Extrahepatic synthesis of apolipoprotein E

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Abstract Apolipoprotein E (apoE) synthesis has been examined in rat and guinea pig tissues using in vitro translation and [^{35}S]methionine labeling of tissue slices. A number of tissues not involved in lipoprotein synthesis synthesize a protein very similar to apoE, including the spleen, adrenal, kidney, testis, ovary, heart, and lung. Although the intestine is involved in lipoprotein synthesis, apoE synthesis could not be detected in intestinal mucosa. The protein synthesized by the extrahepatic tissues was identified as apoE by its electrophoretic mobility, its immunologic reactivity with a monospecific antibody and by limited proteolysis mapping with *Staphylococcus aureus* V8 protease. ApoE represented between 0.02 and 0.7% of the total protein synthesized in the extrahepatic tissues, indicating that apoE mRNA is a fairly abundant mRNA in these tissues. ApoE mRNA was also detected by hybridization with a rat apoE cDNA clone, which hybridized to a single mRNA 1250 nucleotides in length in rat liver and in extrahepatic tissues. Hybridization of the apoE clone to rat genomic DNA demonstrated that the apoE gene was more heavily methylated in intestinal mucosa, which did not synthesize apoE, than in liver, testis, or kidney. ^{35}S labeling of peritoneal macrophages revealed that both rat and guinea pig macrophages synthesized and secreted apoE in vitro. Rhesus aortic smooth muscle cells also synthesized and secreted apoE. The possible functions of apoE synthesized in the peripheral tissues are considered.—**Driscoll, D. M., and G. S. Getz.** Extrahepatic synthesis of apolipoprotein E. *J. Lipid Res.* 1984. **25:** 1368–1379.

Supplementary key words lipoprotein • reverse cholesterol transport • in vitro translation • RNA hybridization • DNA methylation

Apolipoprotein E (apoE) is a 35,000 dalton glycoprotein found in several classes of the plasma lipoproteins (1, 2). ApoE is important because of its critical role in the recognition and uptake of lipoproteins from the plasma. This includes the mediation of lipoprotein binding, uptake, and cholesterol delivery via the B,E receptor, as well as the interaction of chylomicron remnants with the hepatic remnant receptor (3–5). Plasma apoE levels are increased in a variety of pathologic conditions including hypothyroidism, diabetes, type III hyperlipoproteinemia, and cholesterol feeding (6).

Given the important role of apoE in cholesterol metabolism, the regulation of the synthesis and secretion of apoE is physiologically important. Previous studies have shown that although the liver and intestine are the sites of lipoprotein biosynthesis (7, 8) apoE is synthesized predominantly in the liver (9). Recently, however apoE synthesis has been demonstrated in mouse macrophages (10, 11) and in tissue slices of human adrenal and kidney (12). In this study we have used [^{35}S]methionine labeling of tissue slices, in vitro translation, and hybridization of RNA with a rat apoE cDNA clone to identify a number of tissues in the rat and guinea pig which synthesize apoE. Hybridization of the apoE clone to genomic DNA demonstrated that the apoE gene is more heavily methylated in rat intestinal mucosa (a tissue that does not synthesize apoE) than it is in rat liver, kidney, and testis (all organs which are actively involved in the synthesis of apoE). In addition to macrophages, we have also identified another nonhepatic cell type, smooth muscle cells from rhesus aorta, that synthesizes and secretes apoE in vitro.

MATERIALS AND METHODS

Animals

Rats were purchased from Sprague-Dawley (Madison, WI) and Hartley guinea pigs were obtained from Locke-Erickson (Chicago, IL). Animals were maintained in a continuously lighted room and fed normal chow ad libitum. Antibodies were raised in a female goat or in New Zealand white rabbits.

Antibody preparation

Rat apoE was purified from plasma VLDL by Sepharose 6B column chromatography and by preparative gel electrophoresis (13), followed by heparin Sepharose 4B affinity chromatography (14). Antibody to rat apoE

Abbreviations: apo, apolipoprotein; VLDL, very low density lipoprotein; HDL, high density lipoprotein; SDS, sodium dodecyl sulfate; DMEM, Dulbecco's Modified Eagle's Medium; PMSF, phenylmethylsulfonylfluoride; DMSO, dimethylsulfoxide; SSC, standard saline citrate; BSA, bovine serum albumin; PVP, polyvinylpyrrolidone; bp, base pairs.

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was prepared in a female goat and affinity-enriched by adsorption of the immune plasma to a rat HDL_3-Sepharose 4B column (13). The antibody was monospecific for apoE as determined by double immunodiffusion and by immunodecoration of electrophoretically separated apolipoproteins transferred to nitrocellulose paper (15). Preimmune serum was purified by ammonium sulfate fractionation and chromatography on a tandem DEAE-cellulose/carboxy-methylcellulose column (16).

Immunodecoration of proteins transferred to nitrocellulose

Cross-reactivity of the goat anti-rat apoE antibody with guinea pig and rhesus monkey apoE was determined by immunodecoration of electrophoretically separated apolipoproteins from these species transferred to nitrocellulose paper by the method of Towbin, Staehelin, and Gordon (15). The nitrocellulose paper was incubated with the monospecific goat anti-rat apoE antibody and then with peroxidase-coupled Fab fragments of rabbit anti-goat IgG. The color reaction was performed as described (15) except that 4-chloro-1-naphthol was used as the chromogenic substrate.

RNA isolation

Tissues were frozen in liquid nitrogen and RNA was extracted from 1-gram pieces using guanidine thiocyanate and guanidine hydrochloride according to Chirgwin et al. (17). Poly A RNA was isolated using Poly U Sepharose chromatography (13).

In vitro translation

Rabbit reticulocyte lysate was prepared from phenylhydrazine-injected rabbits and treated with micrococcal nuclease (18). Total RNA (3 μg) or poly A RNA (0.18 μg) was translated in a 22-μl assay mixture containing 75 mM KCl, 1 mM MgOAc, 10 mM creatine phosphate, 2 mM dithiothreitol, 20 mM HEPES, pH 7.4, 0.5 mM spermidine, 0.2 mM GTP, 0.06 mM ATP, 0.06 mM unlabeled amino acids, and either 5 μCi of [^{3}H]leucine or 3 μCi of [^{35}S]methionine. Translation was terminated by chilling on ice and adjusting the assay mixture to 10 mM methionine, 10 mM Na phosphate, pH 7.4, 15 mM NaCl, and 1% triton-deoxycholate. Total protein synthesis was determined by TCA precipitation after decolorization with hydrogen peroxide (18).

