Processing of rat liver apoprotein E primary translation product

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Abstract The primary translation product of rat liver apoE mRNA was isolated from wheat germ cell-free translation systems. Plasma apoE and the primary translation product migrated similarly on SDS-polyacrylamide gels, had similar partial proteolytic peptide maps, and bound to and coeluted from heparin-Sepharose columns. Comparison of the partial amino acid sequence of the primary translation product with the amino-terminal sequence of plasma apoE indicated that rat apoE is initially synthesized with an 18 amino acid amino-terminal extension. This entire segment was removed cotranslationally by canine microsomes possessing signal peptidase activity. The microsome-processed translation product did not contain an endoglycosidase H-sensitive oligosaccharide, suggesting that rat apoE is O-glycosylated.—**Reardon, C. A., R. V. Hay, J. I. Gordon, and G. S. Getz.** Processing of rat liver apoprotein E primary translation product. *J. Lipid Res.* 1984. **25:** 348–360.

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ApoE is a component of several plasma lipoproteins including VLDL, chylomicrons, chylomicron remnants, certain subclasses of HDL, and in cholesteryl ester-rich lipoproteins that become prominent in the plasma of experimental animals following cholesterol feeding (1, 2). ApoE is a glycoprotein (3, 4) with a molecular weight between 34,000 and 39,000 (5, 6). Human apoE exhibits a polymorphism on IEF gels which is due both to genetic variability at a single gene locus and to post-translational addition of sialic acid residues (7, 8). The complete amino acid sequence of one isoprotein has been determined (9) and several sites of amino acid substitutions in the apoE isoproteins encoded by different alleles have been identified (10, 11).

ApoE functions as a major recognition signal mediating the clearance of triglyceride-rich lipoproteins. Two classes of lipoprotein receptors that recognize apoE-containing lipoproteins have been identified (12–16). Interaction of apoE- and cholesteryl ester-rich lipoproteins with these receptors results in delivery of cholesterol to the liver for excretion and to other body cells for growth or other specific functions.

ApoE is synthesized primarily by the liver, while little or none is synthesized by the intestine (17, 18). It is now becoming evident that certain peripheral cells are also capable of synthesizing apoE (19–21). The apoE synthesized by the peripheral cells is believed to participate in reverse cholesterol transport whereby excess cellular cholesterol may be transported between peripheral cells and the liver for excretion (19).

In view of the important role of apoE in receptor-mediated catabolism of lipoproteins, regulation of its synthesis and secretion is likely to be physiologically significant. Relatively little is known about the molecular events involved in apoprotein biosynthesis, presecretory processing, and assembly into lipoprotein particles. The earliest biosynthetic precursor of proteins, the primary translation product, can be synthesized in cell-free translation systems. The primary translation product of most secretory proteins has been shown to contain a signal peptide that mediates the cotranslational transfer of the polypeptide into the lumen of the RER (22). In this report, we present data on the characterization of the primary translation product of rat liver apoE mRNA. Its subsequent cotranslational modification is discussed and these findings are compared with current knowledge of the processing and compartmentalization of other apolipoproteins. A preliminary account of this work has been presented (23).

Abbreviations: apo, apolipoprotein; BSA, bovine serum albumin; HDL, high density lipoproteins; IEF, isoelectric focusing; poly(A), polyriboadenylic acid; poly(U), polyribouridylic acid; RER, rough endoplasmic reticulum; SDS, sodium dodecyl sulfate; TCA, trichloroacetic acid; VLDL, very low density lipoproteins.

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MATERIALS AND METHODS

Animals

Male rats of the Sprague-Dawley strain were obtained from ARS-Sprague-Dawley (Madison, WI) or Holtzman (Madison, WI). They were maintained in a continuously lighted room and fed Rockland-Purina Rat and Mouse Chow and water ad libitum. Goats and New Zealand White rabbits were used for antibody preparation.

Materials

Radioisotopes of the highest specific activity available were purchased from Amersham Corporation. EN^{3}Hance was obtained from New England Nuclear. All electrophoretic reagents were obtained from Bio-Rad. Poly(U) Sepharose 4B was obtained from Pharmacia and Protein A Sepharose C1-4B was purchased from Pharmacia Fine Chemicals or Sigma Chemicals. The sources of the following reagents are indicated in parentheses: micrococcal nuclease (*Staphylococcus aureus*) (P-L Biochemicals), creatine kinase (Boehringer Mannheim), dansyl chloride (Aldrich Chemical Company), endoglycosidase H and *Staphylococcus aureus* V8 protease (Miles Laboratories Inc.). All other reagents were obtained from Sigma or were of the best grade commercially available. Fresh commercial wheat germ was obtained from Dixie Portland Flour Mills (Chicago, IL) or General Mills (Chicago, IL). The wheat germ was stored dry under vacuum at 4°C prior to extraction. Rabbit reticulocyte lysates were the generous gift of Dr. Martin Gross (University of Chicago).

Isolation of lipoproteins

All lipoproteins were isolated by ultracentrifugal flotation. VLDL was isolated from chylomicron-free plasma at density 1.006 g/ml. HDL was isolated by flotation within the density range of 1.063–1.21 g/ml or was separated into light (d 1.063–1.75 g/ml) and heavy (d 1.12–1.21 g/ml) subfractions. The isolated lipoproteins were dialyzed against 150 mM NaCl, 1 mM EDTA, pH 7.4, and 0.01% sodium azide or other appropriate buffers prior to use.

Preparation of antigens and antibodies

Rat plasma apoE was prepared from VLDL as described by Hay and Getz (24) and antibodies to apoE were prepared in rabbits and a goat. Alternatively, rat apoE was isolated from ethanol–ether–delipidated HDL (25) by heparin-Sepharose 4B affinity chromatography (26), with final purification accomplished by electroelution from preparative 10–22.5% polyacrylamide-SDS gradient slab gels (27). The anti-apoE antibodies were affinity-enriched by adsorption to an HDL_3 Sepharose column (24, 28). Antibodies to be used for the immunoprecipitation of rat liver polysomes were freed of RNase by tandem CM- and DEAE-cellulose chromatography (29). The specificity of the antibodies was established by Ouchterlony immunodiffusion against whole serum and isolated lipoproteins, by the singular immunoprecipitation of radiolabeled apoE from among the labeled apoproteins of VLDL and HDL, and by the immunodecoration of electrophoretically separated apolipoproteins transferred to nitrocellulose (30).

