57%, chicken and human 62.4%, rat and human 64%. A similar finding has been described for chicken apoA-I: chicken and rat 42%, chicken and human 49%, rat and human 64% (39).

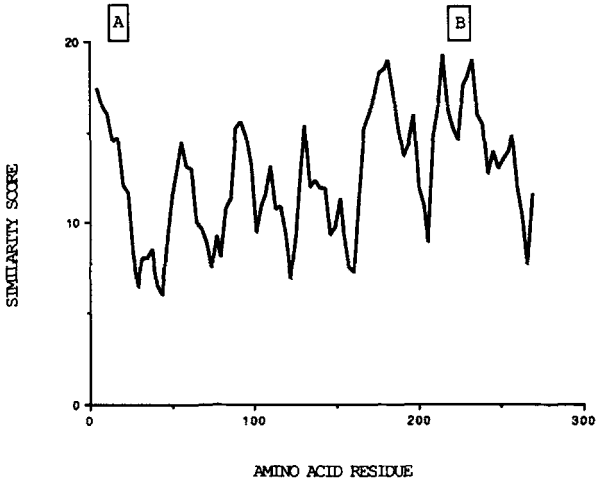


Fig. 3. Similarity profile of the aligned amino acid sequences of the apoB LDL receptor binding domain of seven species: human, pig, rabbit, Syrian hamster, rat, mouse, and chicken. The similarity score was derived using a Venn diagram scoring method developed as a compromise between the mutation data from Dayhoff's mutation matrix and the amino acid physicochemical data (34–36). At each residue position the similarity score indicates the relative significance of amino acid substitutions. Conservation of the same residue provides the maximum score of 20, whereas the score for complete lack of conservation is 0. Therefore, the substitution of one hydrophobic residue for another has a higher relative similarity score than the substitution of a hydrophilic for a hydrophobic residue. The amino acid residue position is plotted on the horizontal axis; the region compared corresponds to the amino acid alignment in Fig. 2, but is numbered 1–275, as there are deletions and insertions in the human sequence. To smooth out the curve, a moving average of nine residues was calculated and every third value was plotted. Amino acid residue 0 corresponds to residue 3139 of the human apoB-100 sequence.

The basic region B of apoB-100 (residues 3359–3367), which in humans is homologous in both primary and secondary structure to the apoE receptor binding domain (residues 140–150) (16), is conserved in the species compared (Fig. 2). The net positive charge is 5 for the human, rat, hamster, and mouse and 4 for the pig, rabbit, and chicken. There is also conservation of region B in secondary structure prediction (Figs. 4 and 5). It forms an amphipathic α-helix with hydrophobic residues along one face and hydrophilic, basic residues along the other face. This secondary structure motif strongly suggests that this sequence is surface-associated, with the hydrophobic face anchoring the helix to the lipid components of LDL and the hydrophilic face orienting the basic residues towards the aqueous phase. The other apoB-100 basic region A (residues 3147–3157), which has been included in this model of the human apoB LDL receptor binding domain (3), does not retain its basic charge across species. The net positive charge in the human is 7; pig, rabbit, rat, and hamster 5; mouse 4; and chicken 1. Secondary structural features of region A also make it less likely to be involved in receptor binding. Region A has neither strongly conserved amphipathic α-helix nor β-sheet potential. However, the hydrophilicity of region A is maintained across species (Fig. 6).

The disulfide bond between residues 3167 and 3297 in humans (3) (R. Pease and J. Scott, unpublished observation) that may bring the two basic regions together to form a single receptor binding domain is not conserved across species (Fig. 2). Both cysteine residues are found in regions with least similarity between species (Fig. 3, residues 29 and 160). This does not exclude the possibility that regions A and B are in proximity in other species, as this configuration may be the way the apolipoprotein folds when associated with lipid; however these data suggest that these cysteine residues are not essential for stabilizing this configuration.