Immunoprecipitation of translation products

Translation products were incubated for 1 hr with 20 μg of preimmune serum or with affinity-enriched goat anti-rat apoE. SDS was added to achieve a final concentration of 0.2% and the immunoprecipitated translation products were isolated by binding to Protein A Sepharose in 10 mM Na phosphate, pH 7.4, 15 mM NaCl, 10 mM methionine, and 1% triton-deoxycholate (buffer A) overnight at 4°C (19). The Sepharose beads were then washed 4 to 5 times with buffer A and the bound translation products were eluted by boiling for 2 min in 62.5 mM Tris, pH 6.8, 2% SDS, 5% mercaptoethanol. The samples were electrophoresed on an SDS/5–22% polyacrylamide gel (20). Rat and guinea pig VLDL were iodinated using iodine monochloride (21), immunoprecipitated, and electrophoresed to identify plasma apoE.

Peptide mapping

Iodinated plasma VLDL and apoE translation products were subjected to limited proteolysis mapping using *Staphylococcus aureus* V8 protease as described by Cleveland et al. (22).

Tissue slices

Tissue slices (10–30 mg wet weight) were incubated in 95% O_2, 5% CO_2 at 37°C for 60 min in 0.2 ml of DMEM containing 50 μCi of [^{35}S]methionine. The tissue slices were rinsed, homogenized in 20 mM Na phosphate, pH 7, 150 mM NaCl, 5 mM EDTA, 1% Triton-X 100, and 100 μg/ml PMSF, and centrifuged at 100,000 *g* (23). The supernatants from the tissue extracts were immunoprecipitated with the goat anti-rat apoE antibody. Because of nonspecific binding of both nonimmune and immune antibodies to proteins in the tissue slices, particularly to actin, a different method was used for immunoprecipitation. SDS was added to the extract to a final concentration of 3.3% and the samples were heated at 95°C for 2 min. The sample was diluted with buffer A so that the final SDS concentration was 0.2%. Twenty micrograms of either nonimmune globulin or goat anti-rat apoE antibody was added and incubated for 2 hr at room temperature. The immunoprecipitated proteins were isolated by binding to Protein A Sepharose as described above.

Cell cultures

Unstimulated peritoneal macrophages were isolated from rats or guinea pigs (24). The cells were incubated at 37°C for 2 hr at 2×10^6 cells/ml in serum-free DMEM in 95% O_2, 5% CO_2. After washing to remove nonadherent cells, methionine-free DMEM containing 40 μM methionine and 100 μCi/ml of [^{35}S]methionine was added for 16 hr. The medium was isolated and immunoprecipitated as described for the immunoprecipitation of apoE translation products. In some experiments the medium was pre-cleared with Protein A Sepharose prior to the addition of antibody to eliminate nonspecific precipitation of fibronectin by the nonimmune and anti-apoE globulin. Rhesus monkey aortic smooth muscle cells were obtained from outgrowth of explants from

the thoracic aorta as previously described (25). After four passages, the cells were labeled with [^{35}S]methionine as above.

Northern blots

A rat apoE cDNA clone (pALE124) was generously provided by Dr. J. Taylor (Gladstone Foundation, San Francisco, CA). This clone contains a 600 base pair insert of rat apoE cDNA cloned into the Pst I site of pBR322 (26). Plasmid DNA was isolated by alkaline lysis and purified on cesium chloride/ethidium bromide density gradients (27). Purified plasmid DNA was then digested with Pst I and electrophoresed in a 1% agarose gel to isolate insert DNA. To generate a radioactive probe for hybridization, insert DNA was eluted from the gel according to Maxam and Gilbert (28) and radiolabeled with a [^{32}P]dCTP using a nick translation kit (Bethesda Research Laboratories, Bethesda, MD).

Ten micrograms of total RNA or 0.5 μg of poly A RNA were denatured at 50°C for 60 min in 1 M glyoxal, 50% DMSO, and 10 mM Na phosphate, pH 7.0. The samples were electrophoresed in a 1% agarose gel in 10 mM Na phosphate, pH 7.0, and the gel was transferred to nitrocellulose in 20 × SSC (3 M NaCl, 0.3 M Na citrate) according to Thomas (29). After baking for 2 hr at 80°C under vacuum, the nitrocellulose sheet was pre-incubated for 16 hr at 42°C in 50% formamide, 5 × SSC, 50 mM Na phosphate, pH 6.5, 0.02% BSA, 0.02% PVP, and 0.02% Ficoll, 0.1% SDS, and 250 μg/ml of denatured salmon sperm DNA. The filters were hybridized at 42°C for 24–30 hr in 50% formamide, 5 × SSC, 20 mM Na phosphate, pH 6.5, 0.004% BSA, 0.004% PVP, 0.004% Ficoll, 0.1% SDS, 200 μg/ml of denatured salmon sperm DNA, and 10^5 cpm/ml of denatured ^{32}P-labeled probe. After hybridization, the filters were washed twice at room temperature in 2 × SSC, 0.1% SDS, and twice at 50°C in 0.1 × SSC, 0.1% SDS (29). The filters were air-dried and exposed to X-Omat AR-5 film with an intensifying screen at −80°C.

Southern blots of genomic DNA

Genomic DNA was isolated from rat liver, kidney, testis, and intestinal mucosa as described by Blin and Stafford (30). Ten to twenty micrograms of genomic DNA were digested with various restriction enzymes using the conditions suggested by the manufacturer, and the DNA fragments were electrophoresed in 2.0% agarose gels. The gel was transferred to nitrocellulose paper by the method of Southern (31) and the nitrocellulose was baked at 80°C under vacuum. The nitrocellulose paper was pre-incubated at 37°C for 16 hr at 50% formamide, 5 × SSC, 50 mM Na phosphate, pH 6.5, 0.1% SDS, 0.02% BSA, 0.02% PVP, 0.02% Ficoll, and 300 μg/ml of denatured salmon sperm DNA. The sheets were then hybridized at 37°C for 48 hr in 50% formamide, 5 × SSC, 20 mM Na phosphate, pH 6.5, 0.1% SDS, 0.004% BSA, 0.004% PVP, 0.004% Ficoll, 200 μg/ml of denatured salmon sperm DNA, and 10^5 cpm/ml ^{32}P-labeled denatured DNA probe. The filters were washed twice at room temperature in 2 × SSC, 0.5% SDS, twice at room temperature in 2 × SSC, 0.1% SDS, and twice at 65°C in 0.1 × SSC, 0.5% SDS. The sheets were air-dried and exposed to film as described above.

Quantitation of radioautograms

For fluorography, gels were impregnated with En^{3}Hance and exposed to Kodak X-Omat AR-5 film with a DuPont Lightening Plus Intensifying Screen (32). Radioautograms were quantitated by three different methods. Bands were eluted from the dried gel in 30% hydrogen peroxide at 50°C and the amount of radioactivity was determined by scintillation counting as described by Young and Fulhorst (33). Alternatively, the bands on the film itself were cut out of the film and incubated in 1 M NaOH for 2 hr. The absorbance of the eluted silver grains was then determined at 500 nm (34). Gels and fluorograms were also scanned using an LKB scanning densitometer.

