Lipoproteins in pinnipeds: analysis of a high molecular weight form of apolipoprotein E

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Abstract We have examined the apolipoprotein content of the lipoproteins of several marine mammals by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Their apolipoprotein (apo) B-100, apoB-48, and apoA-I migrated to virtually the same position as the comparable human apolipoproteins. In cetaceans (bottlenose dolphins and killer whales), the molecular mass of the apoE was identical to that of human apoE (35 kDa). In contrast, in the lipoproteins of pinnipeds (harbor seals, sea lions, and walrus) there was no protein comparable in size to human apoE; however, there were two proteins in the 40- to 44-kDa range. The protein with the higher apparent molecular weight (44 kDa) was apoA-IV, as determined by NH_2-terminal amino acid sequencing. Sequencing of the NH_2-terminal 15 amino acids of the lower molecular weight protein (40–42 kDa) revealed no obvious homology with human apoE. However, a human apoE-specific monoclonal antibody, 1D7, bound to the 40- to 42-kDa protein, allowing us to identify that protein as apoE. Sequencing of sea lion apoE cDNA clones demonstrated that sea lion apoE is 311 amino acids in length, 12 residues longer than human apoE. All 12 additional residues are located in the NH_2-terminal 31 amino acids, a region that has extremely low homology with the NH_2-terminal portion of human apoE. The remainder of the sea lion apoE sequence is 74% homologous to human apoE. The sea lion apoE cDNA was expressed in Chinese hamster ovary (CHO) cells as well as CHO ldlD cells, a cell line that is deficient in *O*-glycosylation of proteins. The size of the sea lion apoE secreted by these two cell lines, compared with the apoE in sea lion plasma, indicated that the predominant form of apoE in sea lion plasma must be posttranslationally modified. Thus, our studies have demonstrated that the higher apparent molecular weight of pinniped (sea lion) apoE is due to a longer polypeptide chain as well as posttranslational modification of the protein. —**Davis, R. W., V. R. Pierotti, S. J. Lauer, S. T. Hubl, J. W. McLean, J. L. Witztum, and S. G. Young.** Lipoproteins in pinnipeds: analysis of a high molecular weight form of apolipoprotein E. *J. Lipid Res.* 1991. **32:** 1013–1023.

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Atherosclerosis is rare in sea mammals, despite the fact that these animals consume a fish diet that is very rich in fats and cholesterol (1). Because of the apparent protection from atherosclerotic disease and because of our long-standing interest in sea mammal physiology (2, 3), we began to study sea mammal lipoproteins and apolipoproteins. We found that the apparent molecular weights of sea mammal apolipoprotein (apo) B-100, apoB-48, and apoA-I, as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), were almost identical to those of the comparable human apolipoproteins. The apoE of cetaceans (killer whale and dolphin) had an apparent molecular weight of 35,000, virtually identical to that of human apoE. In pinniped lipoproteins, however, no protein comparable in size to human apoE could be detected, initially suggesting that the pinnipeds might be deficient in apoE (4). However, further experiments, described here, establish that species from all three genera of the order Pinnipedia, harbor seals (*Phoca vitulina*), sea lions (*Zalophus californianus*), and walrus (*Odobenidi rosemarius*), have an unusually large apoE molecule with an apparent molecular weight of 40,000–42,000. On stained SDS-gels, the apoE in pinnipeds is the lower protein band of a double band in the 40- to 44-kDa size range; the upper protein band is apoA-IV. In this paper, we report the results of our studies on the biochemical basis of the large apoE molecule in pinnipeds.

Abbreviations: apo, apolipoprotein; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; VLDL, very low density lipoproteins; LDL, low density lipoproteins; HDL, high density lipoproteins; CMV, cytomegalovirus; CHO, Chinese hamster ovary.

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MATERIALS AND METHODS

Blood and tissue samples

Blood samples were obtained from 11 harbor seals (*Phoca vitulina*), 6 sea lions (*Zalophus californianus*), a walrus (*Odobenidi rosemarius*), 4 bottlenose dolphins (*Tursiops truncatus*), and 5 killer whales (*Orcinus orca*) at Sea World in San Diego, California. All of the animals were fed a mixed diet of fresh-frozen herring, mackerel, and smelt (3–5% body weight per day) that was supplemented with vitamins. The composition of the diet is approximately 67% water, 15% fat, 18% protein, and negligible carbohydrate (5). The cholesterol content in the diet is approximately 0.08% (w/w) (5). Therefore, an average 70-kg harbor seal or lion consumes about 500 g of fat and 2800 mg of cholesterol daily.

Samples of liver and brain were taken from an adult female sea lion at necropsy. The sea lion had been euthanized because of chronic illness in accordance with the animal care policies of Sea World. Several tissues were frozen in liquid nitrogen and then stored at −70°C; these tissues were used to construct cDNA libraries (see below).

Analysis of lipoproteins

Blood samples were collected in tubes containing EDTA (1 mg/ml final concentration). The very low density lipoproteins (VLDL) (d < 1.006 g/ml), low density lipoproteins (LDL) (d 1.025–1.063 g/ml), and high density lipoproteins (HDL) (d 1.063–1.21 g/ml) were isolated from plasma by sequential ultracentrifugation, using a Beckman 50.3 Ti rotor (6). The lipoprotein fractions were dialyzed against phosphate-buffered saline (0.154 M NaCl, 21 mM Na_2HPO_4, 15 mM NaH_2PO_4, 0.3 mM EDTA, pH 7.35). The protein content of the lipoprotein fractions was measured according to the method of Lowry et al. (7). Cholesterol and triglyceride concentrations were measured in whole plasma and in lipoprotein fractions using a bichromatic analyzer (ABA 200, Abbott Laboratories) according to standardized techniques of the Lipid Research Clinics Program (8). Lipoprotein electrophoresis was performed on 1% agarose gels (No. AC470100, Fisher Scientific, Pittsburgh, PA) and the Corning electrophoresis system (Palo Alto, CA), according to the manufacturers' instructions; the gels were stained with fat red 7B.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

Lipoproteins were delipidated with a 3:1 mixture of ethanol and ethyl ether. The apolipoproteins were then dissolved in a sample buffer containing 10 mM Tris-HCl, 30% glycerol, 2% SDS, and 2% 2-mercaptoethanol and loaded onto a 3–15% gradient polyacrylamide slab gel containing 0.1% SDS (9). After electrophoresis, the separated apolipoproteins were made visible by staining the gel with 0.1% Coomassie Brilliant Blue R250.