The hydrophobicity profiles of these seven sequences are similar (Fig. 6). The two regions corresponding to the basic regions A and B of the human sequence have a similar profile in all the sequences compared. The amphipathic stretch between the two basic regions is con-

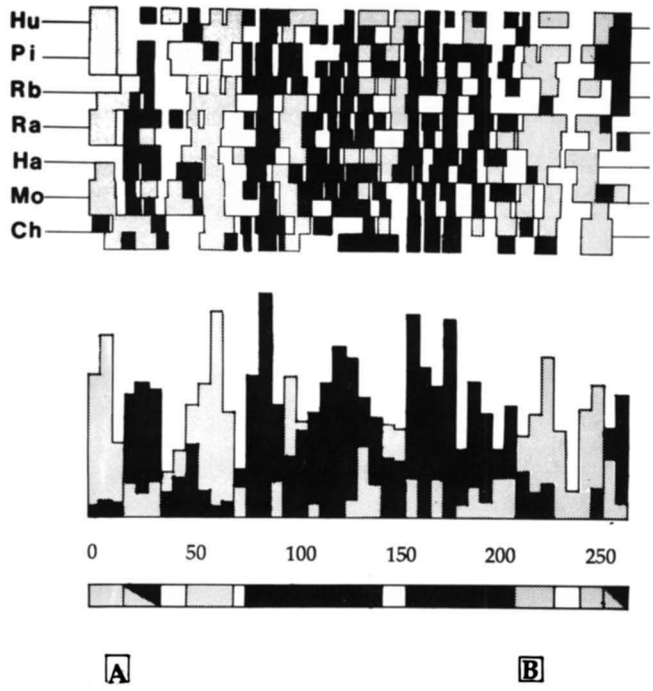


Fig. 4. Consensus secondary structure prediction of the apoB LDL receptor binding domain. This is based upon the Chou-Fasman and Garnier-Osguthorpe-Robson predictions of the secondary structure of the seven species studied (25, 26). In the top panel the seven species secondary structure predictions are aligned (Hu-human; Pi-pig; Rb-rabbit; Ra-rat; Ha-hamster; Mo-mouse; Ch-chicken). Above the line next to the species name is the Chou-Fasman prediction, below the line is the Garnier-Osguthorpe-Robson prediction. Black boxes indicate β-sheet, speckled boxes α-helix, and white areas random coil or β-turn. The second panel is a histogram constructed from the top panel by plotting the total area of β-sheet or α-helix in five amino acid residue cuts along the sequence. The third panel indicates the consensus secondary structure prediction based upon the predominant secondary structure in the histogram. Basic regions A and B are represented by the boxes at the bottom of the diagram. Amino acid residue 0 corresponds to residue 3139 of the human apoB-100 sequence.

served and may be a region that is associated with lipid. This region has a strong β-sheet potential, which is homologous across species and contains many conserved proline residues (Fig. 4).

This comparative structural analysis assumes that the interaction between apoB-100 and the apoB,E (LDL) receptor is conserved in the species compared. Changes in the function of this receptor-ligand system in different species may explain the differences in the structure observed. Comparative data suggest that apoB-100 functions as a ligand for the apoB,E (LDL) receptor throughout the mammalian kingdom. Quantitative kinetic studies of LDL catabolism in the apoB,E (LDL) receptor pathway in the intact animal demonstrate that the receptor-dependent transport is responsible for 61% human, 58% rabbit, 81% rat, and 70% hamster LDL clearance (40). The K_m for the receptor-dependent transport is similar in all these species. Heterologous mammalian LDL competes with human LDL for binding to the human apoB,E (LDL) receptor, however there is great interspecies variation in binding affinities (41, 42). Epitopes of anti-apoB-100 receptor blocking monoclonal antibodies are conserved across mammlian species compared to anti-apoB-100 nonreceptor blocking monoclonal antibodies (41, 42), which suggests that the region of apoB-100 responsible for binding to the apoB,E (LDL) receptor is conserved.