RESULTS

Cross-reactivity of goat anti-rat apoE antibody

We have previously characterized a monospecific antibody to rat apoE (35). To determine whether this antibody would cross-react with guinea pig and rhesus monkey apoE, lipoproteins from each of these species were electrophoresed on SDS-polyacrylamide gels and the proteins were transferred to nitrocellulose paper for immunodecoration with the monospecific antibody. **Fig. 1** compares the Coomassie Blue-stained gel with the immunoblot which was decorated with goat anti-rat apoE antibody. In addition to reacting with rat apoE (lanes b, m), the antibody also reacted with apoE from guinea pig (lanes c–h) and rhesus monkey (lanes i–l). The antibody did not react with any of the other apolipoproteins present in the lipoproteins, including apoB, apoA-I, apoA-II, apoA-IV, and the C-peptides. No immunologic reactivity was seen when the immunoblots were decorated with the nonimmune goat globulin. As has been previously noted for rat apoE (36), both guinea pig and rhesus monkey apoE often separate on electrophoresis as a broad band or as a doublet or triplet on SDS-polyacrylamide gels. The basis for this heterogeneity is not known.

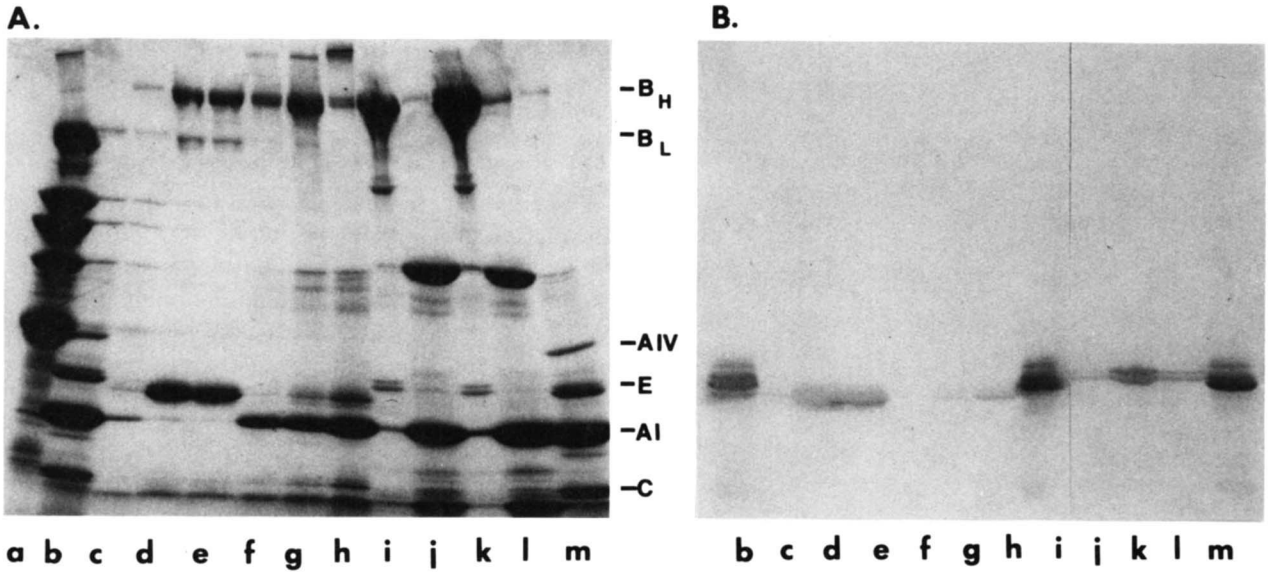

Fig. 1. Cross-reactivity of goat anti-rat apoE antibody. Lipoproteins were transferred to nitrocellulose paper from an SDS/5–22.5% polyacrylamide gel for immunodecoration. The paper was incubated with monospecific goat anti-rat apoE antibody at 5 μg/ml and then with peroxidase-coupled rabbit anti-goat IgG. The color reaction was performed using 4-chloro-1-naphthol at 0.5 mg/ml in 0.015% H_2O_2. Fig. 1A, the Coomassie blue-stained gel after transfer; Fig. 1B, the immunoblot. Lane a (molecular weight markers), lanes b, m (rat HDL), lanes c–e (guinea pig VLDL), lanes f–h (guinea pig HDL), lanes i, k (rhesus LDL), lanes j, l (rhesus HDL). The electrophoretic mobilities of rat apolipoproteins are indicated.

Identification of the apoE translation product

Using a monospecific goat anti-rat apoE antibody, we have previously characterized the primary translation product of rat apoE (35). To identify the guinea pig apoE translation product, total rat and guinea pig liver RNAs were translated in an in vitro reticulocyte lysate system containing [^{35}S]methionine. The concentrations of MgOAc (1 mM) and KCl (75 mM) required in the translation mixture for optimal protein synthesis and for the optimal synthesis of apoE by rat and guinea pig liver RNAs were determined in initial experiments. Both total unfractionated and poly A RNA were translated with equal efficiency in the reticulocyte lysate system. Incorporation of radioactivity into TCA-precipitable proteins was linear with respect to RNA concentration from 0 to 3 μg of total RNA and 0 to 0.18 μg of poly A RNA per 22-μl assay.

After translation was terminated, the translation products were precipitated with either nonimmune globulin or the cross-reactive goat anti-rat apoE antibody and separated by electrophoresis. The fluorogram in **Fig. 2** compares the total (lane a) and immunoprecipitated (lane b) translation products of guinea pig liver RNA. A single band was immunoprecipitated by the anti-apoE antibody which had the same electrophoretic mobility as guinea pig plasma apoE (lane d). Guinea pig apoE is a 32,500 dalton protein, approximately 2500 daltons smaller than the rat apoE translation product (lane e) or rat plasma apoE (lane g). Nonimmune globulin failed to precipitate apoE from either guinea pig (lane c) or rat (lane f) translation products. The identity of the apoE translation products was confirmed by limited proteolysis mapping (see below).