SDS-polyacrylamide gel electrophoresis and fluorography

Protein samples and cell-free translation immunoprecipitates were analyzed on polyacrylamide gradient gels as described by O'Farrell (31). After destaining, the gels were treated with EN^{3}Hance according to the manufacturer's instructions. The impregnated gels were then exposed to preflashed Kodak X-Omat R or Kodak X-Omat AR film at −40°C (32).

Protein estimation

Protein concentrations were determined by the method of Lowry et al. (33) using BSA as the standard. After the color reaction was completed, SDS was added to a final concentration of 0.15% in order to eliminate interference by lipoprotein lipids (34).

RNA isolation

Livers were obtained from decapitated normal male rats with body weights of 125–150 g. Total cytoplasmic rat liver RNA was isolated using phenol chloroform (24). Impurities in the RNA that inhibit in vitro translation were removed by sequential washing of the precipitated RNA (28). The $A_{260/280}$ ratio was ~2.0. Poly(A)-containing RNA was isolated by chromatography on Poly(u)-Sepharose columns (24).

RNA enriched for apoE mRNA was isolated by indirect immunoprecipitation of rat liver polysomes essentially as described by Shapiro et al. (28) and Hay and Getz (24). Total rat liver polysomes were isolated by the magnesium precipitation procedure of Palmiter (35). Polysomes containing apoE mRNA were precipitated on ice with 10–15 μg of affinity-enriched, RNase-free goat anti-apoE per A_{260} unit of polysomes and RNase-free rabbit anti goat IgG. Approximately 40% of the mRNA activity of the enriched RNA was attributable to apoE mRNA.

Cell-free translation assays

The wheat germ S-30 extract was prepared from floated (36) wheat germ as described by Roberts and Paterson (37) with the following modifications. The supernatant was centrifuged twice at 30,000 *g* for 10 min (38). After preincubation, the supernatant was applied to a 1.5 × 60 cm column of Sephadex G-25 (medium) (39) equilibrated with 20 mM HEPES, pH 7.6, 120 mM KCl, 5 mM magnesium acetate, and 6 mM 2-mercapto-

ethanol. The most turbid fractions were pooled, centrifuged at 30,000 *g* for 20 min (38), and stored in liquid nitrogen.

Cell-free translation assays performed with wheat germ extracts contained 10 μl of RNA or water and 30 μl of mixed reagents whose final concentrations in the assays were 20 mM HEPES, pH 7.4, 84 mM KCl, 1.8 mM magnesium acetate, 2 mM DTT, 1 mM ATP, 0.02 mM GTP, 8 mM creatine phosphate, 0.05 units of creatine phosphokinase, 0.06 mM of each unlabeled L-amino acid, 0.048 mM spermine, 5 μl of wheat germ S-30, 4 μCi of [^{3}H]leucine or 5 μCi of [^{35}S]methionine, and 450 ng of rat liver poly(A)-mRNA or 5 μg of total RNA. The concentration of KCl, magnesium acetate, and spermine and the pH were optimized for the synthesis of rat apoE (40). The assay mixtures were incubated for 2.5 hr at 25°C. Total protein synthesis was determined after TCA precipitation (24).

Rabbit reticulocyte lysates were prepared from New Zealand White rabbits injected with phenylhydrazine (41) and treated with micrococcal nuclease (42) prior to use.

Cell-free translation assays using reticulocyte lysate contained 4 μl of RNA or water, 8 μl of mixed reagents, and 10 μl of nuclease-treated lysate. The final reagent concentrations in the assays were 75 mM KCl, 1 mM magnesium acetate, 10 mM creatine phosphate, 0.05 units of creatine phosphokinase, 0.5 mM ATP, 0.2 mM GTP, 0.06 mM each of the appropriate unlabeled amino acids, 1.6 μCi of [^{3}H]leucine or 3 μCi of [^{35}S]methionine, and either 180 ng of rat liver poly(A)mRNA or 3 μg of total rat liver RNA. The assays were optimized for KCl and magnesium acetate concentrations. The reaction was incubated for 90 min at 25°C. Total protein synthesis was determined by TCA precipitation after decolorization with hydrogen peroxide (42).

Protein synthesized in the cell-free translation assays was isolated by indirect immunoprecipitation as described (24) with the following modification. Polysomes were removed by centrifugation in a Beckman airfuge at 28 psi (100,000 *g*) for 35 min prior to the addition of antibodies.

When the cell-free translation products were to be analyzed by SDS-polyacrylamide gel electrophoresis, they were isolated by immunobinding with Protein A-Sepharose beads. Following incubation of the cell-free translation assays with the first antibody, SDS was added to a final concentration of 0.2% and 30 μl of 1:1 diluted Protein A-Sepharose beads per 0.01 A_{280} unit of first antibody were added. The samples were rotated for 1 hr at room temperature and then overnight at 4°C. The beads were washed four times with immunobinding buffer (10 mM Na phosphate, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1% DOC, 10 mM unlabeled amino acid, 0.2% azide, and 0.2% SDS) and boiled for 2 min in SDS-PAGE sample buffer to dissociate the antigen-antibody-Protein A complex, prior to electrophoresis.

Cotranslational processing by canine microsomes

Canine microsomes were isolated from the pancreas of a normal adult mongrel dog by the procedure of Scheele, Jacoby, and Carne (43). Microsome membranes were treated with micrococcal nuclease (42) and added to the cell-free translation assays at a final concentration of 20 A_{260} units/ml.

Isolation of the apoE translation product

For radiochemical sequencing of the translation product, the preparation and isolation procedure was modified as follows. Total rat liver RNA or rat liver RNA enriched for apoE mRNA by immunoprecipitation of rat liver polysomes was translated in wheat germ lysates. The translation products were doubly labeled with [^{35}S]methionine and one or more of the following tritiated amino acids. Wheat germ cell-free systems contained 3 μCi of [^{35}S]methionine and either 4 μCi of [^{3}H]leucine, 3 μCi of [^{3}H]glutamic acid, 3 μCi of [^{3}H]glutamine, 4 μCi of [^{3}H]valine, or 4 μCi of [^{3}H]lysine per 40 μl of reaction mixture.

Final purification of the apoE translation product was accomplished by electroelution from preparative 10% polyacrylamide-SDS gels as described by Stephens (27) and modified as follows. The elution buffer comprised 25 mM Tris, 190 mM glycine, pH 8.3, and 0.01% SDS. A current of 3 mA per gel was applied for 12–15 hr. Dansylated plasma apoE was used as a fluorescent marker to locate the position of the apoE translation production in the polyacrylamide gel.