The genetics, structure, and function of the pig apoB LDL receptor binding site has been studied in detail (33, 43, 44). Eight alleles (Lpb^{1-8}) of pig apoB have been identified immunologically, one of which (Lpb^5) is associated with a phenotype similar to familial hypercholesterolemia, except that there is no apoB,E (LDL) receptor defect. In vivo studies of this mutant allele reveal that there is a catabolic defect of apoB clearance, and in vitro binding and competition studies using pig fibroblast apoB,E (LDL) receptors demonstrate that there is defective binding (2–6 × reduced binding affinity) of this mutant ligand (43). The cloning and characterization of pig apoB (corresponding to human residues 3133 to 3497) for this mutant and the Lpb^2 Lpb^7, and Lbp^3 alleles revealed 10 base substitutions. Four of these substitutions resulted in an alteration in amino acid residue, and only one (Gly to Asp corresponding to human residue 3164) was nonconservative (33). None of these amino acid changes were unique to Lpb^5. It was concluded that a mutation outside of this region of apoB was responsible for the difference

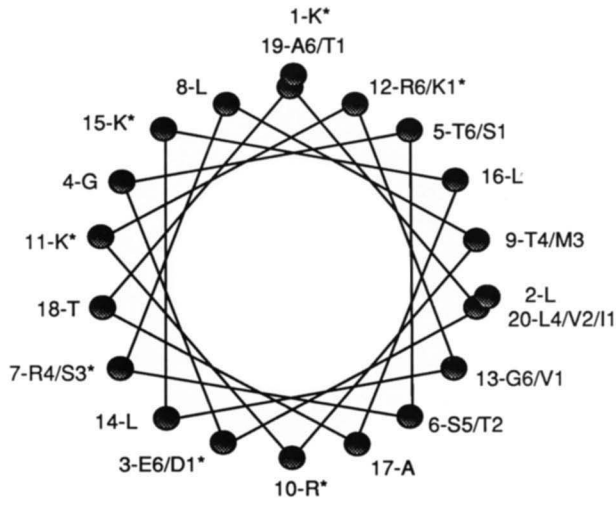


Fig. 5. Edmundson-wheel diagram of basic region B of the apoB LDL receptor binding domain of seven species (37). The amino acid residues of basic region B corresponding to human sequence residues 3352–3371 were plotted on the Edmundson-wheel. The number indicates the amino acid residue (1 = 3352), the first letter the consensus amino acid residue at that position, followed by the frequency of this consensus residue at this position across the seven species. The consensus residue is separated from alternate residues found in other species, together with their frequency. Asterisks indicate basic residues.