To determine the relative abundance of the apoE translation products in the various tissues, each translation mixture was divided into three aliquots containing equal amounts of TCA-precipitable counts. One aliquot of the total translation products was reserved for electrophoresis. The other aliquots were incubated with either nonimmune globulin or goat anti-rat apoE as described and the three samples were then electrophoresed in parallel. Preliminary experiments established that the amount of anti-apoE antibody added to the total translation mixture synthesized by liver and extrahepatic RNAs was sufficient to precipitate all of the apoE translation product. The amount of radioactivity incorporated into the immunoprecipitated apoE translation product relative to the amount incorporated into total translation products was determined by two methods as described in Methods. Radioactive bands were cut out of the dried gel and the radioactivity was eluted from the gel slices with H_2O_2. The amount of radioactivity in each sample was then quantitated by scintillation counting. Alternatively, the bands on the radioautogram were cut out of the film and incubated in NaOH to

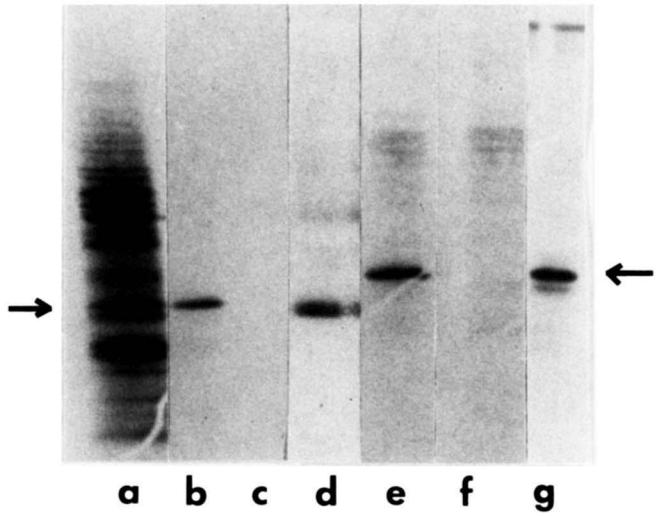

Fig. 2. Identification of guinea pig apoE translation product. Guinea pig or rat liver RNAs were translated in a reticulocyte lysate system in the presence of [^{35}S]methionine and the translation products were immunoprecipitated and analyzed by electrophoresis on a SDS/5–22% polyacrylamide gel followed by fluorography. The total guinea pig liver translation products are shown in lane a. The guinea pig (lane b) or rat (lane e) apoE translation products were precipitated using a goat anti-rat apoE antibody. Control precipitations with nonimmune globulin are shown in lanes c (rat) and f (guinea pig). Guinea pig (lane d) and rat (lane g) plasma VLDL were iodinated, immunoprecipitated, and electrophoresed to identify plasma apoE. The electrophoretic mobility of guinea pig apoE is indicated by the arrow on the left and of rat apoE by the arrow on the right.

elute the silver grains. The absorbance of the eluted silver grains was determined at 500 nm. Both methods of quantitation gave similar results. In four animals, apoE represented 1.02% (±0.22) of the total translation products synthesized by rat liver RNAs and 0.55% (±0.18) by guinea pig liver RNAs.

As has been observed previously for both rat and monkey apoE (35), the guinea pig apoE translation product unexpectedly comigrated with the mature plasma protein. This comigration was observed when proteins were electrophoresed on 10 or 15% polyacrylamide gels or on 5 to 22.5% or 10 to 22.5% polyacrylamide gradient gels (data not shown).

Extrahepatic synthesis of apoE

To identify extrahepatic sites of apoE synthesis in the normal rat and guinea pig, RNAs from different tissues were translated in vitro and the translation products were precipitated with either nonimmune globulin (**Fig. 3A**) or with the goat anti-rat apoE antibody (Fig. 3B). A single band which comigrated with plasma apoE was precipitated by the monospecific antibody from the total translation products synthesized by RNAs from rat liver (lane a), spleen (lane b), adrenal (lane c), kidney (lane d), testis (lane e), ovary (lane f), heart (lane g), and lung (lane h). The apoE translation product was undetectable in rat intestinal mucosa (lane i). Nonimmune globulin did not immunoprecipitate the apoE translation product in any of these tissues. In vitro translation of guinea pig RNAs demonstrated that these same tissues, except intestinal mucosa, also synthesize apoE in the guinea pig (data not shown).

The percentage of radioactivity incorporated into the apoE translation product was determined as described above and the results are summarized in **Table 1.** The apoE translation product represented between 0.02 to 0.7% of the total protein synthesized in these peripheral tissues. In both the rat and guinea pig, the apoE translation product was most abundant in the liver and kidney and undetectable in the intestinal mucosa. In similar experiments using a monospecific anti-rat apoA-I antibody, the apoA-I translation product was identified among the translation products of liver and intestinal RNAs, but was undetectable in any of the extrahepatic tissues of the rat or guinea pig (data not shown).

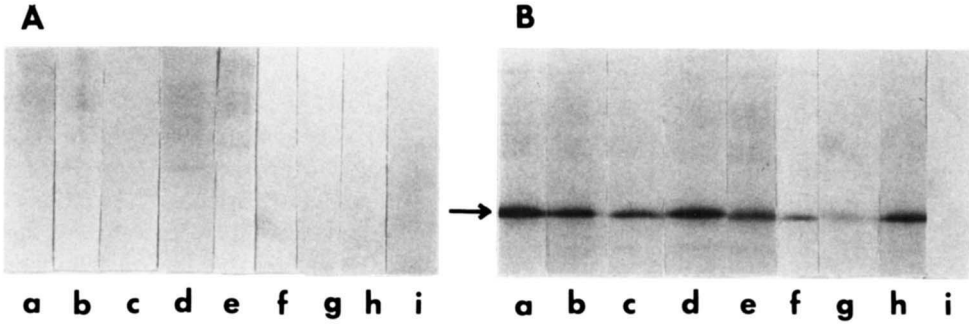

Fig. 3. ApoE translation products specified by RNAs from extrahepatic tissues. RNAs from rat tissues were translated in rabbit reticulocyte lysate in the presence of [^{35}S]methionine and the translation products were incubated with either nonimmune (Fig. 3A) or goat anti-rat apoE globulin (Fig. 3B). The precipitated proteins were electrophoresed on a SDS/5–22% polyacrylamide gel and fluorographed. The position of rat plasma apoE is indicated by the arrow. Lane a (liver), lane b (spleen), lane c (adrenal), lane d (kidney), lane e (testis), lane f (ovary), lane g (heart), lane h (lung), and lane i (intestinal mucosa).

TABLE 1. Abundance of apoE in translation products specified by rat and guinea pig RNAs[a]

Tissue	% of Total Protein Synthesis	
	Rat	Guinea Pig
Liver	0.85–1.12	0.43–0.62
Kidney	0.59–0.73	0.33–0.57
Spleen	0.37–0.49	0.04–0.12
Lung	0.40–0.46	0.11–0.17
Testis	0.19–0.36	0.29–0.37
Adrenal	0.12–0.25	0.16–0.20
Ovary	0.07–0.14	0.09–0.21
Heart	0.02–0.08	0.01–0.03
Intestinal mucosa	N.D.[b]	N.D.

[a] RNAs were translated in a reticulocyte lysate system containing [^{35}S]methionine and the translation products were incubated with nonimmune or goat anti-rat apoE globulin. The total and immunoprecipitated translation products were analyzed by SDS-polyacrylamide gel electrophoresis and fluorography as described in Methods. The results are expressed as the percentage of radioactivity incorporated into the apoE translation product relative to the amount incorporated into total protein. This was determined by measuring radioactivity in gel slices by scintillation counting or by the elution of silver grains from the radioautogram as described in Methods. The range of results represents duplicate experiments on RNAs from two animals.