Protein sequencing

The protein bands within the VLDL and LDL of pinnipeds that migrated in the 40- to 45-kDa range were subjected to NH_2-terminal amino acid sequencing. For these studies, the VLDL proteins were separated on 3–15% gradient SDS-polyacrylamide slab gels. The separated proteins were then electrophoretically transferred to a polyvinyl difluoride membrane (Millipore, Bedford, MA); NH_2-terminal amino acid sequencing was then performed using an Applied Biosystems 477A pulsed-liquid sequencer (Applied Biosystems, Foster City, CA).

Antibodies and Western blots

The 40- to 42-kDa protein band from the VLDL of a harbor seal was excised from an SDS-polyacrylamide slab gel; the protein was electroeluted from the gel slice and then used to develop an antiserum in both a rabbit and a guinea pig. Western blots (9) were used to characterize the antisera; both antisera bound strongly to both of the 40- to 42-kDa bands in harbor seal and sea lion VLDL. The guinea pig antiserum also bound to human apoE in human VLDL samples. Rabbit antisera specific for human apoE were kindly provided by Drs. E. Krul and K. Weisgraber, a monoclonal antibody specific for human apoE, WUE4, by Dr. Krul; and a monoclonal antibody specific for human apoE, 1D7, by Drs. R. Milne and Y. Marcel.

Northern blot

Total cellular RNA was isolated from sea lion brain and liver and from Hep3B (a cultured human hepatoma cell line) by standard techniques (10). The presence of apoE mRNA in the sea lion organs was assessed by Northern blot analysis (11). The Northern blot was probed with a 990-base pair ^{32}P-labeled human apoE cDNA fragment (human apoE cDNA nucleotides 35-1024).

Construction and screening of sea lion brain and liver cDNA libraries

Sea lion brain and liver poly(A)+RNA samples were isolated by using messenger-affinity paper according to the manufacturer's instructions (HYBOND-mAP, Amersham, Arlington Heights, IL). Double-stranded cDNAs were synthesized by priming with oligo(dT) (12, 13). The cDNAs larger than 600 base pairs were then ligated into the lambda GT10 bacteriophage vector (14).

A total of 1.2 million plaques of the unamplified brain and liver cDNA libraries were screened, using the 990-base pair human ^{32}P-labeled human apoE cDNA probe according to the techniques outlined by Huynh, Young, and Davis (14). Three apoE clones were isolated from the liver library and two from the brain library. The DNA in-

TABLE 1. Values for total cholesterol and triglycerides in plasma very low density lipoproteins, low density lipoproteins, and high density lipoproteins from the five marine mammal species examined[a]

Species	N	Plasma		Cholesterol		
		TC[b]	TG[b]	VLDL	LDL	HDL
Harbor seal	11	258 ± 9	43 ± 7	4 ± 0.6	70 ± 10	184 ± 6
Sea lion	6	323 ± 26	92 ± 18	12 ± 2	93 ± 18	217 ± 16
Walrus	1	293	16	17	113	72
Bottlenose dolphin	4	190 ± 2	99 ± 37	15 ± 7	22 ± 2	152 ± 29
Killer whale	6	245 ± 47	118 ± 20	37 ± 6	113 ± 44	95 ± 6

[a]All values are mg/dl ± SD.
[b]TC, total cholesterol; TG, triglycerides.

serts of three lambda clones were cloned into the *Eco*RI site of M13 in both orientations. Two of these three liver cDNA clones contained the entire coding sequence for the mature apoE protein and the signal peptide. Both strands of the apoE cDNA insert were sequenced using the dideoxynucleotide chain termination technique of Sanger, Nicklen, and Coulson (15).

Construction of a sea lion apolipoprotein E expression vector, and transfection of cell lines

To construct a sea lion apoE expression vector, a fragment of the sea lion apoE M13 cDNA clone spanning from the *Eco*RI site (on the M13 polylinker 5' to the sea lion apoE insert) to the *Apa*I site at sea lion apoE cDNA nucleotide 1049 was inserted into the *Eco*RI and *Apa*I sites of Bluescribe SKII (Stratagene, La Jolla, CA). An *Eco*RI-*Kpn*I fragment was then removed from this plasmid and inserted into the polylinker of pCMV5, a mammalian cDNA expression vector given to us by Dr. David Russell (Department of Molecular Genetics, University of Texas, Southwestern Medical Center, Dallas, TX). This plasmid contains the sequences of the cytomegalovirus (CMV) promoter, SV40 enhancer, and human growth hormone terminator (16). This plasmid, designated pCMV-SLE, was used to transfect COS-7 cells (obtained from the American Type Culture Collection), wild-type Chinese hamster ovary (CHO) cells, and mutant CHO ldlD cells. The CHO and CHO ldlD cells were obtained from Dr. Monty Krieger (Massachusetts Institute of Technology, Cambridge, MA). The cells were maintained as previously described (16). The mutant CHO ldlD cells lack the capacity for *O*-linked glycosylation of proteins when grown in serum-free medium in which glucose is the only sugar source, but are capable of glycosylation when the medium is supplemented with galactose and *N*-acetylgalactosamine (17, 18). Transient transfections of COS-7 cells were performed as described (16, 19). Mixed pools of stably transformed CHO cells and CHO ldlD cells expressing sea lion apoE were developed by cotransfecting these cells with pCMV-SLE and the neomycin-resistance gene pSV2neo and then selecting for cells capable of growing in the presence of the neomycin analogue G418 (16). Both the extracts of transfected cells and the medium in which transfected cells were grown were analyzed for evidence of sea lion apoE expression by Western blot analysis using the guinea pig antiserum to the 40- to 42-kDa protein in harbor seal VLDL.

RESULTS

The plasma lipid and lipoprotein levels for the different sea mammals are listed in **Table 1**. Most of the cholesterol in the harbor seals, sea lions, and bottlenose dolphins was in the HDL; in contrast, a greater proportion of the cholesterol was in the LDL fraction of the walrus and the killer whales. We fasted four harbor seals for 15 h, then fed them a meal containing 2 kg of herring and mackerel (approximately 300 g fat and 1600 mg of cholesterol). The postprandial cholesterol levels did not change significantly compared with the fasting levels (both 240-280 mg/dl). The triglyceride levels reached a peak of 115 mg/dl 2–4 h after the meal, then gradually returned to baseline levels (~45 mg/dl) after approximately 14 h.