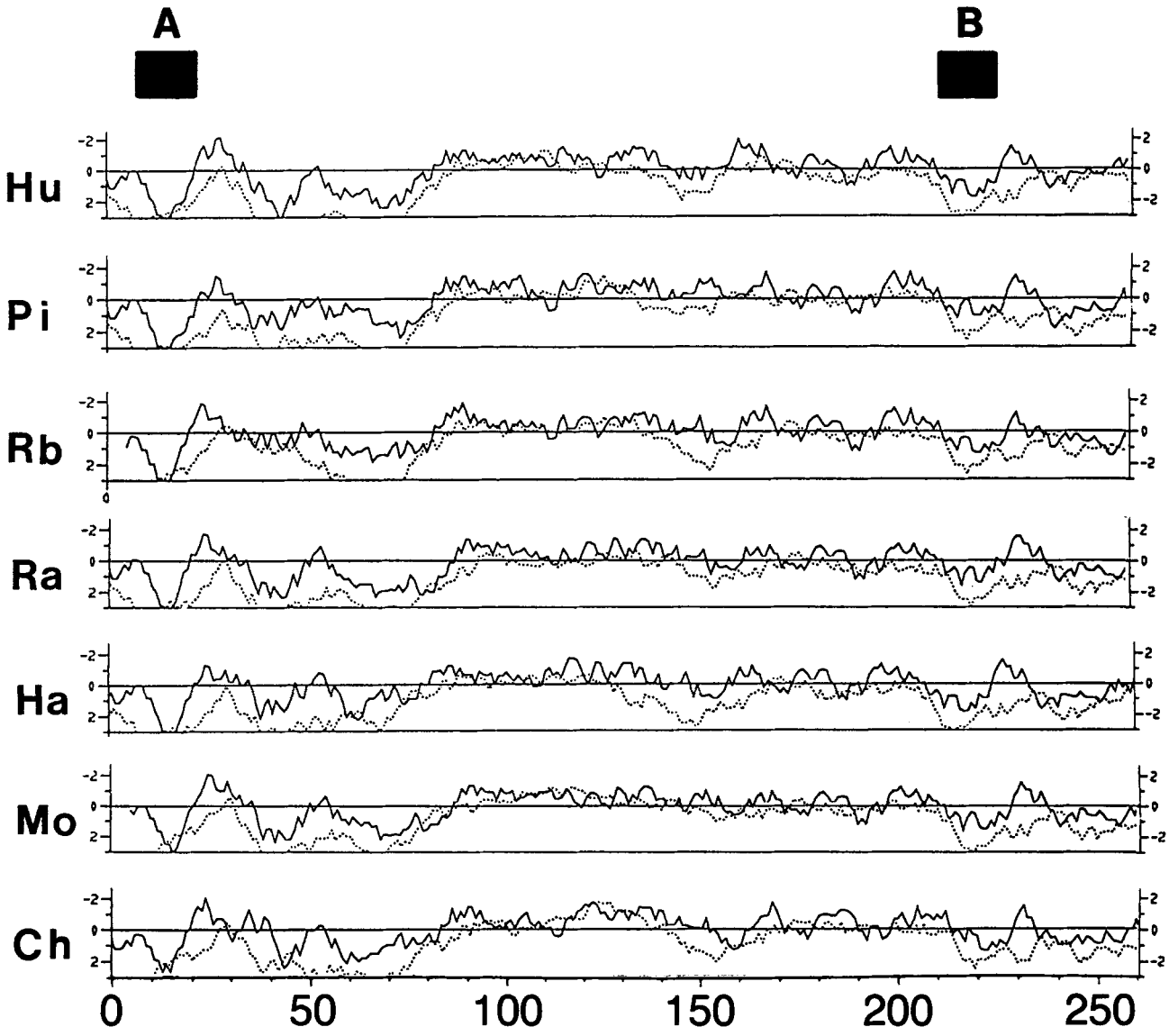


Fig. 6. Composite hydrophobicity plots of the apoB LDL receptor binding domains of seven species. The hydrophobicity plots of each of the species were produced using the PepPlot program of the Wisconsin sequence analysis program. The species are labeled on the left of their plot as in Fig. 4. The solid line indicates the Kyte-Doolittle hydrophobicity plot (27) and is the average of residue specific hydrophobicity index over a window of nine amino acid residues. Above the horizontal line the plot is hydrophobic, whereas below the line it is hydrophilic. The dotted line is the Goldman-Engelman-Steiz transbilayer helix prediction (28). The curve is fitted as a residue specific hydrophobicity scale plotted with a window of 20 residues. The boxes indicate the positions of the basic regions A and B. Amino acid residue 0 corresponds to residue 3139 of the human apoB-100 sequence.

in apoB metabolism of this and the other Lpb alleles. The pig and human apoB sequences were compared and the reduced net positive charge of basic regions A and B was noted, together with the absence of the cysteine residues at the positions equivalent to human residues 3167 and 3297. To determine whether the difference in the primary structure of apoB between the human and pig affected the interaction with the apoB,E (LDL) receptor, pig and human LDL were compared in competitive binding assays to both human and pig fibroblast apoB,E (LDL) receptors (44). Pig LDL had a higher binding affinity to both human and pig fibroblast receptors compared to human LDL. The authors suggest that residues outside the domain containing these two basic regions of apoB may play an important role in high affinity binding of LDL to its receptor.

Chicken lipoprotein metabolism is different from that found in mammals. In particular, intestinal lipoproteins are absorbed directly into hepatic portal system, forming portomicrons, rather than chylomicrons (45). Also, VLDL and LDL play an important role in oogenesis. There is a second protein present in apoB-containing