Radiochemical sequencing

Automated sequential Edman degradation of the purified apoE translation products was performed using a Beckman 890C sequenator and either a 0.1 M or a 0.3 M Quadrol program (44). The butyl chloride wash of each cycle was dried and counted for radioactivity in PCS or 3a70 scintillation cocktail as described below. Repetitive yields were determined by quantitating the recovery of selected residues obtained from degradation of lysozyme, myoglobin, or lactoglobin.

Measurement of radioactivity

Radioactivity was measured in a Searle Model 6872 Isocap 1300 scintillation counter. The efficiency of counting ^{3}H and ^{35}S in our system was 38% and 64%, respectively.

Amino-terminal sequencing of rat HDL-associated apoE

Sequential Edman degradation of 2 nmol of apoE was performed using a 0.3 M Quadrol program (44) and the Beckman spinning cup sequenator. Anilinothiazolinones derived from each cycle were converted to phenylthio-

hydantoins and subsequently identified by high performance liquid chromatography (45).

RESULTS

Characterization of the apoE translation product

The proteins synthesized in wheat germ lysates in response to rat liver mRNA that were immunobound by the anti-apoE antibody and Protein A-Sepharose were analyzed by electrophoresis on SDS-polyacrylamide gradient slab gels (**Fig. 1**). The major protein bound by the antibody (lane B) had the same electrophoretic mobility as mature plasma apoE (band at M_r = 35,000 in lane A) on this 5–22.5% polyacrylamide-SDS slab gel, as well as on a 10% polyacrylamide-SDS gel (data not shown). The purified full length apoE translation product is depicted in lane C (Fig. 1). The major translation product and the majority of the lower molecular weight proteins were shown to be related to apoE by the ability of plasma apoE purified by heparin-Sepharose affinity chromatography to inhibit the binding of these nascent proteins to the antibody (data not shown). The lower molecular weight proteins are probably incomplete apoE polypeptides produced by premature termination of translation of the apoE mRNA (40).

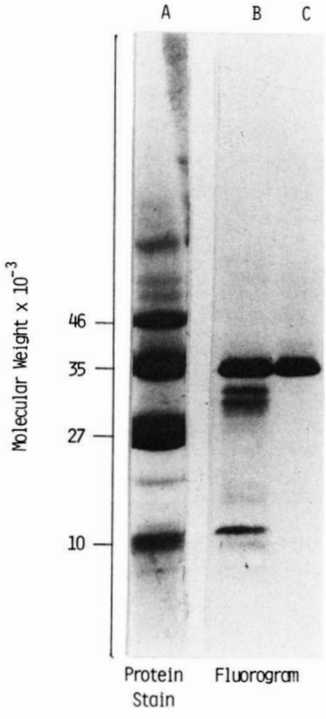

Fig. 1. Immunobinding and purification of the apoE translation product from wheat germ cell-free translation assays. Total rat liver poly(A) mRNA was translated in wheat germ cell-free systems in the presence of [^{3}H]leucine and the apoE translation product was isolated by immunobinding with goat anti-apoE and Protein A-Sepharose. The apoE translation product was purified by electroelution from preparative 10% polyacrylamide gels using dansylated plasma apoE as a fluorescent marker. The labeled proteins were analyzed on 5–22.5% polyacrylamide-SDS gradient slab gels and fluorographed as described in the text. Coomassie Blue stain of plasma apoHDL (20 μg) (A), anti-apoE immunobound proteins (B), and electroeluted purified apoE translation product (C). In lane (A), the peptides of HDL with M_r of 46,000, 35,000, 27,000, and 10,000 are A-IV, E, A-I, and C apoproteins, respectively.

TABLE 1. Indirect immunoprecipitation of wheat germ cell-free translation assays[a]

Rat Liver RNA	Anti-ApoE	Net Percent[b]	Standard Deviation
Poly(A) mRNA	Rabbit K-6	1.06%	0.54
	Goat 377	0.96%	0.43
Total RNA	Goat 377	1.04%	0.23

[a] RNA was translated in wheat germ cell-free translation systems. Aliquots of the translation mixture were first incubated with anti-apoE or nonimmune IgG and then with the appropriate anti-antibody as described in Materials and Methods. The immunoprecipitates were washed and counted for radioactivity as described.

[b] Net percent precipitated is equal to the amount of radioactivity specifically precipitated by anti-apoE divided by the amount of radioactivity incorporated into total rat liver proteins as measured by TCA insoluble radioactivity. Anti-apoE specific radioactivity was determined by subtracting nonspecific precipitated radioactivity as measured by nonimmune IgG precipitation from the radioactivity precipitated by anti-apoE. Nonspecific precipitation was less than 1% of TCA-insoluble radioactivity.

The amount of immunoprecipitable apoE synthesized in wheat germ lysates stimulated with different rat liver RNA preparations was determined by indirect immunoprecipitation using two different monospecific anti-apoE antibodies. This provides an approximation of the relative abundance of apoE mRNA in total cytoplasmic rat liver mRNA. The results are presented in **Table 1.** When poly(A) mRNA was translated in wheat germ lysates, approximately 1% of total rat liver proteins synthesized was immunoprecipitated by these two antibodies. Each of six different poly(A) mRNA preparations translated in the wheat germ lysates yielded quantitatively similar results. When total cytoplasmic RNA was translated in wheat germ lysates, again approximately 1% of total protein synthesis was devoted to apoE.

In order to confirm that the translation product that comigrated with plasma apoE on SDS-polyacrylamide gels was in fact the apoE translation product, their peptide maps were compared. Plasma apoE and the purified translation product were partially digested with *Staphylococcus aureus* V8 protease (46). The peptide fragments generated were separated on 15% polyacrylamide-SDS slab gels (**Fig. 2**). The peptide pattern was identical for both the plasma protein and the translation product. The pattern of the peptide fragments generated from bovine serum albumin digested under the same conditions was considerably different (not shown).