The VLDL, LDL, and HDL fractions from all five species of sea mammals had predominantly pre-β, β, and α mobility, respectively, on agarose gels. However, some α-migrating lipoproteins were always detectable in the LDL fraction of the sea mammals' plasma (data not shown). The latter observation is consistent with the SDS-PAGE finding that there was a significant amount of apoA-I in the LDL fraction (**Fig. 1A**). By SDS-PAGE analysis, the lipoproteins of sea mammals contained apoB-100, apoB-48, and apoA-I bands, and each of these proteins had an apparent molecular weight that was virtually identical to that of the corresponding human protein. However, no protein similar in size to human apoE (35 kDa) was detectable in any of the lipoprotein fractions of the pinnipeds. Instead, in the VLDL of harbor seals and the walrus, there were proteins of 42 and 44 kDa. On a stained SDS-polyacrylamide gel, these proteins migrated as a double band (Fig. 1). In the sea lion, the VLDL frequently contained a double band consisting of proteins of 40 and 44 kDa; however, in some samples, only the 40-kDa band

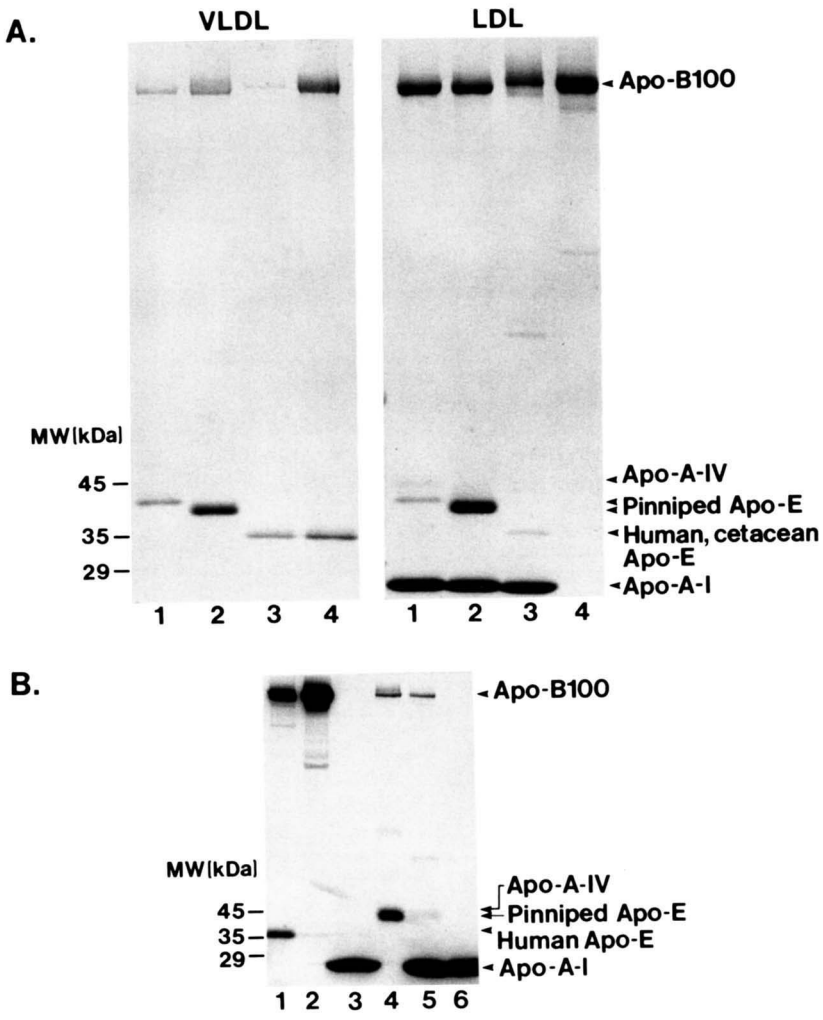

Fig. 1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the delipidated apolipoproteins of the different lipoprotein fractions of the five sea mammal species. The lipoprotein density fractions of the sea mammals were prepared by ultracentrifugation, then delipidated with a 3:1 mixture of ethanol and ether (v/v). Twenty to 40 μg of delipidated apolipoproteins were dissolved in a 2% SDS sample buffer and then electrophoresed on a 3–15% gradient SDS-polyacrylamide slab gel. The gel was stained with 0.1% Coomassie Brilliant Blue R250, then destained, and photographed. Panel A shows gels of VLDL and LDL samples: for each gel, lane 1 shows the apolipoproteins of harbor seal; lane 2, sea lion; lane 3, bottlenose dolphin; lane 4, human. When a larger amount of protein was applied to the gel, apoB-48 was visible in the VLDL fraction of all species (not shown). Panel B shows a comparison of human and walrus lipoproteins: lane 1, human VLDL; lane 2, human LDL; lane 3, human HDL; lane 4, walrus VLDL; lane 5, walrus LDL; lane 6, walrus HDL. MW, molecular weight.

was observed. NH_2-terminal amino acid sequencing of the 44-kDa protein in the double band revealed 95%, 75%, and 96% homology with human apoA-IV for harbor seals, sea lions, and walrus, respectively (**Table 2**). The NH_2-terminal amino acid sequence of the lower molecular weight band (i.e., 40 kDa) had very little amino acid homology with human apoE (Table 2). However, 9 of the NH_2-terminal 14 residues in these species were identical to those previously reported for dog apoE, suggesting that the 40-kDa protein in sea lion lipoproteins was apoE (Table 2). The VLDL and LDL fractions of bottlenose dolphins did not contain detectable amounts of apoA-IV; however, these fractions did contain a protein that co-migrated with human apoE. An amino acid analysis was performed on the dolphin VLDL 35-kDa protein; it was very similar to that of human apoE (data not shown).