lipoproteins termed VLDLII which has been cloned and characterized (46, 47). Two chicken apoB-specific receptors have recently been identified. One is an oocyte receptor with a molecular mass of 95 kDa (48). The other is a fibroblast receptor with a molecular mass of 130 kDa (49). The chicken oocyte receptor demonstrates many of the ligand binding characteristics of the mammalian apoB,E (LDL) receptor, which include sensitivity to EDTA and suramin and the inability to bind reductively methylated chick LDL. Ligand blotting experiments demonstrate that rabbit β-VLDL binds to the oocyte receptor. Polyclonal antibodies raised against the bovine apoB,E (LDL) receptor recognized the chicken oocyte receptor (48). The chick fibroblast receptor resembles the mammalian apoB,E (LDL) receptor in size and also in function (49). The chicken fibroblast receptor binds, internalizes, and degrades LDL and VLDL. The number of these receptors on the surface of chick fibroblasts is down-regulated by the presence of apoB-containing lipoproteins and the rate of cholesterol synthesis is also reduced. These data suggest that the apoB/LDL receptor system predates the mammalian radiation, although the molecular details of this system in the chicken remain to be investigated.

Although it is likely that the region of apoB-100 compared in this study represents the apoB LDL receptor binding domain, other regions of apoB-100 may be important for the interaction of LDL and the apoB,E (LDL) receptor. The epitopes for the receptor blocking anti-apoB-100 monoclonal antibodies flank the two basic regions but are separated by more than 100 amino acid residues on either side. A mutation of apoB-100 that reduces the affinity of LDL for its receptor has been identified as an amino acid change at residue 3500 Arg to Gln (50–52). The affinity of the receptor blocking monoclonal antibody MB47 for this LDL variant is increased by approximately 60%, implying that the mutation affects the epitope for this monoclonal antibody (52). This basic residue at position 3500 may be directly involved in ligand binding to the apoB,E (LDL) receptor; alternatively a conformational change has to be invoked to explain its effect or else a second mutation elsewhere in apoB may be associated with this base substitution. The identification of further mutations in this region affecting ligand binding will help characterize the apoB LDL receptor binding domain.

In conclusion, a comparative study of the region of apoB-100 thought to be involved in binding to the apoB,E (LDL) receptor reveals that the cluster of basic residues between 3359 and 3367 is phylogenetically the primary region involved in this interaction. A second cluster of hydrophilic residues, between 3147 and 3157, has become more basic from chicken to man and may have evolved to fulfill an important role in humans in the binding of LDL to its receptor. Binding studies of full-length apoB-100 constructs reconstituted into LDL with mutations in the binding domain are necessary to resolve this issue.

We thank Howard Eder, Karin Gaensler, Tim Knott, Nobuyo Maeda, Richard Pease, Lyn Powell, Clive Pullinger, Sally Robertson, Lesley Sargeant, William Taylor, Simon Wallis, and Gary Williams for their contribution to this project, and P. Tucker, G. Samenza, and B. L. M. Hogan for supplying libraries. A. L. was supported by a Medical Research Council research training fellowship.

Manuscript received 22 November 1989 and in revised form 25 January 1990.