[b] N.D., not detectable.

Synthesis of apoE by tissue slices

To determine whether the apoE mRNA detected by in vitro translation is translated in tissues, tissue slices were labeled with [^{35}S]methionine. Incorporation of radioisotope into TCA-precipitable protein was linear for 60 min. Following incubation, the tissue slices were homogenized and the tissue extracts were reacted with the nonimmune globulin or the goat anti-apoE antibody. Preliminary experiments demonstrated that several cell proteins, particularly actin, were precipitated from the tissue extracts by both the nonimmune and anti-apoE globulins under the conditions used to immunoprecipitate the apoE translation product. When tissue extracts were first boiled in 3.3% SDS before immunoprecipitation as described in Methods, the nonspecific precipitation of these proteins was reduced although not completely eliminated.

Fig. 4A illustrated the electrophoretic profile of the total ^{35}S-labeled cellular proteins from the liver (lane a), adrenal (lane b), kidney (lane c), testis (lane d), and ovary (lane e). Many of the proteins synthesized in the tissue slices are of higher molecular weight than the proteins synthesized in the in vitro translation system. This is to be expected, however, since proteins are not glycosylated or modified post-translationally in the reticulocyte lysate system unlike in the tissue slices. In addition, large molecular weight RNAs may not be efficiently translated in the reticulocyte lysate system. A relatively prominent protein, which comigrated with plasma apoE and which was immunoprecipitated by the goat anti-apoE antibody, was detected in tissue slices from all five tissues (Fig. 4B). Although both the nonimmune and anti-apoE antibody still precipitated actin, the nonimmune antibody failed to immunoprecipitate apoE (Fig. 4C).

The percentage of radioactivity incorporated into apoE in the tissue slices was determined as described above. ApoE represented approximately 0.52% of the total protein synthesized in the liver, 0.13% in adrenal, 0.49% in kidney, 0.16% in testis, and 0.09% in the ovary.

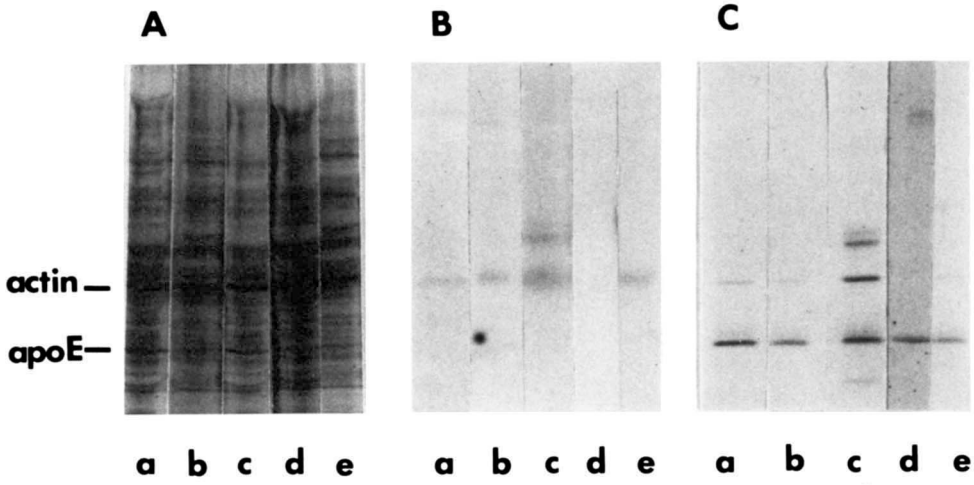

Fig. 4. ApoE synthesis by tissue slices. Guinea pig tissue slices were labeled in vitro with [^{35}S]methionine, and the tissue extracts were subjected to electrophoresis on a SDS/5–22% polyacrylamide gel and fluorography. The total radiolabeled proteins are shown in Fig. 4A from the liver (lane a), adrenal (lane b), kidney (lane c), testis (lane d), and ovary (lane e). These tissue extracts were reacted with either nonimmune globulin (Fig. 4B) or with goat anti-rat apoE (Fig. 4C). The electrophoretic mobilities of actin and plasma apoE are indicated.

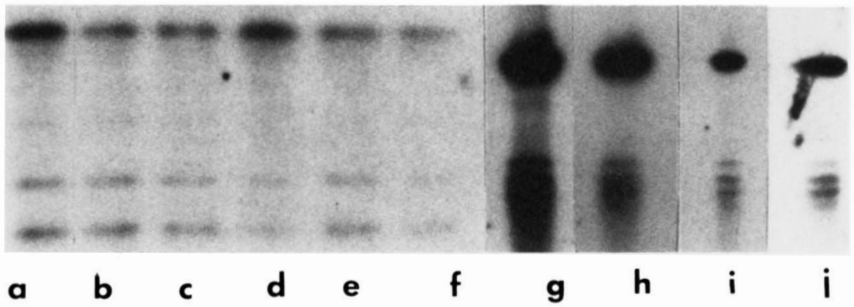

Fig. 5. Limited proteolysis mapping of apoE translation products. Proteins were immunoprecipitated with the goat anti-rat apoE antibody. After electrophoresis on a SDS/5–22% polyacrylamide gel, the appropriate bands were cut out of the gel and digested with *Staphylococcus aureus* V8 protease. The digested proteins were electrophoresed on a SDS/15% polyacrylamide gel and fluorographed: iodinated rat VLDL (lane a) and rat translation products specified by RNAs from liver (lane b), adrenal (lane c), testis (lane d), kidney (lane e), and spleen (lane f); iodinated guinea pig VLDL (lane g) and guinea pig translation products specified by RNAs from liver (lane h), adrenal (lane i), and testis (lane j).

Peptide mapping

To confirm the identity of the protein detected in the extrahepatic tissues, *Staphylococcus aureus* V8 protease was used for limited proteolysis mapping as shown in **Fig. 5.** Identical cleavage patterns were generated from rat plasma apoE (lane a), and the apoE translation products from rat liver (lane b), adrenal (lane c), testis (lane d), kidney (lane e), and spleen (lane f). The cleavage pattern of the rat apoE differed from that of guinea pig apoE. Digestion of guinea pig plasma apoE (lane g) and the apoE translation products of guinea pig liver (lane h), adrenal (lane i), and testis (lane j) with V8 protease resulted in identical patterns.