A rabbit antiserum to human apoB-100 bound to the apoB-100 and apoB-48 of these sea mammals on Western blots. However, a rabbit antiserum to human apoE did not bind to any protein in these sea mammals' lipoproteins (data not shown). Similarly, a monoclonal antibody to human apoE, WUE4, failed to bind to sea mammal apoE (**Fig. 2A**). However, another human apoE-specific

TABLE 2. NH_2-terminal amino acid sequence data[a] for the apolipoprotein E and apolipoprotein A-IV of harbor seals, sea lions, and walrus

	Amino Acid Number																						
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
ApoE																							
Human[b]	K	V	E	Q	A	V	E	T	E	P	E	P	E	L	R	Q	Q	T	E	W			
Dog[c]	D	V	Q	P	E	P	E	L	E	R	E	L	E	P	K	V	Q	Q	E	L			
HS[d]	D	V	E	P	E	S	P	L	Q	G	K	L	E	P	D	P	E	S					
SL[d]	D	V	E	P	E	S	P	L	E	E	N	L	E	P	E	L	E	P	K				
Wal[d]	D	V	E	P	E	S	P	L	Q	G	K	L	E	P	D								
ApoA-IV																							
Human[b]	E	V	S	A	D	Q	V	A	T	V	M	W	D	Y	F	S	Q	L	S	N	N	A	K
HS	E	V	S	A	D	Q	V	A	T	V	V	W	D	Y	F	S	Q	L	S	N	N	A	
SL	E	V	N	A	N	Q	V	A	T	V	V	W											
Wal	E	V	S	A	[e]	Q	V	A	T	V	[e]	L	D	Y	F	S	Q	L	S	N	N	A	K

[a]One-letter code: A, alanine; C, cysteine; D, aspartic acid; E, glutamic acid; F, phenylalanine; G, glycine; H, histidine; I, isoleucine; K, lysine; L, leucine; M, methionine; N, asparagine; P, proline; Q, glutamine; R, arginine; S, serine; T, threonine; V, valine; W, tryptophan; Y, tyrosine.

[b]Sequence of human apoE and human apoA-IV taken from refs. 20 and 21.

[c]Sequence of canine apoE taken from ref. 22.

[d]HS, harbor seal; SL, sea lion; Wal, walrus.

[e]Identity of residue not determined with certainty.

monoclonal antibody, 1D7 (23), bound to the 40- and 42-kDa bands of sea lion and harbor seal VLDL, respectively (Fig. 2B). Antibody 1D7 binds to the LDL receptor-binding domain of human apoE and has been shown to bind to the apoE of other mammalian species (24). Antibody 1D7 did not, however, bind to dolphin apoE (Fig.

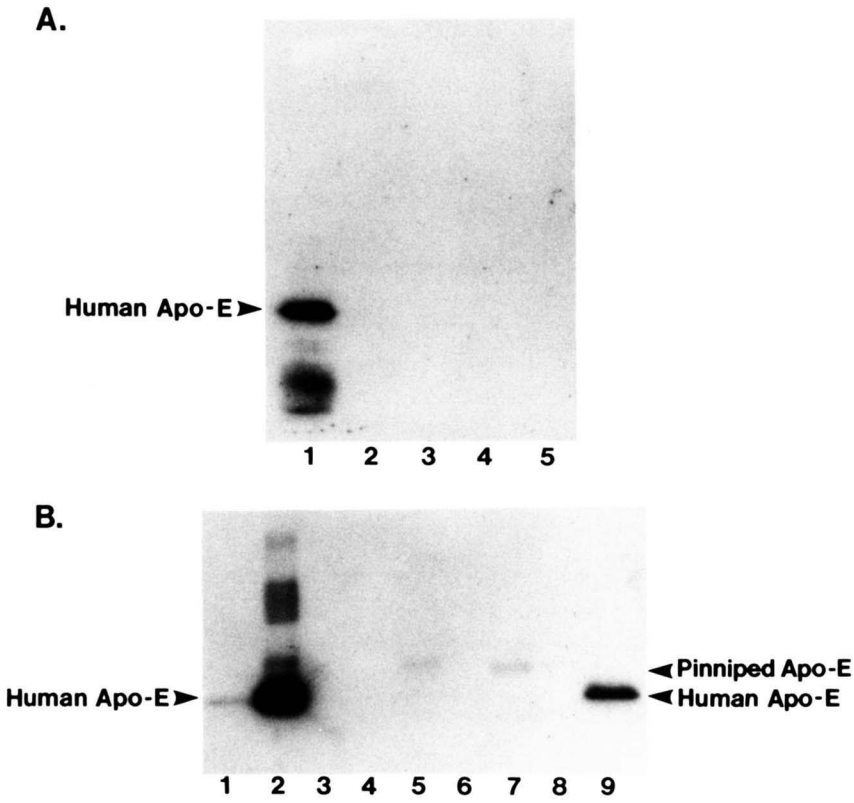

Fig. 2. Western blots of marine mammal apolipoproteins using apoE-specific monoclonal antibodies. The SDS-PAGE of VLDL and LDL apolipoproteins was performed as described in the legend for Fig. 1. The separated apolipoproteins were then electrophoretically transferred to nitrocellulose membranes. Immunoblots were then performed using the human apoE-specific monoclonal antibodies WUE4 (panel A) and 1D7 (panel B). Panel A: lane 1 shows human VLDL; lane 2, harbor seal VLDL; lane 3, harbor seal LDL; lane 4, sea lion VLDL; and lane 5, sea lion LDL. Panel B: lane 1 shows human LDL; lane 2, human chylomicrons; lane 3, bottlenose dolphin VLDL; lane 4, bottlenose dolphin LDL; lane 5, sea lion VLDL; lane 6, sea lion LDL; lane 7, harbor seal VLDL; lane 8, harbor seal LDL; and lane 9, human VLDL.

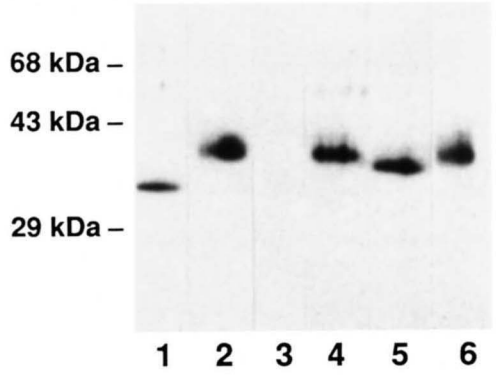

Fig. 3. Expression of sea lion apoE in cultured cells. Transfected cells were grown for 24 h in 5 ml of serum-free medium exactly as previously described (16). Glucose was the only sugar source in the medium. The medium in which the transfected cells were grown was then extensively dialyzed against 10 mM NH_4HCO_3 and lyophilized. One-tenth of the lyophilized protein was loaded onto a 10–20% gradient SDS-polyacrylamide slab gel. The separated proteins were then electrophoretically transferred to a sheet of nitrocellulose membrane for immunoblotting with a guinea pig antiserum specific for harbor seal apoE (1:500 dilution). The blot was then washed and incubated with ^{125}I-labeled goat anti-guinea pig immunoglobulin G. Lane 1 shows human VLDL (10 μg of delipidated protein loaded); lane 2, sea lion VLDL (1 μg of delipidated protein loaded); lane 3, medium in which CHO cells were transfected with the CMV vector only; lane 4, medium in which CHO cells were transfected with pCMV-SLE; lane 5, medium in which CHO ldlD cells were transfected with pCMV-SLE; lane 6, medium in which COS-7 cells were transiently transfected. Sea lion apoE was also found in the cell extracts (data not shown). When the CHO ldlD cells were grown in serum-free medium supplemented with 10 μM galactose and 100 μM *N*-acetylgalactosamine (16), the sea lion apoE in the culture medium migrated at a higher molecular weight than that observed when the cells were in a medium containing glucose as the sole sugar source (data not shown).