Plasma apoE is capable of binding to the sulfated glycosaminoglycan heparin (26). The only other apolipo-

protein with affinity for heparin is apoB. To determine if the putative apoE translation product also had affinity for heparin, purified [^{3}H]leucine-labeled translation product was next applied to a heparin-Sepharose column along with solubilized apoproteins from ethanol–ether-delipidated HDL (**Fig. 3**). Of the HDL apoproteins, only apoE was adsorbed to the column. As the salt concentration of the buffer was increased, a second protein peak containing primarily apoE was eluted. Eighty percent of the putative apoE translation product adsorbed to the heparin-Sepharose column and was co-eluted with plasma apoE. The immunological reactivity, the pattern of partial proteolytic digestion, the ability to bind to a heparin-Sepharose column, as well as the inability of the protein to bind to anti-apoE in the presence of excess purified plasma apoE, all suggested that the polypeptide bound by the anti-apoE antibody was the primary translation product of apoE mRNA.

Radiochemical sequencing

The comigration of the apoE translation product and plasma apoE on SDS-polyacrylamide gel electrophoresis was an unexpected result. The vectorial translocation of most secreted proteins into the lumen of the rough endoplasmic reticulum is initiated by a signal peptide located on the amino-terminus of the primary translation product.

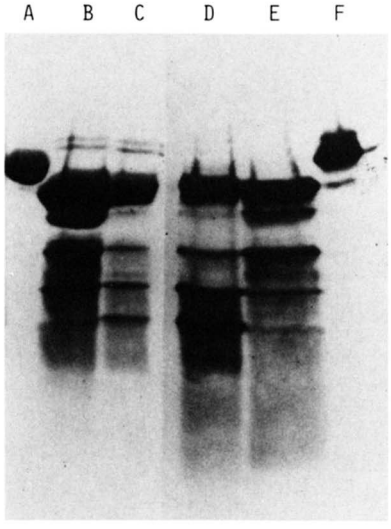

Fig. 2. Partial proteolytic mapping of plasma apoE and its translation product. Purified plasma apoE and purified [^{3}H]leucine-labeled apoE translation product were digested with 140 μg/ml *Staphylococcus aureus* V8 protease for 30 min at 37°C, as described by Cleveland et al. (46). The samples were analyzed by electrophoresis on a 15% polyacrylamide-SDS gel. The Coomassie Blue-stained gel of 9 μg of undigested plasma apoE (A), *S. aureus* V8 protease digest of 70 μg (B), and 17.5 μg (C) of purified plasma apoE, and the fluorogram of the *S. aureus* V8 protease digest of the purified apoE translation product alone (D), or in the presence of 10 μg of BSA (E), and the undigested purified translation product (F).

The presence of the signal peptide is usually recognized by the slower electrophoretic mobility and the larger apparent molecular weight of the primary translation product compared to the mature protein.

In order to determine if the primary translation product of apoE mRNA contained a signal peptide, its amino terminal sequence, determined radiochemically, was compared with that of mature apoE derived from plasma HDL. The results of Edman degradation of 2 nmol of the mature plasma protein are presented in **Table 2.** No amino acid could be clearly assigned to position 14 because of an ambiguous signal. The assignment of glutamic acid to position 1 is consistent with results obtained earlier by Swaney, Braithwaite, and Eder (5) using dansylation. The yield of glutamic acid at this position was 44% (875 pmol recovered from 2 nmol of polypeptide). The high yield plus the single sequence observed for residues 1–14 indicated the apoE recovered from plasma HDL was pure and a unique species.

The results of the radiochemical sequencing of the primary translation product are shown in **Fig. 4.** A single methionine residue, at cycle 1, was detected in the first 40 residues of the translation product. This amino acid is likely to be the initiator methionine. In all subsequent radiochemical sequencing, except with lysine, the translation products were double-labeled with [^{35}S]methionine and tritiated amino acids. The methionine residue served as an internal standard. [^{35}S]Radioactivity was always released only at cycle 1 and is not included on the histograms. Leucine was the most abundant amino acid and was released at cycles 4, 7, 8, 9, 12, 13, 17, 22, and 28. Glutamic acid or glutamine residues were released at cycles 19, 21, 23, and 27, lysine at cycle 2, and valine at cycle 10 and, in an extended sequence analysis (not shown), also at cycle 24. The amino-termini of the mature protein and cell-free product are compared in **Table 3.** Overlapping areas of sequence are boxed. The sequence of plasma apoE begins at residue 19 of the primary translation product. From these comparisons we concluded that the cell-free product that was immunoprecipitated by our monospecific antibody and bound by heparin-Sepharose was, in fact, a precursor of plasma apoE. This precursor contained an 18 amino acid amino-terminal extension that was not represented in the plasma protein.

Processing of the apoE translation product by canine microsomes

When added to cell-free translation systems during translation, canine pancreas microsomes efficiently remove the signal peptide of all secreted proteins without removing propeptides. A cotranslational cleavage assay (47) was utilized to determine if the entire amino-terminal oligopeptide extension on the apoE precursor was a signal