REFERENCES

1. Esser, V., L. E. Limbird, M. S. Brown, J. L. Goldstein, and D. W. Russell. 1988. Mutational analysis of the ligand binding domain of the low density lipoprotein receptor. *J. Biol. Chem.* **263:** 13282–13290.
2. Mahley, R. W. 1988. Apolipoprotein E: cholesterol transport protein with expanding role in cell biology. *Science.* **240:** 622–630.
3. Knott, T. J., R. J. Pease, L. M. Powell, S. C. Wallis, S. C. Rall, Jr., T. L. Innerarity, B. Blackhart, W. H. Taylor, Y. Marcell, R. Milne, D. Johnson, M. Fuller, A. J. Lusis, B. J. McCarthy, R. W. Mahley, B. Levy-Wilson, and J. Scott. 1986. Complete protein sequence and identification of structural domains of human apolipoprotein B. *Nature.* **323:** 734–738.
4. Scott, J. 1989. The molecular and cell biology of apolipoprotein-B. *Mol. Biol. Med.* **6:** 65–80.
5. Weisgraber, K. H., and S. C. Rall, Jr. 1987. Human apolipoprotein B-100 heparin-binding sites. *J. Biol. Chem.* **262:** 11097–11103.
6. Marcel, Y. L., P. K. Weech, P. Mitthorp, F. Tercé, C. Vézina, and R. W. Milne. 1984. Monoclonal antibodies and the characterization of apolipoprotein structure and function. *Prog. Lipid Res.* **23:** 169–195.
7. Curtiss, L. K., and T. S. Edgington. 1982. Immunochemical heterogeneity of human plasma apolipoprotein B-I. Apolipoprotein B binding of mouse hybridoma antibodies. *J. Biol. Chem.* **257:** 15213–15221.
8. Schonfeld, G., and E. S. Krul. 1986. Immunologic approaches to lipoprotein structure. *J. Lipid Res.* **27:** 583–601.
9. Krul, E. S., Y. Kleinman, M. Kinoshita, B. Pfleger, K. Oida, A. Law, J. Scott, R. Pease, and G. Schonfeld. 1988. Regional specificities of monoclonal anti-human apolipoprotein B antibodies. *J. Lipid Res.* **29:** 937–947.
10. Pease, R. J., R. W. Milne, W. K. Jessup, A. Law, P. Provost, J-C. Fruchart, R. T. Dean, Y. L. Marcel, and J. Scott. 1990. Use of bacterial expression cloning to localize the epitopes for a series of monoclonal antibodies against apolipoprotein B-100. *J. Biol. Chem.* **265:** 553–568.
11. Milne, R., R. Théolis, Jr., R. Maurice, R. J. Pease, P. K. Weech, E. Rassart, J-C. Fruchart, J. Scott, and Y. L. Marcel. 1989. The use of monoclonal antibodies to localize the low density lipoprotein receptor-binding domain of apolipoprotein B. *J. Biol. Chem.* **264:** 19754–19760.
12. Powell, L. M., S. C. Wallis, R. J. Pease, Y. H. Edwards, T. J. Knott, and J. Scott. 1987. A novel form of tissue-specific RNA processing produces apolipoprotein B48 in intestine. *Cell.* **50:** 831–840.