2B). The guinea pig antiserum to the 42-kDa protein of harbor seal VLDL bound strongly to the 40-kDa band in sea lion VLDL (**Fig. 3**) and to the 42-kDa band in harbor seal VLDL (not shown); it also bound to the apoE in human VLDL (Fig. 3).

The experiments with monoclonal antibody 1D7 and the guinea pig antiserum to the harbor seal 42-kDa protein strongly suggested that the 40- to 42-kDa proteins in pinnipeds were apoE. To verify that an apoE gene is expressed in these animals, we analyzed sea lion liver and brain for the presence of apoE mRNA. A Northern blot of sea lion brain and liver RNA probed with a ^{32}P-labeled human apoE cDNA fragment revealed a single band of ~1400 bases in both tissues; this mRNA was slightly larger than the human apoE mRNA in Hep3B cells (**Fig. 4**).

To gain a further understanding of apoE structure in pinnipeds, we constructed sea lion brain and sea lion liver cDNA libraries and then isolated clones from both libraries containing the entire coding sequence for sea lion apoE. The longest cDNA clone contained a 5' untranslated region of 30 nucleotides and a 3' untranslated region of 148 nucleotides. This sea lion apoE cDNA codes for a signal peptide of 18 amino acids and a mature protein of 311 amino acids, 12 residues longer than human apoE (**Fig. 5**). The extra 12 amino acids in the sea lion sequence are located within the NH_2-terminal 31 amino acids of sea lion apoE. The differences between the human and sea lion apoE amino acid sequences are also illustrated in Fig. 4.

Sea lion apoE is predicted to have a molecular weight of 36,053, approximately 1750 greater than that of human apoE. Thus, the primary structure only partially explains why sea lion apoE migrates at 40 kDa on SDS-gels. To examine this phenomenon further, we expressed the full-length sea lion apoE clone in CHO cells and CHO ldlD cells exactly as performed previously by Wernette-Hammond et al. (16). The CHO ldlD cells are deficient in *O*-linked glycosylation of proteins when grown in a medium in which glucose is the only sugar (17, 18). Sea lion apoE was easily detectable within the medium of transfected cells by Western blot analysis using the guinea pig antiserum to the harbor seal 42-kDa protein (Fig. 3). The sea lion apoE secreted by transiently transfected COS-7 cells and stably transformed wild-type CHO cells co-migrated with the apoE in sea lion VLDL. The sea lion apoE secreted by stably transformed CHO ldlD cells migrated

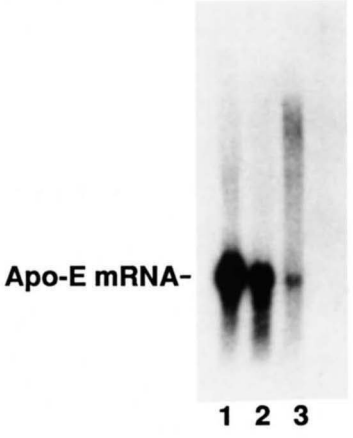

Fig. 4. A Northern blot of RNA from sea lion liver and brain probed with a ^{32}P-labeled human apoE cDNA fragment. RNA was prepared from sea lion liver and brain and from the human hepatoma cell line Hep3B using previously described techniques (10). Twenty micrograms of each sample was electrophoresed on an agarose gel, and a Northern blot was performed. The blot was probed with a ^{32}P-labeled human apoE cDNA fragment (see Materials and Methods). The blot was washed at medium stringency, 1 × SSC at 56°C. Lane 1 shows sea lion liver RNA; lane 2, sea lion brain RNA; lane 3, Hep3B RNA. The sea lion apoE mRNA band was estimated to be ~1400 nucleotides in length by comparison with size markers. In this experiment as well as several subsequent experiments, the sea lion apoE mRNA appeared to be slightly larger than the human apoE mRNA. In one subsequent experiment, we probed similar Northern blots with a ^{32}P-labeled sea lion apoE cDNA fragment and then washed the blot at high stringency (0.1 × SSC at 56°C). The same mRNA bands were identified with the ^{32}P-labeled sea lion cDNA probe.

Fig. 5. Nucleotide sequence of a sea lion apoE cDNA clone, with the predicted amino acid sequence. Both strands of the sea lion apoE cDNA clone were sequenced completely. Nucleotides are numbered 1–1169 along the left-hand side. Amino acids of the signal peptide are numbered −18 through −1; amino acids of the mature protein are numbered 1–311. At amino acid residues where the human apoE (the E3 isoform) amino acid sequence differed from the sea lion sequence, the homologous human amino acid is indicated below the sea lion amino acid. Human apoE amino acids 4–19 are indicated in brackets below sea lion apoE amino acids 4–31. The nucleotide sequence coding for the NH_2-terminal 75 amino acids of sea lion apoE was verified in M13 clones derived from several independent lambda bacteriophage cDNA clones.

at a distinctly lower position. This finding strongly suggests that the apoE in sea lion plasma, as well as the apoE synthesized by COS-7 cells and wild-type CHO cells, migrates with a higher apparent molecular weight on SDS-gels partly because of posttranslational modification of the protein. Thus, there appear to be two reasons for the fact that sea lion plasma apoE migrates at a high molecular weight: posttranslational modification (glycosylation), and the fact that the primary structure contains 12 extra amino acids. Glycosylation of sea lion apoE must be *O*-linked, because there are no *N*-linked glycosylation sites in the sea lion apoE sequence.