Identification of apoE mRNA in extrahepatic tissues

These experiments demonstrated that a protein very similar or identical to apoE was synthesized by a number of extrahepatic tissues. To extend these observations, RNAs were probed by hybridization with a ^{32}P-labeled cDNA clone of rat apoE to identify apoE mRNA in these tissues. In **Fig. 6,** total RNAs from rat liver (lane a), spleen (lane b), adrenal (lane c), kidney (lane d), ovary (lane e), testis (lane f), lung (lane g), heart (lane h), and intestinal mucosa (lane i) were denatured with DMSO and glyoxal and electrophoresed in an agarose gel. The gel was transferred to nitrocellulose paper and the paper was hybridized to ^{32}P-labeled insert DNA from a rat apoE cDNA clone pALE124. In agreement with the results of MacLean, Fukazawa, and Taylor (26), the clone hybridized to a mRNA in rat liver that was 1250 nucleotides in length. The clone also hybridized to a single mRNA of the same size in all the extrahepatic tissues except intestinal mucosa. The relative amount of hybridizable apoE mRNA in extrahepatic tissues was compared to the amount in the liver by elution of the silver grains from the radioautogram as described in Methods and the results are summarized in **Table 2.** A standard curve was constructed using increasing concentrations of total rat liver RNA for electrophoresis and hybridization. The abundance of hybridizable apoE mRNA in the extrahepatic tissues ranged from 2% to 53% of that found in the liver.

ApoE synthesis in primary cultures

The only two cell types known to synthesize apoE are the hepatocyte (37) and the mouse macrophage (10, 11). To identify other cell types capable of synthesizing apoE, rat and guinea pig peritoneal macrophages and rhesus aortic smooth muscle cells were examined. The cells were incubated overnight in serum-free media containing [^{35}S]methionine. The medium was then isolated, reacted with the nonimmune globulin or goat anti-rat apoE antibody, and electrophoresed to identify

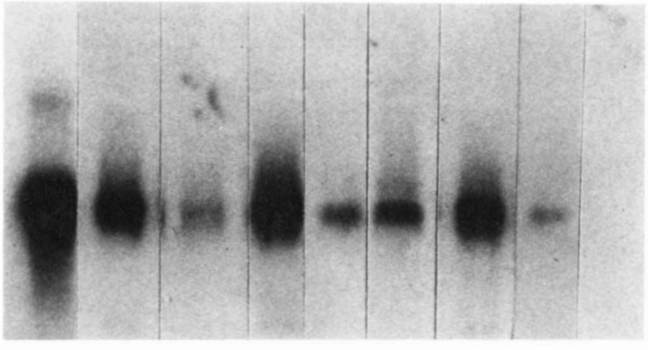

Fig. 6. Identification of rat apoE mRNA. Total RNA (5 μg) from rat tissues was denatured with DMSO and glyoxal, electrophoresed on a 1.0% agarose gel, and transferred to nitrocellulose paper (29). The paper was incubated with 10^5 cpm/ml of ^{32}P-labeled insert DNA from rat apoE cDNA clone pALE 124 as described in Methods. Lane a (rat liver), lane b (spleen), lane c (adrenal), lane d (kidney), lane e (ovary), lane f (testis), lane g (lung), lane h (heart), and lane i (intestinal mucosa).

TABLE 2. Relative abundance of hybridizable apoE mRNA in rat extrahepatic tissues[a]

Tissue	ApoE mRNA Abundance
Liver	100%
Kidney	47–58%
Lung	42–60%
Spleen	28–43%
Testis	12–26%
Ovary	9–18%
Adrenal	2–15%
Heart	4–11%
Intestinal mucosa	N.D.[b]

[a] RNAs (5 μg) were denatured with DMSO and glyoxal, separated by electrophoresis, and transferred to nitrocellulose paper. The nitrocellulose was incubated with 10^5 cpm/ml of ^{32}P-labeled DNA from rat apoE cDNA clone pALE124 and exposed to X-Omat AR5 film as described in the text. To determine the relative amounts of apoE mRNA in each tissue, the bands on the film corresponding to apoE mRNA were cut out and incubated in NaOH. The absorbance of the eluted silver grains was determined at 500 nm. The amount of apoE mRNA in each tissue is expressed relative to the amount in the liver. The range of results represents duplicate experiments on RNAs from two animals.

[b] N.D., not detectable.

newly synthesized and secreted apoE. Preliminary experiments established that the amount of anti-apoE antibody added was sufficient to precipitate all the apoE from macrophage and smooth muscle media (data not shown). The fluorogram in **Fig. 7** shows the total proteins secreted by normal rat (lane a) and guinea pig (lane f) macrophages. Preliminary experiments (lane b) demonstrated that both the nonimmune globulin and the anti-apoE antibody precipitated a 250,000 dalton protein, presumably fibronectin which is known to bind nonspecifically to Protein A (11). Preclearing of the medium with Protein A Sepharose prior to the addition of nonimmune globulin eliminated the nonspecific precipitation of this band (lanes c, g) and this step was included in subsequent experiments. The monospecific anti-apoE antibody precipitated a protein secreted by rat (lane d) and guinea pig (lane h) macrophages that comigrated with plasma apoE (lanes e, i). Macrophages from both animals secrete apoE, demonstrating that this property is not unique to the mouse (10, 11). Unlike the experiments done in the mouse however, these macrophages were not cholesteryl-ester enriched or extensively cultured prior to labeling, but instead were incubated overnight with isotopic precursor immediately after isolation. The percentage of radioactivity in the secreted protein that was incorporated into apoE was determined as described above. The apoE produced by both rat and guinea pig macrophages represented approximately 1% of the total secreted protein.

Fig. 8 shows that aortic smooth muscle cells isolated from the thoracic aorta of a rhesus monkey also synthesized and secreted apoE in culture. A very prominent

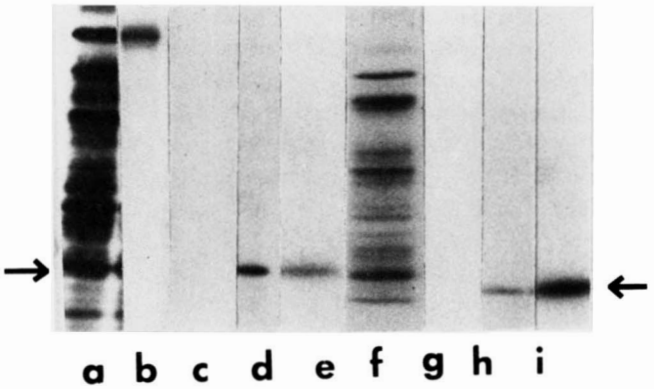

Fig. 7. ApoE synthesis by macrophages. Rat and guinea pig peritoneal marophages were isolated and cultured as described in Methods. The cells were labeled overnight with [^{35}S]methionine in serum-free medium. The medium was isolated, precipitated with either nonimmune or goat anti-rat apoE globulin, and electrophoresed on a SDS/5–22.5% polyacrylamide gel as described in Methods. Total secreted proteins of rat (lane a) and guinea pig (lane f) macrophages; proteins precipitated by nonimmune globulin from rat macrophages without (lane b) and after (lane c) preclearing with Protein A Sepharose and from guinea pig macrophages (lane g); proteins precipitated by anti-apoE antibody from rat (lane d) and guinea pig (lane h) macrophages; rat (lane e) and guinea pig (lane i) ^{125}I-labeled plasma apoE. The electrophoretic mobility of rat apoE is indicated by the arrow on the left and guinea pig apoE by the arrow on the right.