13. Chen, S-H., G. Habib, C-Y. Yang, A-W. Gu, B. R. Lee, S-A. Weng, S. R. Silberman, S-J. Cai, J. P. Deslypere, M. Rosseneu, A. M. Gotto, Jr., W-H. Li, and L. Chan. 1987. Apolipoprotein B-48 is the product of a messenger RNA with an organ-specific inframe stop codon. *Science.* **238:** 363–366.
14. Hui, D. Y., T. L. Innerarity, R. W. Milne, and R. W. Mahley. 1984. Binding of chylomicron remnants and β-VLDL to hepatic and extra-hepatic lipoprotein receptors: a process independent of apolipoprotein B-48. *J. Biol. Chem.* **259:** 15060–15068.
15. Young, S. G., F. P. Peralta, B. W. Dubois, L. K. Curtiss, J. K. Boyles, and J. L. Witztum. 1987. Lipoprotein B37, a naturally occurring lipoprotein containing the amino-terminal portion of apolipoprotein B-100, does not bind to the apolipoprotein B,E (low density lipoprotein) receptor. *J. Biol. Chem.* **262:** 16604–16611.
16. De Loof, H., M. Rosseneu, C-Y. Yang, W-H. Li, A. M. Gotto, Jr., and L. Chan. 1987. Human apolipoprotein B: analysis of internal repeats and homology with other apolipoproteins. *J. Lipid Res.* **28:** 1455–1464.
17. Yang, C-Y., S-H. Chen, S. H. Gianturco, W. A. Bradley, J. T. Sparrow, N. Tanimura, W-H. Li, D. A. Sparrow, H. DeLoof, M. Rosseneu, F-S. Li, Z-H. Gu, A. M. Gotto, and L. Chan. 1986. Sequence, structure, receptor binding domain and internal repeats of apolipoprotein B-100. *Nature.* **323:** 738–742.
18. McGuire, K., W. R. Duncan, and P. W. Tucker. 1986. Structure of a class 1 gene from Syrian hamster. *J. Immunol.* **137:** 66–372.
19. Fahrner, K., B. L. M. Hogan, and R. A. Flavell. 1987. Transcription of H-2 and Qa genes in embryonic and adult mice. *EMBO J.* **6:** 1265–1271.
20. Hunziker, W., M. Spiess, G. Semenza, and H. F. Lodish. 1986. The sucrose-isomaltase complex: primary structure, membrane orientation, and evolution of a stalked intrinsic brush border protein. *Cell.* **46:** 227–234.
21. Maniatis, T., E. F. Fritsch, and J. Sambrook. 1982. Molecular Cloning - a Laboratory Manual. Cold Spring Harbour Laboratory.
22. Feinberg, A. P., and B. Vogelstein. 1983. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. *Anal. Biochem.* **132:** 6–13.
23. Helms, C., M. Y. Graham, J. E. Dutchik, and M. V. Olson. 1985. Laboratory methods. A new method for purifying lambda DNA from phage lysates. *DNA.* **4:** 39–49.
24. Sanger, F., and A. R. Coulson. 1978. The use of thin acrylamide gels for DNA sequencing. *FEBS Lett.* **87:** 107–110.
25. Chou, P. Y., and G. D. Fasman. 1974. Conformational parameters for amino acids in helical, β-sheet and random coil regions calculated from proteins. *Biochemistry.* **13:** 211–245.
26. Garnier, J., D. J. Osguthorpe, and B. Robson. 1978. Analysis of the accuracy and implications of simple methods for predicting the secondary structure of globin proteins. *J. Mol. Biol.* **120:** 97–120.
27. Kyte, J., and R. F. Doolittle. 1982. A simple method for displaying the hydropathic character of a protein. *J. Mol. Biol.* **157:** 105–132.
28. Engelman, D. M., T. A. Steitz, and A. Goldman. 1986. Identifying nonpolar transbilayer helices in amino acid sequences of membrane proteins. *Annu. Rev. Biophys. Biophys. Chem.* **15:** 321–353.
29. Knott, T. J., S. C. Rall, Jr., T. L. Innerarity, S. F. Jacobson, M. S. Urdea, B. Levy-Wilson, L. M. Powell, R. J. Pease, R. Eddy, H. Nakai, M. Beyers, L. M. Priestley, E. Robertson, L. B. Rall, C. Betsholtz, T. B. Shows, R. W. Mahley, and J. Scott. 1985. Human apolipoprotein B: structure of carboxyl-terminal domains, sites of gene expression, and chromosomal localization. **230:** 37–43.
30. Knott, T. J., S. C. Wallis, L. M. Powell, R. J. Pease, A. J. Lusis, B. Blackhart, B. J. McCarthy, R. W. Mahley, B. Levy-Wilson, and J. Scott. 1986. Complete cDNA and derived protein sequence of human apoB-100. *Nucleic Acids Res.* **14:** 7501–7503.
31. Law, S. W., S. M. Grant, K. Higuchi, A. Hospattankar, K. Lackner, N. Lee, and H. B. Brewer, Jr. 1986. Human liver apolipoprotein B-100 cDNA: complete nucleic acid and derived amino acid sequence. *Proc. Natl. Acad. Sci. USA.* **83:** 8142–8146.
32. Carlsson, P., C. Darnfors, S-O. Olofsson, and G. Bjursell. 1986. Analysis of the human apoB gene; complete structure of the B74 region. *Gene.* **49:** 29–51.
33. Maeda, N., D. L. Ebert, T. M. Doers, M. Newman, J. Hasler-Rapacz, A. D. Attie, J. Rapacz, and O. Smithies. 1988. Molecular genetics of the apolipoprotein B gene in pigs in relation to atherosclerosis. *Gene.* **70:** 213–229.
34. Dayhoff, M. O. 1972. Atlas of Protein Sequence and Structure. Vol V. National Biomed. Foundation. Silver Spring, Maryland.
35. Taylor, W. R. 1986. The classification of amino acid conservation. *J. Theor. Biol.* **119:** 205–218.
36. Taylor, W. R. 1987. Protein structure prediction. *In* Nucleic Acid and Protein Sequence Analysis: a Practical Approach. M. J. Bishop and C. J. Rawlings, editors. IRL Press, Oxford. 285–322.
37. Schiffer, M., and A. B. Edmundson. 1967. Use of helical wheels to represent the structures of proteins and to identify segments with helical potential. *Biophys. J.* **7:** 121–125.
38. Kirchgessner, T. G., C. Heinzmann, K. L. Svenson, D. A. Gordon, M. Nicosia, H. G. Lebherz, A. J. Lusis, and D. L. Williams. 1987. Regulation of chicken apolipoprotein B: cloning, tissue distribution, and oestrogen induction of mRNA. *Gene.* **59:** 241–251.
39. Rajavashisth, T. B., P. A. Dawson, D. L. Williams, J. Shahelford, H. G. Lebherg, and A. J. Lusis. 1987. Structure, evolution and regulation of chicken apoA-I. *J. Biol. Chem.* **262:** 7058–7065.
40. Dietschy, J. M., D. K. Spady, and J. B. Meddings. 1988. A quantitative approach to low density lipoprotein metabolism in man and in various experimental animals. *In* Hyperlipidaemia and Atherosclerosis. K. E. Suckling and P. H. Groot, editors. Academic Press, New York. 17–32.
41. Nelson, C. A., M. A. Tasch, M. Tikkanen, R. Dargar, and G. Schonfeld. 1984. Evolution of low density lipoprotein structure probed with monoclonal antibodies. *J. Lipid Res.* **25:** 821–830.
42. Young, S. G., J. C. Witztum, D. C. Casal, L. K. Curtiss, and S. Bernstein. 1986. Conservation of the low density lipoprotein receptor binding domains of apoprotein B. *Arteriosclerosis.* **6:** 178–187.
43. Lowe, S. W., W. J. Checovich, J. Rapacz, and A. D. Attie. 1988. Defective receptor binding of low density lipoprotein from pigs possessing mutant apolipoprotein B alleles. *J. Biol. Chem.* **263:** 15467–15473.
44. Ebert, D. L., N. Maeda, S. W. Lowe, J. Hasler-Rapacz, J. Rapacz, and A. D. Attie. 1988. Primary structure comparison of the proposed low density lipoprotein (LDL) receptor binding domain of human and pig apolipoprotein B: implications for LDL-receptor interactions. *J. Lipid Res.* **29:** 1501–1509.