A. Hexanucleotide Repeats within the 5′ Portions of Sea Lion and Human Apo-E cDNA Sequences

	1		2		3		4		5	
Sea Lion	Val-2	Glu-3	Pro-4	Glu-5	Ser-6	Pro-7	Leu-8	Glu-9	Glu-10	Asn-11
	CTG	GAG	CCG	GAG	TCG	CCG	CTG	GAG	GAG	AAC
Human	GTG	GAG	CAA	GCG	---	---	GTG	GAG	ACA	GAG
	Val-2	Glu-3	Gln-4	Ala-5			Val-6	Glu-7	Thr-8	Glu-9

	6		7		8		9		10	
Sea Lion	Leu-12	Glu-13	Pro-14	Glu-15	Leu-16	Glu-17	Pro-19	Lys-19	Arg-20	Glu-21
	CTG	GAG	CCC	GAG	CTG	GAG	CCC	AAG	CGG	GAG
Human	CCG	GAG	CCC	GAG	---	---	---	---	---	---
	Pro-10	Glu-11	Pro-12	Glu-13						

	11		12		13		14		15	
Sea Lion	Leu-22	Glu-23	Gln-24	Glu-25	Val-26	Glu-27	Pro-28	Glu-29	Ala-30	Gly-31
	CTG	GAG	CAG	GAG	GTG	GAG	CCC	GAG	GCC	GGG
Human	CTG	CGC	CAG	CAG	---	---	---	---	ACC	GAG
	Leu-14	Arg-15	Gln-16	Gln-17					Thr-18	Glu-19

B. Percentage of Nucleotides Conforming to the Consensus Hexanucleotide Repeat Sequence (C $\frac{C}{T}$ G G A G)

	C	$\frac{C}{T}$	G	G	A	G
Sea Lion (15 repeats)	73%	80%	73%	80%	87%	93%
Human (9 repeats)	56%	78%	56%	78%	78%	89%

Fig. 6. Repetitive elements in the 5' region of the human and the sea lion apoE cDNA sequences. Panel A shows the cDNA nucleotide sequences for sea lion apoE amino acids 2-31 and human apoE amino acids 2-19, aligned according to hexanucleotide repeats. Within this region, there are 15 hexanucleotide repeats (consensus sequence C$\frac{C}{T}$GGAG) in the sea lion sequence, and 9 in the human sequence. Panel B shows the percentage of nucleotide conforming to the C$\frac{C}{T}$GGAG consensus repeat in the 15 sea lion repeats and the 9 human repeats.

DISCUSSION

We studied the plasma lipoproteins of two cetacean species (bottlenose dolphins and killer whales) and three species of pinnipeds (walrus, harbor seal, and sea lion). The chemical and physical properties of marine mammal lipoproteins have been described (25). None of these five species of marine mammals had high levels of LDL cholesterol or triglycerides, despite the fact that they consume a diet that is extremely rich in cholesterol and fats (5). The harbor seal and sea lion had very high levels of HDL cholesterol. If HDL cholesterol is protective against atherosclerosis in these animals, as it seems to be in humans, it might help to explain why atherosclerosis is rarely reported for pinnipeds. Although Stout (1) described lesions in the aortas of seals (*Phoca vitulina*) and sea lions (*Zalophus californianus*), generally they were fibrous plaques devoid of stainable lipid and may not truly have been atherosclerosis. One case of atherosclerosis in a harbor seal with severe hypercholesterolemia was reported by Davis et al. (26). In contrast to pinnipeds, there have been several well-documented cases of atherosclerosis in cetaceans (27-29) (personal communication, J. McBain, Sea World, San Diego).

The apoB-100, apoB-48, apoA-I, and apoA-IV of sea mammals co-migrated on SDS-gels with the comparable human apolipoproteins. The apoE of dolphins and killer whales co-migrated with human apoE, but pinniped apoE was much larger than human apoE on SDS-gels. The apoE of the harbor seal and the walrus had an apparent molecular weight of ~42,000; that of sea lion apoE was ~40,000.

To understand the structural basis for the larger apoE in sea lions, we cloned and sequenced the sea lion apoE cDNA. The mature sea lion apoE is composed of 311 amino acids, compared with the 299 of human apoE. All 12 extra amino acids are located within the NH_2-terminal 31 amino acids of the protein. These extra amino acids appear to result from six additional copies of an imperfect hexanucleotide repeat, whose consensus sequence is

C[C/T]GGAG. The hexanucleotide repeat occurs within the initial portion of exon 3 in the human apoE gene 5' to the 11- and 33-nucleotide repeats that have previously been identified in the 3' portion of exon 3 and exon 4 (30–32). A comparison of the hexanucleotide repeats within the 5' portion of the human and sea lion apoE cDNA sequences (coding for human apoE amino acids 2–19 and sea lion apoE amino acids 2–31) are shown in **Fig 6**. The amino acid sequences of sea lion and human apoE within this region are extremely poorly conserved. Within sea lion apoE residues 2–31, there are 12 negatively charged amino acids and only 2 positively charged amino acids. The functional importance of this negatively charged and evolutionally divergent NH_2-terminal region of apoE is not yet known. Beginning with tryptophan-32 of the sea lion apoE and tryptophan-20 of human apoE, there are no gaps or insertions in the alignment of the human and sea lion apoE sequences (Fig. 5). When amino acids 4–31 of sea lion and 4–19 of human apoE are excluded from the analysis, the homology between the two species on the nucleotide and amino acid level is 84% and 74%, respectively. Of the various mammalian apoE sequences that have been reported to date (20, 30, 33–39), the sea lion sequence is most homologous to that of the dog—83% on the amino acid level (exclusive of sea lion residues 4–31).

The sea lion and human apoE sequences were very highly conserved in the region of the molecule that is thought to bind to the cellular LDL receptor. Sea lion apoE amino acids 129–184, a region spanning the putative receptor-binding region of the molecule (40), were 88% homologous to amino acids 117–172 of the human sequence. The positively charged amino acids lysine and arginine are thought to be very important in receptor binding (40); each lysine and arginine residue between amino acids 117 and 172 in the human sequence was conserved in the sea lion sequence. Although we did not purify sea lion apoE and directly test its binding to the LDL receptor, we have demonstrated that the harbor seal VLDL fraction competes quite well with ^{125}I-labeled rabbit β-VLDL for binding to both human and harbor seal fibroblasts (unpublished observations, R. Davis, J. Witztum, S. Young). Because rabbit β-VLDL binding to the LDL receptor is mediated by apoE (41–44), we believe that the apoE of pinnipeds is capable of high-affinity binding to the LDL receptor.

Two other regions of sea lion apoE were noteworthy for high levels of amino acid conservation relative to human apoE. Sea lion amino acids 32–72 were 85% conserved, and amino acids 265–302 were 95% conserved. The functional significance of these regions is unclear; however, it seems very likely that the carboxy-terminal region of apoE is involved in lipid binding (45, 46).