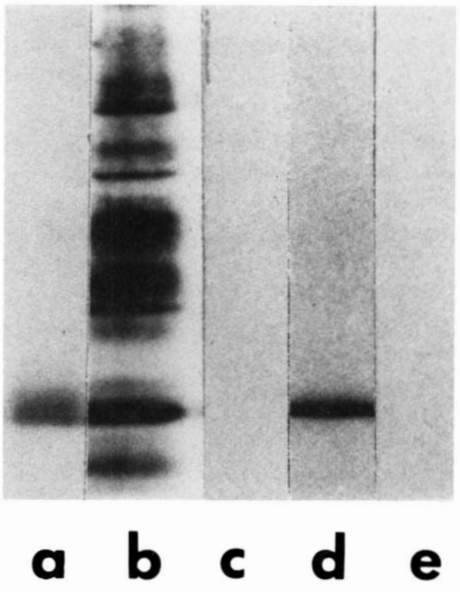

Fig. 8. ApoE synthesis by rhesus aortic smooth muscle cells. Smooth muscle cells were isolated from the thoracic aorta of a rhesus monkey and cultured as described in Methods. After labeling the cells in [^{35}S]methionine, the medium was isolated and precipitated with nonimmune or goat anti-rat apoE antibody. The proteins were analyzed by electrophoresis on a SDS/5–22.5% polyacrylamide gel followed by fluorography. Lane a, ^{125}I-labeled rhesus plasma apoE; lane b, total media proteins; lane c, proteins precipitated by nonimmune globulin; lane d, proteins precipitated by goat anti-rat apoE; lane e, proteins precipitated by goat anti-rat apoE in the presence of 50 μg of purified rhesus plasma apoE.

band was secreted into the media (lane b) that comigrated with plasma apoE (lane a) and reacted with the monospecific anti-rat apoE antibody (lane d). This antibody was previously shown to cross-react with rhesus plasma apoE (Fig. 1). This protein secreted by the smooth muscle cells was shown to be related to apoE by its inability to bind to the anti-apoE antibody in the presence of excess cold plasma apoE (Fig. 8, lane e). ApoE was not precipitated by the nonimmune globulin (lane c). Quantitation of the radioautogram as described in Methods demonstrated that apoE represented 5% of the total protein secreted from these cells. Experiments using monospecific antibodies to apoA-I or to apoB did not detect the synthesis of these two apolipoproteins by the rat or guinea pig macrophages or by the aortic smooth muscle cells (data not shown).

Methylation of the rat apoE gene

Every tissue examined synthesized apoE except intestinal mucosa. Because methylation of a gene is often inversely correlated with gene expression, the methylation pattern of the rat apoE gene was examined in a variety of tissues. In **Fig. 9,** genomic DNA was isolated from normal adult male rat liver (lane a), kidney (lane b), testis (lane c), and intestinal mucosa (lane d) and digested with the restriction enzymes MspI (Fig. 9A) or HpaII (Fig. 9B). These two enzymes are isoschizomers in that they both recognize the same nucleotide sequence (5′ CCGG 3′) but differ in their ability to digest this sequence if the internal C is methylated. MspI will cleave the sequence with a methylated C whereas HpaII will not. The digested DNA fragments were electrophoresed in agarose gels and transferred to nitrocellulose by Southern blotting. The nitrocellulose paper was then probed with ^{32}P-labeled cDNA insert from a rat apoE cDNA clone to identify apoE gene fragments. As shown in Fig. 9A, the clone hybridized to the same two DNA fragments (1450bp and 650bp) in MspI-digested DNAs from all four tissues. When these DNAs were digested with HpaII (Fig. 9B), the clone hybridized to these same two fragments as well as to a third fragment of 2100bp in the liver, kidney, and testis. However, only the 2100bp fragment was detected in DNA from intestinal mucosa digested with HpaII. When these DNAs were digested with other restriction enzymes such as EcoRI and BamHI, the restriction map of the apoE gene was identical in all four tissues. These results suggest that the apoE gene is more heavily methylated in the rat intestinal mucosa than in the other tissue since the HpaII digest of intestinal DNA only contained the 2100bp fragment. The methylation pattern of the apoE gene appeared to be heterogeneous in the liver, kidney, and testis, since all three fragments were detected in the DNAs from these tissues.

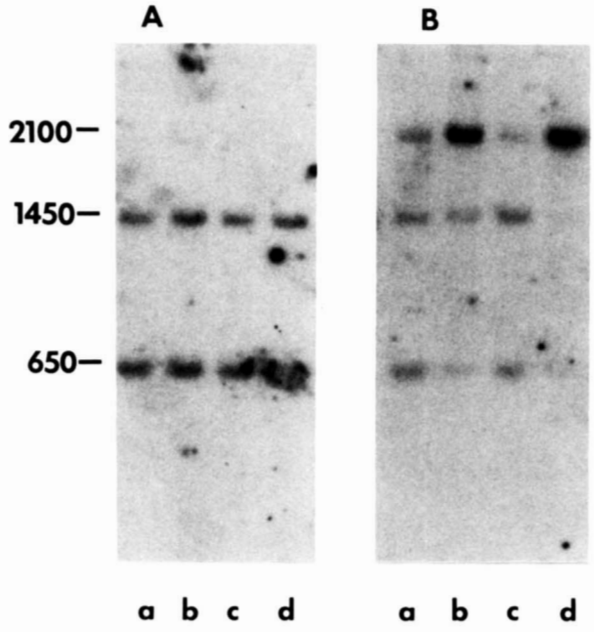

Fig. 9. Methylation of the rat apoE gene. Genomic DNAs were isolated from rat liver (lane a), kidney (lane b), testis (lane c), and intestinal mucosa (lane d). Twenty μg of DNA was digested with the restriction enzyme MspI (Fig. 9A) or HpaII (Fig. 9B) and then electrophoresed in a 2.0% agarose gel. After the gel was transferred to nitrocellulose paper, the nitrocellulose was probed with ^{32}P-labeled insert DNA from a rat apoE cDNA clone pALE 124 as described in Methods. The size of each fragment in base pairs is listed on the left.