45. Chapman, M. J. 1980. Animal lipoproteins: chemistry, structure, and comparative aspects. *J. Lipid Res.* **21:** 789–853.
46. van het Schip, A. D., F. C. Meijlink, R. Strijker, M. Gruber, A. J. van Vliet, J. A. van de Klundert, and G. Ab. 1983. The nucleotide sequence of the chicken apo very low density lipoprotein II gene. *Nucleic Acids Res.* **11:** 2529–2540.
47. Haché, R. J., R. Wiskocil, M. Vasa, R. N. Roy, P. C. Lau, and R. G. Deeley. 1983. The 5' noncoding and flanking regions of the avian very low density apolipoprotein II and serum albumin genes. *J. Biol. Chem.* **258:** 4556–4564.
48. George, R., D. L. Barber, and W. J. Schneider. 1987. Characterization of the chicken oocyte receptor for low and very low density lipoproteins. *J. Biol. Chem.* **262:** 16838–16847.
49. Hayashi, K., J. Nimpf, and W. J. Schneider. 1989. Chicken oocytes and fibroblasts express different apolipoprotein-B-specific receptors. *J. Biol. Chem.* **264:** 3131–3139.
50. Vega, G. L., and S. M. Grundy. 1986. In vivo evidence for reduced binding of low density lipoproteins to receptors as a cause of primary moderate hypercholesterolaemia. *J. Clin. Invest.* **78:** 1410–1414.
51. Innerarity, T. L., K. H. Weisgraber, K. S. Arnold, R. W. Mahley, R. M. Kram, G. L. Vega, and S. M. Grundy. 1987. Familial defective apolipoprotein B-100: low density lipoproteins with abnormal receptor binding. *Proc. Natl. Acad. Sci. USA.* **84:** 6919–6923.
52. Weisgraber, K. H., T. L. Innerarity, Y. M. Newhouse, S. G. Young, K. S. Arnold, R. M. Kraus, G. L. Vega, S. M. Grundy, and R. W. Mahley. 1988. Familial defective apolipoprotein B-100: enhanced binding of monoclonal antibody to abnormal low density lipoprotein. *Proc. Natl. Acad. Sci. USA.* **85:** 9758–9762.