The calculated molecular weight of sea lion apoE is 36,053, yet apoE in sea lion VLDL has an apparent molecular weight, by SDS-PAGE, of ~40,000 (Figs. 1 and 2). We suspected that posttranslational modification of sea lion apoE might account for this difference. To evaluate this possibility, we performed expression studies modeled on those of Wernette-Hammond et al. (16). Wernette-Hammond et al. (16) determined that both human plasma apoE and the human apoE expressed by CHO cells is glycosylated at threonine-194. They showed that CHO ldlD cells, a cell line defective in *O*-glycosylation of proteins (17, 18), failed to glycosylate apoE (16). When threonine-194 was eliminated from the human apoE sequence by mutagenesis, wild-type CHO cells did not glycosylate any alternate site in the human apoE sequence. We expressed the sea lion apoE cDNA in COS-7 cells, wild-type CHO cells, and CHO ldlD cells. The sea lion apoE expressed by COS-7 cells and wild-type CHO cells co-migrated with sea lion plasma apoE at an apparent molecular weight of ~40,000, whereas the sea lion apoE secreted by the CHO ldlD cells was significantly smaller, ~37,000–38,000 (Fig. 3). These studies demonstrated that the sea lion apoE secreted by CHO cells, which comigrates with sea lion plasma apoE, is glycosylated. Based on the precedent established by Wernette-Hammond and co-workers' studies of human apoE (16), we suspect that both the apoE in sea lion plasma and the sea lion apoE secreted by stably transformed CHO cells are posttranslationally modified by *O*-linked glycosylation of a serine or threonine residue. Interestingly, the glycosylated threonine residue in the human sequence (threonine-194) is not conserved in the sea lion sequence. We do not yet have any data regarding alternate sites of *O*-linked glycosylation of sea lion apoE.

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REFERENCES

1. Stout, C. 1969. Atherosclerosis in exotic carnivora and pinnipedia. *Am. J. Pathol.* **57:** 673–687.
2. Davis, R. W. 1983. Lactate and glucose metabolism in the resting and diving harbor seal (*Phoca vitulina*). *J. Comp. Physiol.* **153:** 275–288.
3. Davis, R. W., M. A. Castellini, T. H. Williams, and G. L. Kooyman. 1990. Fuel homeostasis in the harbor seal during submerged swimming. *J. Comp. Physiol.* **160:** 627–635.

4. Davis, R. W., S. G. Young, M. A. Castellini, J. L. Witztum, and L. Cornell. 1987. Apolipoprotein E deficiency in harbor seals and sea lions. *Arteriosclerosis.* **7:** 506a (Abstr.).
5. Diem, K., and C. Lentner, editors. 1970. Scientific Tables. 7th edition. Geigy Pharmaceuticals, New York. 515.
6. Havel, R. J., H. A. Eder, and J. H. Bragdon. 1955. The distribution and chemical composition of ultracentrifugally separated lipoproteins in human serum. *J. Clin. Invest.* **34:** 1345–1353.
7. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193:** 265–275.
8. Lipid Research Clinics Program. 1974. Manual of Laboratory Operations. Lipid and Lipoprotein Analysis. 2nd edition. Vol. 1. DHEW Publication no. 75-628. Government Printing Office, Washington, DC.
9. Young, S. G., S. J. Bertics, T. M. Scott, B. W. Dubois, L. K. Curtiss, and J. L. Witztum. 1986. Parallel expression of the MB19 genetic polymorphism in apoprotein B-100 and apoprotein B-48. Evidence that both apoproteins are products of the same gene. *J. Biol. Chem.* **261:** 2995–2998.
10. Maniatis, T., E. F. Fritsch, and J. Sambrook. 1982. Molecular Cloning. A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
11. Thomas, P. S. 1983. Hybridization of denatured RNA transferred or dotted to nitrocellulose paper. *Methods Enzymol.* **100:** 255–266.
12. Okayama, H., and P. Berg. 1982. High-efficiency cloning of full-length cDNA. *Mol. Cell. Biol.* **2:** 161–170.
13. Gubler, U., and B. J. Hoffman. 1983. A simple and very efficient method for generating cDNA libraries. *Gene.* **25:** 263–269.
14. Huynh, T. V., R. A. Young, and R. W. Davis. 1985. Construction and screening of cDNA libraries in λgt10 and λgt11. *In* DNA Cloning Techniques. A Practical Approach. Vol. 1. D. M. Glover, editor. IRL Press, Oxford. 49–78.
15. Sanger, F., S. Nicklen, and A. R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA.* **74:** 5463–5467.
16. Wernette-Hammond, M. E., S. J. Lauer, A. Corsini, D. Walker, J. M. Taylor, and S. C. Rall, Jr. 1989. Glycosylation of human apolipoprotein E. The carbohydrate attachment site is threonine 194. *J. Biol. Chem.* **264:** 9094–9101.
17. Krieger, M., M. S. Brown, and J. L. Goldstein. 1981. Isolation of Chinese hamster cell mutants defective in the receptor-mediated endocytosis of low density lipoprotein. *J. Mol. Biol.* **150:** 167–184.
18. Kingsley, D. M., K. F. Kozarsky, L. Hobbie, and M. Krieger. 1986. Reversible defects in *O*-linked glycosylation and LDL receptor expression in a UDP-Gal/UDP-GalNAc 4-epimerase deficient mutant. *Cell.* **44:** 749–759.
19. Wigler, M., R. Sweet, G. K. Sim, B. Wold, A. Pellicer, E. Lacy, T. Maniatis, S. Silverstein, and R. Axel. 1979. Transformation of mammalian cells with genes from procaryotes and eucaryotes. *Cell.* **16:** 777–785.
20. Rall, S. C., Jr., K. H. Weisgraber, and R. W. Mahley. 1982. Human apolipoprotein E. The complete amino acid sequence. *J. Biol. Chem.* **257:** 4171–4178.
21. Elshourbagy, N. A., D. W. Walker, M. S. Boguski, J. I. Gordon, and J. M. Taylor. 1986. The nucleotide and derived amino acid sequence of human apolipoprotein A-IV mRNA and the close linkage of its gene to the genes of apolipoproteins A-I and C-III. *J. Biol. Chem.* **261:** 1998–2002.
22. Weisgraber, K. H., R. F. Troxler, S. C. Rall, and R. W. Mahley. 1980. Comparison of the human, canine, and swine E apoproteins. *Biochem. Biophys. Res. Commun.* **95:** 374–380.
23. Milne, R. W., P. Douste-Blazy, Y. L. Marcel, and L. Retegui. 1981. Characterization of monoclonal antibodies against human apolipoprotein E. *J. Clin. Invest.* **68:** 111–117.
24. Weisgraber, K. H., T. L. Innerarity, K. J. Harder, R. W. Mahley, R. W. Milne, Y. L. Marcel, and J. T. Sparrow. 1983. The receptor-binding domain of human apolipoprotein E. Monoclonal antibody inhibition of binding. *J. Biol. Chem.* **258:** 12348–12354.
25. Puppione, D. L., and A. V. Nichols. 1970. Characterization of the chemical and physical properties of the serum lipoproteins of certain marine mammals. *Physiol. Chem. Phys.* **2:** 49–58.
26. Davis, R. W., T. E. Carew, J. McBain, S. G. Young, and J. L. Witztum. 1988. Sudden death associated with hypercholesterolemia and severe atherosclerosis in a 15 month old harbor seal. *Arteriosclerosis.* **8:** 612a–613a (Abstr.).
27. Roberts, J. C., Jr., R. C. Boice, R. L. Brownell, Jr., and D. H. Brown. 1965. Spontaneous atherosclerosis in Pacific toothed and baleen whales. *In* Comparative Atherosclerosis. The Morphology of Spontaneous and Induced Atherosclerotic Lesions in Animals and Its Relation to Human Disease. J. C. Roberts, Jr., R. Straus, and M. S. Cooper, editors. Harper and Row, New York. 151–155.
28. Cowan, D. F. 1966. Pathology of the pilot whale *Globicephala melaena*. A comparative survey. *Arch. Pathol.* **82:** 178–189.
29. Truex, R. C., F. G. Nolan, R. C. Truex, Jr., H. P. Schneider, and H. I. Perlmutter. 1961. Anatomy and pathology of the whale heart with special reference to the coronary circulation. *Anat. Rec.* **141:** 325–353.
30. Rajavashisth, T. B., J. D. Kaptein, K. L. Reue, and A. J. Lusis. 1985. Evolution of apolipoprotein E: mouse sequence and evidence for an 11-nucleotide ancestral unit. *Proc. Natl. Acad. Sci. USA.* **82:** 8085–8089.
31. Li, W. H., M. Tanimura, C. C. Luo, S. Datta, and L. Chan. 1988. The apolipoprotein multigene family: biosynthesis, structure, structure-function relationships, and evolution. *J. Lipid Res.* **29:** 245–271.
32. Boguski, M. S., M. Freeman, N. A. Elshourbagy, J. M. Taylor, and J. I. Gordon. 1986. On computer-assisted analysis of biological sequences: proline punctuation, consensus sequences, and apolipoprotein repeats. *J. Lipid Res.* **27:** 1011–1034.
33. Luo, C. C., W. H. Li, and L. Chan. 1989. Structure and expression of dog apolipoprotein A-I, E., and C-I mRNAs: implications for the evolution and functional constraints of apolipoprotein structure. *J. Lipid Res.* **30:** 1735–1746.
34. McLean, J. W., C. Fukazawa, and J. M. Taylor. 1983. Rat apolipoprotein E mRNA. Cloning and sequencing of double-stranded cDNA. *J. Biol. Chem.* **258:** 8993–9000.
35. Marotti, K. R., B. E. Whitted, C. K. Castle, H. G. Polites, and G. W. Melchior. 1989. Nucleotide sequence of cynomolgus monkey apolipoprotein E cDNA. *Nucleic Acids Res.* **17:** 1778.
36. Matsushima, T., G. S. Getz, and S. C. Meredith. 1990. Primary structure of guinea pig apolipoprotein E. *Nucleic Acids Res.* **18:** 202.
37. McLean, J. W., N. A. Elshourbagy, D. J. Chang, R. W. Mahley, and J. M. Taylor. 1984. Human apolipoprotein E mRNA. cDNA cloning and nucleotide sequencing of a new variant. *J. Biol. Chem.* **259:** 6498–6504.
38. Hao, Q. L., T. T. Yamin, T. C. Pan, S. L. Chen, B. S. Chen, P. A. Kroon, and Y. S. Chao. 1987. Isolation and characterization of a full-length rabbit apolipoprotein E