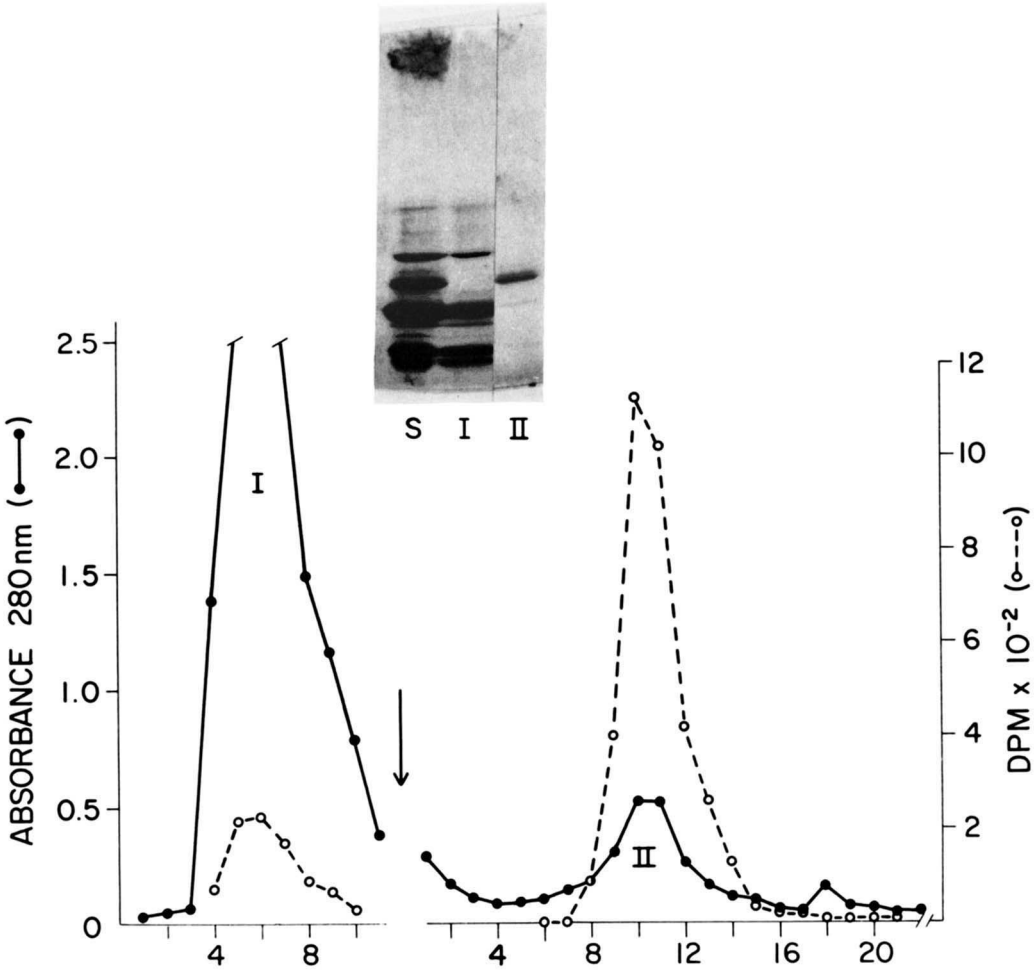

Fig. 3. Heparin-Sepharose 4B affinity chromatography of the apoE translation product. Purified [^{3}H]leucine-labeled apoE translation product was combined with 1 mg of solubilized apoHDL. The sample was applied to a 10 cm × 0.7 cm column of heparin-Sepharose 4B equilibrated with 0.12 M NaCl in column buffer (0.002 M Na phosphate, pH 7.4, and 0.01% Na azide). Five ml of equilibration buffer was passed through the column before an 18-ml linear salt gradient from 0.05 to 1.5 M NaCl in column buffer was started (arrow) to elute the adsorbed proteins. Fractions (0.5 ml) were collected throughout. Absorbance at A_{280} nm was monitored (●——●) and 0.2-ml aliquots of each fraction were counted for radioactivity (○- - -○). Aliquots from the highest absorbing fraction in both protein peaks (I and II) were analyzed by electrophoresis on 5–22.5% polyacrylamide-SDS gels, along with an aliquot of the unfractionated HDL (S).

sequence or if it contained a "pro" in addition to a presegment.

Canine microsomes prepared by the method of Scheele et al. (43) inhibited the translational activity of the wheat germ lysates used in this study by 60 to 70%. Similar inhibition of wheat germ translational activity by canine microsomes has been observed by others (43, 48). Due to the high degree of inhibition of protein synthesis, the processing of the apoE translation product by canine microsomes was examined with rabbit reticulocyte lysates. The predominant polypeptide isolated by immunobinding with anti-apoE from reticulocyte lysates stimulated with rat liver RNA was identical in size to that isolated from wheat germ assays (**Fig. 5**). The major difference was that fewer lower molecular weight proteins were isolated from reticulocyte lysates. This was to be expected if the lower molecular weight proteins were products of premature termination of translation, which is less frequently encountered with reticulocyte lysates. The addition of canine microsomes to reticulocyte lysates had a slight (twofold) stimulatory effect on translational activity.

Canine microsomes appeared to cotranslationally remove the signal peptide of the apoE translation product. As illustrated in Fig. 5, the apoE product synthesized in the presence of canine microsomes (lane B) had a slightly faster mobility on SDS-polyacrylamide gels and lower ap-

TABLE 2. Amino-terminal sequence analysis of rat plasma apoE obtained from HDL

Cycle Number	pmol Phenylthiohydantoin Amino Acid Recovered/Cycle[a]								Assigned Residue
	Asp	Glu	Gly	Leu	Pro	Gln	Thr	Val	
1	—	[875][b]	170	151	—[c]	—	—	52	Glu
2	—	230	[920]	—	—	—	—	—	Gly
3	—	[744]	103	—	—	—	—	—	Glu
4	—	197	—	[646]	—	—	—	—	Leu
5	—	[587]	—	—	—	—	—	—	Glu
6	—	—	—	—	—	—	—	[574]	Val
7	—	—	—	—	—	—	[84]	—	Thr
8	[414]	—	—	—	—	—	—	—	Asp
9	130	91	—	—	—	[207]	—	—	Gln
10	—	74	—	[351]	—	—	—	—	Leu
11	—	—	—	—	[90]	—	—	—	Pro
12	—	—	[397]	—	—	—	—	—	Gly
13	—	106	—	—	—	[187]	—	—	Gln
14	54	—	—	—	—	—	—	—	X
15	[264]	—	—	—	—	—	—	—	Asp

[a] The amount of PTH-amino acid recovered was calculated by integrating the area under a peak and comparing it to that obtained from known quantities of PTH-amino acid standards.

[b] Boxes are included to indicate amino acid assignments made for each cycle of Edman degradation.

[c] Less than 50 pmol of PTH-amino acid detected.

parent molecular weight than the translation product synthesized in the absence of microsomes (lane A). The putative incomplete translation product of apoE migrating at M_r = 10,000 is also processed by canine microsomes (lane B, Fig. 5). This is compatible with processing at the N-terminus. Processing of the translation product was a cotranslational event; addition of microsomes after translation was complete produced no change in the mobility of the translation product (lane C). This cleavage reaction was examined in greater detail by sequencing immunoreactive, cotranslationally cleaved apoE labeled with [^{3}H]leucine and [^{35}S]methionine. The [^{3}H]leucine-labeled, processed protein yielded peaks of radioactivity at cycles 4 and 10 (**Fig. 6**). This distribution of leucine residues differed completely from that observed in the intact primary translation product (see Fig. 4) but corresponded exactly to the leucines at positions 4 and 10 of plasma apoE (Table 2). Thus the entire 18 amino acid amino-terminal extension was removed by microsomal signal peptidase. The cleavage reaction was quite efficient; based on the recovery of [^{35}S]methionine at cycle 1 of the cleaved peptide, it appeared that 94% was cotranslationally processed. Thus, based on this in vitro assay, nascent apoE protein is not processed through a propeptide intermediate.