DISCUSSION

These results demonstrate that a broad spectrum of peripheral tissues including the spleen, adrenal, kidney, testis, ovary, heart, and lung can synthesize appreciable amounts of apoE. Extrahepatic synthesis of apoE occurs both in the normal rat, in which apoE is the major plasma apolipoprotein, and in the normal guinea pig, which has extremely low levels of plasma apoE. The protein synthesized in the extrahepatic tissues is identical or remarkably similar to plasma apoE as judged by its electrophoretic mobility, its immunologic reactivity with a monospecific antibody, and by limited proteolysis mapping. In addition, apoE mRNA has been detected in these same tissues in the rat by hybridization with a rat apoE cDNA clone. The clone hybridized to a single mRNA of 1250 nucleotides in the liver and in the extrahepatic tissues. This is the size of the apoE mRNA in the rat liver reported by MacLean et al. (26).

Three techniques that essentially measure different phenomena (in vitro translation of purified RNAs, protein synthesis in tissue slices, and hybridization of a rat apoE cDNA clone to RNAs) were used to measure the

relative abundance of apoE mRNA in extrahepatic tissues. Translational control mechanisms and factors that influence the translational efficiency of a mRNA in vivo may be eliminated in the in vitro translation assay. The rat apoE clone will detect any hybridizable apoE mRNA independent of whether that mRNA is functional, i.e., translatable in vitro or in vivo. Despite the differences between these measurements, the ordering of the various extrahepatic tissues with respect to their apoE synthesis is similar whether determined by in vitro translation or by Northern hybridization (Tables 1 and 2). Although the relative abundance of apoE mRNA detected in a particular tissue may vary slightly depending on which of the three methods is used, it is clear that apoE mRNA is a fairly abundant mRNA in all the extrahepatic tissues, except intestinal mucosa.

A monospecific anti-rat apoE antibody was used in these studies to identify the primary translation product of guinea pig apoE, which comigrated with the mature plasma protein. Rat apoE is initially synthesized as a larger precursor containing an 18 amino acid signal peptide (35). However, in spite of the presence of this signal peptide, we (35) and others (38) have observed that the primary translation product of rat apoE has the same electrophoretic mobility as the plasma protein. This may be due in part to the fact that plasma apoE often migrates as a broad band or even a doublet or triplet on SDS-polyacrylamide gels (36). In addition, plasma apoE contains carbohydrate moieties not present in the primary translation product, which apparently balances the effect of the removal of the signal peptide on electrophoretic mobility (35). The similar electrophoretic mobility of plasma apoE and the apoE primary translation product in the guinea pig is presumably also accounted for by the post-translational removal of the signal peptide and addition of oligosaccharide.

Although the liver and intestine are the sites of lipoprotein synthesis, peripheral synthesis of some of the apolipoproteins has been reported. In the rooster apoB is synthesized by the kidney (39) and apoA-I is synthesized by the kidney, vein, artery, and skeletal muscle (40). ApoA-I is also synthesized in chick breast muscle especially around the time of hatching (41). ApoE synthesis has been demonstrated in mouse peritoneal (10) and bone marrow macrophages (11), in tissue slices of human adrenal and kidney (12), and most recently in mouse lung, kidney, spleen, and heart (42). In our study, although apoE synthesis was readily detected in a wide variety of normal rat and guinea pig tissues, we did not detect any extrahepatic synthesis of apoA-I or apoB.

Both our in vitro translation and hybridization studies failed to detect apoE mRNA in rat or guinea pig intestinal mucosa. This is consistent with in vivo studies that showed that the rat intestine synthesizes little if any apoE (9). Recently it has been reported that the rat intestine does synthesize apoE mRNA although at a very low level (26). Because whole intestine including the muscularis mucosa was used in this study, it seems likely that smooth muscle cells present in the intestinal wall may account for the small amount of apoE mRNA found in the rat intestine.

DNA methylation has been implicated in the transcriptional regulation of eukaryotic genes. It is now well-established in a number of systems that there is an inverse correlation between the degree of methylation of cytosine residues (particularly in the sequence CpG) in a gene and the level of its expression (43). Our hybridization studies with genomic DNA digested with the restriction enzymes HpaII and MspI demonstrate that the methylation pattern of the rat apoE gene is tissue-specific. The gene was more heavily methylated in the intestinal mucosa, the only tissue examined that did not synthesize apoE. In contrast, the gene is less methylated in the liver, kidney, and testis, three tissues that are actively involved in the synthesis of apoE. The methylation pattern of the apoE gene appeared to be heterogeneous in the liver, testis, and kidney since DNA isolated from these tissues contained both the methylated and nonmethylated fragments of the apoE gene. This pattern may be due to heterogeneity between different members of the same cell population or may reflect the mixed cell population of these tissues.

The fact that peritoneal and bone marrow macrophages can synthesize and secrete apoE in vitro suggests that macrophages may contribute to the synthesis of apoE in extrahepatic tissues. However, the magnitude of the apoE synthesis in the extrahepatic tissues (0.02 to 0.7% of the total protein synthesized) argues that other cell types are likely to be involved. Furthermore, the relative abundance of apoE mRNA among the tissues studied makes a similar argument. Thus, although the spleen and lung almost certainly harbor more macrophages than steroidogenic tissues such as the testis, the latter tissues contain more apoE mRNA than the two macrophage-rich tissues, at least in the guinea pig. Even in the rat, the apoE mRNA contents are very similar in the testis, lung, and spleen (Table 1). In addition, this study shows that rhesus aortic smooth muscle cells synthesize and secrete relatively large quantities of apoE. In studies to be published elsewhere, apoE synthesis has also been demonstrated in rat ovarian granulosa cells.

The synthesis of apoE appears to be a common property of many tissues and perhaps of many cell types as well. However, the function of the apoE synthesized in these peripheral tissues is not known. Whether the apoE synthesized in these tissues contributes to the

plasma pool of apoE remains to be investigated. Because lipoproteins containing apoE are recognized and removed from the plasma by the liver, Basu et al. (10) have suggested that apoE may function to transport excess cholesterol from cholesterol-loaded macrophages to the liver for excretion. Although this mechanism of reverse cholesterol transport may be important in scavenger cells such as macrophages, apoE may play a different role in other cell types. Blue et al. (12) have suggested that apoE functions as a shuttle protein to transport cholesterol between tissues or cells. Alternatively, apoE may function in the uptake rather than the removal of cholesterol from these cells. A cell with a need for cholesterol may secrete apoE, essentially generating its own receptor ligand. The secreted apoE could bind to an apoE-poor lipoprotein, thus converting it to a lipoprotein which could then be recognized by the cell's own receptor. ApoE may even play a role in areas other than cholesterol transport. ApoE has been shown to modulate lymphocyte proliferation (44, 45) and may function as a more general chalone or messenger between cells. Because of its high affinity for phospholipid (46), apoE may play a role in phospholipid synthesis, transport, or metabolism. Although the functions of the apoE synthesized in the peripheral tissues are not yet known, apoE is clearly a more ubiquitous protein than previously suspected.

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