cDNA. *Atherosclerosis.* **66:** 125–130.
39. Hixson, J. E., L. A. Cox, and S. Borenstein. 1988. The baboon apolipoprotein E gene: structure, expression, and linkage with the gene for apolipoprotein C-I. *Genomics.* **2:** 315–323.
40. Mahley, R. W., and S. C. Rall, Jr. 1989. Type III hyperlipoproteinemia (dysbetalipoproteinemia): the role of apolipoprotein E in normal and abnormal lipoprotein metabolism. *In* The Metabolic Basis of Inherited Disease. 6th ed. C. R. Scriver, A. L. Beaudet, W. S. Sly, and D. Valle, editors. McGraw-Hill, New York. 1195–1213.
41. Koo, C., M. E. Wernette-Hammond, Z. Garcia, M. J. Malloy, R. Uauy, C. East, D. W. Bilheimer, R. W. Mahley, and T. L. Innerarity. 1988. The uptake of cholesterol-rich remnant lipoproteins by human monocyte-derived macrophages is mediated by low density lipoprotein receptors. *J. Clin. Invest.* **81:** 1332–1340.
42. Friedman, G., M. E. Wernette-Hammond, D. Y. Hui, R. W. Mahley, and T. L. Innerarity. 1987. Characterization of lipoprotein receptors on rat Fu5AH hepatoma cells. *J. Lipid Res.* **28:** 1482–1494.
43. Wernette-Hammond, M. E., Z. Garcia, K. S. Arnold, and T. L. Innerarity. 1989. β-Very low density lipoprotein uptake in cultured fibroblasts and smooth muscle cells from Watanabe heritable hyperlipidemic rabbits. *Arteriosclerosis.* **9:** 501–510.
44. Innerarity, T. L., K. S. Arnold, K. H. Weisgraber, and R. W. Mahley. 1986. Apolipoprotein E is the determinant that mediates the receptor uptake of β-very low density lipoproteins by mouse macrophages. *Arteriosclerosis.* **6:** 114–122.
45. Wetterau, J. R., L. P. Aggerbeck, S. C. Rall, Jr., and K. H. Weisgraber. 1988. Human apolipoprotein E3 in aqueous solution. I. Evidence for two structural domains. *J. Biol. Chem.* **263:** 6240–6248.
46. Aggerbeck, L. P., J. R. Wetterau, K. H. Weisgraber, C. S. C. Wu, and F. T. Lindgren. 1988. Human apolipoprotein E3 in aqueous solution. II. Properties of the amino- and carboxyl-terminal domains. *J. Biol. Chem.* **263:** 6249–6258.