Endoglycosidase H

In addition to removing the signal peptide, canine microsomes also have the capacity to transfer the oligosaccharide precursor from dolichol phosphate to asparagine residues in glycoproteins yielding an N-linked glycoside (49) with an endoglycosidase H-sensitive bond. In vivo this oligosaccharide precursor is transferred to the nascent polypeptide in the RER (50).

Since the nature of the glycosidic linkages in plasma apoE is unknown, we treated the purified microsome-processed translation product with endoglycosidase H to determine if it contained an N-linked oligosaccharide precursor (**Fig. 7**). Endoglycosidase H treatment of the processed translation product did not change the electrophoretic mobility of the protein on SDS-polyacrylamide gels. On the other hand, ovalbumin, which contains one endoglycosidase H-sensitive oligosaccharide linkage (51), migrated in this electrophoretic system as a smaller protein following enzymatic treatment. Therefore, it appears that the early biosynthetic precursor of apoE does not contain an endoglycosidase H-sensitive N-linked oligosaccharide precursor.

DISCUSSION

The initial synthetic product of most secretory proteins contains an amino-terminal signal peptide. The signal peptide initiates the transfer of the growing nascent secretory protein into the lumen of the RER and then is rapidly removed by a membrane-associated protease, even before the synthesis of the protein is completed. In the

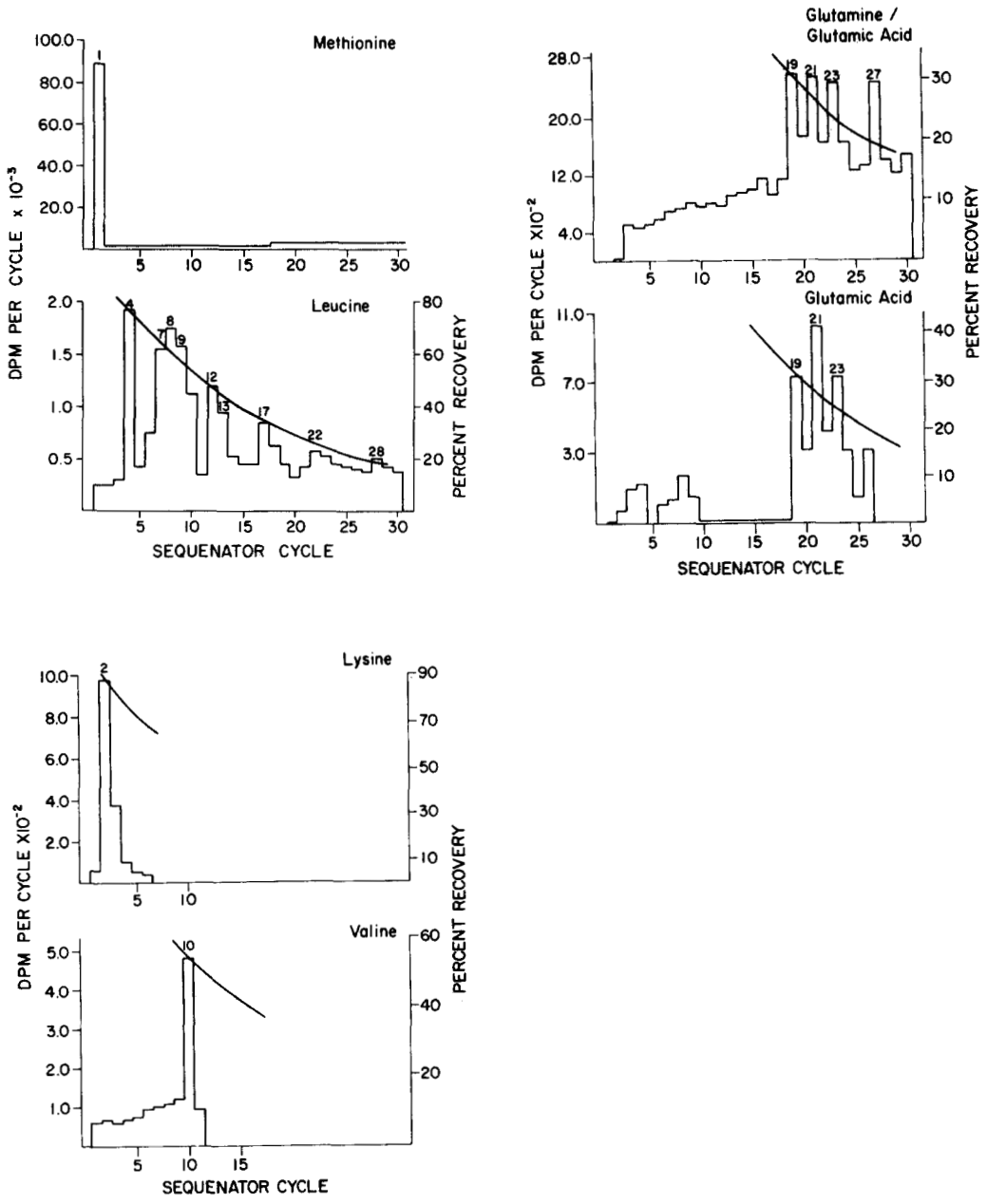

Fig. 4. Radiochemical sequencing of the primary translation product of apoE. The primary translation product of apoE mRNA was synthesized in wheat germ cell-free translation assays containing [^{35}S]methionine and the indicated [^{3}H]amino acid. With the exception of [^{3}H]leucine, the radiolabeled apoE translation products were isolated from wheat germ systems primed with RNA enriched for apoE mRNA prepared by anti-apoE immunoprecipitation of rat liver polysomes. With leucine, total cytoplasmic rat liver RNA was translated. The primary translation product was purified by immunobinding and electroelution from preparative SDS-polyacrylamide gels and subjected to automated sequential Edman degradation as described in Materials and Methods. The percent recovery is a theoretical curve based on 94% repetitive yield and normalized to the first radioactive peak. The amount of radiolabeled preapoE loaded onto the sequenator was: [^{35}S]methionine, 131,000 dpm; [^{3}H]leucine, 124,000 dpm; [^{3}H]glutamic acid and glutamine, 300,000 dpm; [^{3}H]valine, 22,500 dpm; [^{3}H]lysine, 23,500 dpm; and [^{3}H]glutamic acid, 110,000 dpm.

RER and the Golgi, the nascent secretory proteins may be subjected to further post-translational modifications or processing before they are discharged from the cell (52).

The presence of a signal peptide on an in vitro synthesized translation product is usually recognized by its slower electrophoretic mobility in SDS-polyacrylamide gels compared to the mature protein. We observed that plasma apoE and its translation product (Fig. 1) had similar electrophoretic mobility, raising the possibility that apoE may be initially synthesized without an amino-terminal signal peptide. Apoproteins differ from other secretory

TABLE 3. The amino terminal sequence of plasma apoE and the apoE translation product

	−18	−15	−10	−5	−1	+1	+5	+10
Translation product:	Met Lys	X[a] Leu	X X Leu Leu Leu Val	X Leu Leu	X	Glu / Glu / Lys	X Glu Leu Glu Val / Gly Glu Leu Glu Val / Val Gln Ala Val Glu	X Thr Asp Gln Glx / Glu Thr Glu Leu X / Pro Pro Glu Leu Asp
Rat HDL apoE								Pro Gly Gln X Asp
Human plasma apoE[b]								Glu Pro Glu Leu Arg

[a] X indicates that no assignment was made.
[b] From reference 9.

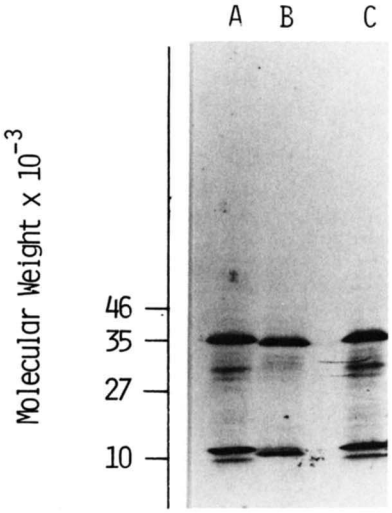

Fig. 5. Cotranslational cleavage of preapoE by canine microsomes. Total rat liver RNA was translated in rabbit reticulocyte lysates containing [^{35}S]methionine in the absence or presence of 20 A_{280} units per ml of micrococcal nuclease-treated canine microsomes and the translation products were immunobound, washed, and prepared for electrophoresis as described in Materials and Methods. The immunobound proteins were analyzed by electrophoresis on 5–22.5% polyacrylamide-SDS slab gel and the gel was fluorographed at −40°C. No microsomes (A), microsomes present during translation (B), and microsomes added after translation was terminated (C).

proteins in that they are secreted mainly as components of larger multimolecular complexes. Intracellular processing for these proteins also involves association with specific lipid complexes and other apoproteins to form the nascent lipoproteins that are secreted from the cells. The lipid binding determinants of apoproteins reside in amphipathic helices containing both a polar and nonpolar face (53). These proteins are capable of spontaneously associating with lipids, especially phospholipids, in vitro to form stable apoprotein-lipid complexes (54). The possibility then existed that the signal for the translocation of this protein into the lumen of the RER may reside in the hydrophobic sequence or amphipathic helices of mature apoE.

However, comparison of the amino terminal sequences of the intact apoE translation product and its cotranslationally cleaved derivative with the amino-terminal sequence of the plasma apoprotein (Table 3) demonstrated that apoE is indeed initially synthesized with an 18 amino acid signal peptide. Although the sequence of the apoE signal peptide has been determined only in part, it is apparent that it contains a positively charged amino terminus and a hydrophobic, leucine-rich central core region which are characteristic of signal peptides reported for many other secretory proteins (22). Since we did not include small side chain amino acids in our radiochemical sequencing, few of the amino acids in the carboxy-terminal region of the amino-terminal extension were identified.

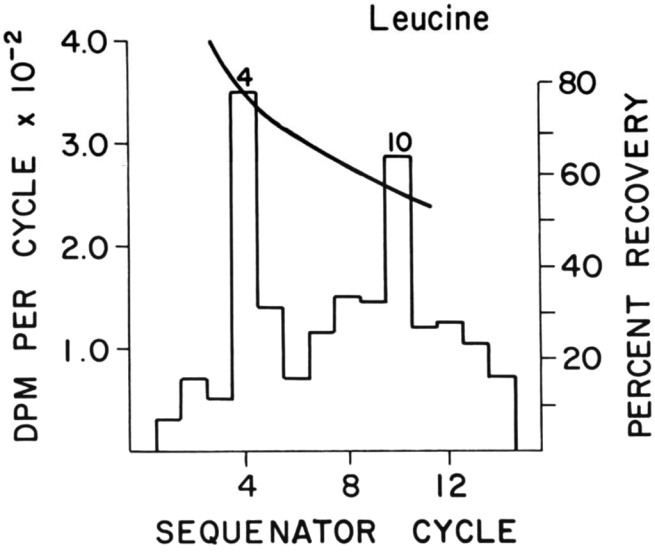

Fig. 6. Radiochemical sequencing of the microsome-processed apoE translation product. RNA enriched for apoE mRNA by immunoprecipitation of rat liver polysomes was translated in wheat germ cell-free translation assays containing 20 A_{280} units per ml of micrococcal nuclease-treated canine microsomes and [^{35}S]methionine and [^{3}H]leucine. The microsome-processed translation product was isolated and subjected to automated sequential Edman degradation as described in the legend to Fig. 4; 37,800 dpm of [^{3}H]leucine and 31,000 dpm of [^{35}S]methionine were applied to the sequenator. Only the leucine results are shown.

Our partial amino acid sequence is entirely consistent with that inferred from the just reported apoE cDNA sequence nucleotide (55). From this latter sequence alanine, as is frequently the case, precedes the signal peptide cleavage site.

In the past three years, several apoproteins have been reported to contain amino-terminal signal peptides (45, 56–59). In all cases the translation products have been shown by SDS-polyacrylamide gel electrophoresis to have a larger apparent molecular weight than the mature protein. Gordon et al. have demonstrated that rat (45) and human (56) intestinal preapoA-I contained an 18 amino acid signal peptide and rat intestinal preapoA-IV (57) contained a 20 amino acid signal peptide. Cockerel preapoVLDL was shown by Chan, Bradley, and Means (58) to contain a 23 amino acid signal peptide. The presence of signal peptides on the translation products of apoproteins indicates that the initial events involved in apoprotein biosynthesis and vectorial transfer are the same as for most other secretory proteins.

Very little amino acid sequence identity has been observed among the signal peptides of secretory proteins, not even among functionally related proteins derived from the same species and secretory tissue (60). On the other hand, intestinal preproapoA-I and preapoA-IV appear to be unique in possessing within their signal peptides a fairly high degree of sequence identity (57), which suggests the possibility that apoprotein signal peptides may share common features that in some way may initiate the targeting of apoproteins to the site or sites of lipoprotein assembly. In the rat, apoE, like apoA-I and apoA-IV, is associated with HDL (61). It was of interest, therefore, to compare the partial sequence of the hepatic preapoE signal peptide with that of intestinal preproapoA-I from the same species in order to determine the extent and possible areas of amino acid sequence identity between them. This comparison revealed very little sequence identity, only 17%, and essentially none within the hydrophobic core. The core regions of the intestinal A preapoproteins were relatively poor in leucine and rich in valine and alanine, while that of preapoE was rich in leucine and poor in valine (alanine was not determined). The sequence of the signal peptide inferred from the complete cDNA sequence (55) reveals additional positions of amino acid identity for a total of 39% identity (7 of 18 residues). The identical residues are all at the two termini of the signal peptide. Strikingly, five of the six C terminal residues are identical for apoE and apoA-I signal peptides.

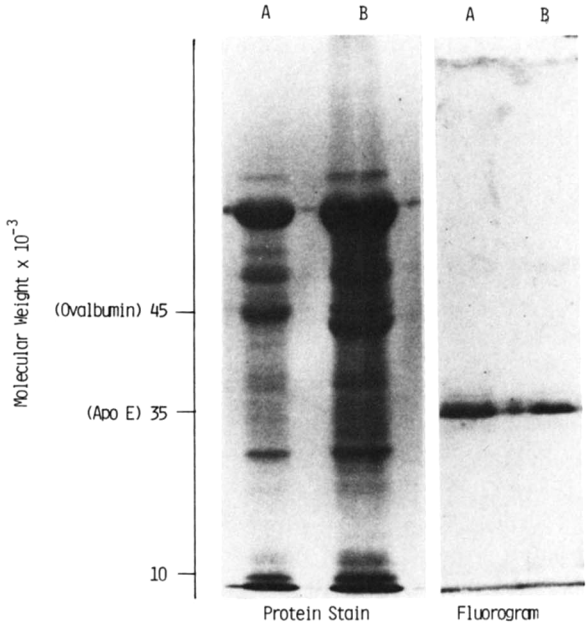

Fig. 7. Endoglycosidase H digestion of microsome-processed apoE translation product. In a total volume of 0.062 ml, purified microsome-processed apoE translation product and ovalbumin (10 μg) were digested with endoglycosidase H (46). The protein samples were heat-denatured at 95°C for 5 min in the presence of 2% SDS and 5% 2-mercaptoethanol, and adjusted to contain 30 mM sodium citrate, pH 5.0, 1 mM PMSF, 800 units Trasylol, and 0.03 units of endoglycosidase H. The samples were incubated for 18 hr at 37°C, precipitated on ice with 12.5% TCA, dissolved in SDS-PAGE sample buffer, and analyzed by electrophoresis on 9–13% polyacrylamide-SDS slab gel. Ovalbumin (Coomassie Blue stain) and the microsome-processed translation product (fluorogram) were incubated in the absence (A) and presence (B) of endoglycosidase H.

Gordon et al. (45, 56) have determined that rat and human intestinal apoA-I, but not rat apoA-IV, are initially synthesized with an unusual hexapeptide propeptide that is apparently removed only after secretion (56). No propeptide was observed on the microsome-processed translation product of hepatic apoE. This suggests that the propeptide may serve a special purpose related to the biosynthesis, assembly into lipoproteins, or physiological function of apoA-I that is either not shared by other apoproteins or is served by an analogous peptide embedded in the mature apoprotein sequence, similar to the internal signal equivalent of ovalbumin (62).

Differences between the glycosylation of the primary translation product and apoE may explain their comigration on SDS-polyacrylamide gels. The molecular weight of the carbohydrate portion of plasma apoE was estimated from the individual sugar residues (4) to be approximately 2100. From the known amino acid composition of the signal peptide, the molecular weight of the signal peptide was estimated to be approximately 2300. Because of the similar molecular weights, maturation of the apoE translation product involving the cleavage of the signal peptide and the attachment of the carbohydrate residues may not result in a significant change in the molecular weight of the protein. The translation product processed by canine microsomes has a faster mobility than either the unprocessed primary translation product or the mature protein. The similarity in electrophoretic mobility of rat apoE and apoE primary translation product has been noted by others (63) and is not unique to the rat. This has been observed also in the guinea pig (21), the rhesus monkey (64) and baboon.[5]

The absence of an endoglycosidase H-sensitive oligosaccharide precursor on the microsome-processed apoE translation product suggests that the carbohydrate moieties are attached to plasma apoE via O- rather than N-glycosidic linkages and involves the addition of individual sugar residues rather than the addition and modification of an oligosaccharide precursor (50). We cannot account for the difference between our results and those of Lin-Lee, Bradley, and Chan (63) who suggested that the translation product processed by canine microsomes was sensitive to endoglycosidase H. Rall et al. (11) have suggested that human apoE contains O-glycosidic linkages. N-glycosylation involves asparagine residues in a particular tripeptide sequence which is absent from the sequences of human (11) and rat (55) apoE. Two other observations from our laboratory argue that N-glycosylation is probably not involved in apoE processing.[6] The intrahepatic apoE precursor found in the rat liver RER is smaller than mature apoE, which would not be the case if the precursor were N-glycosylated. Neither the size nor the amount of apoE secreted by the perfused rat liver was altered by the addition of tunicamycin, which blocks N-glycosylation.

Indirect immunoprecipitation in wheat germ cell-free translation systems indicated that apoE mRNA represented approximately 1% of total rat liver mRNA which is similar to that reported earlier by us (65) and others (63, 66). A similar figure has been found in the guinea pig (21) while that for two monkeys (rhesus and cynomolgus) was about half as much (64). This value can only be regarded as an estimate. It is based upon determining the amount of immunoprecipitable apoE synthesized in the mRNA-dependent cell-free translation systems relative to the synthesis of other liver proteins. Therefore, it only measures translatable mRNAs and is further limited by differences in the efficiency of reinitiation and translation of the mRNAs, stability of the mRNAs, and by the completeness of immunoprecipitation. Nevertheless, this value is in fairly good agreement with other observations in our laboratory which indicated that approximately 0.7% of total hepatic polysomes were immunoprecipitated by anti-apoE and anti-antibody. More accurate determinations of apoE mRNA levels in normal and pathological states could be calculated from Rot curves using the cloned DNA complement of apoE mRNA.

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