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mRNA-DEPENDENT SYNTHESIS OF RAT APOLIPOPROTEIN E *in vitro*: COTRANSLATIONAL PROCESSING AND IDENTIFICATION OF AN ENDOGLYCOSIDASE H-SENSITIVE GLYCOPEPTIDE INTERMEDIATE

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Summary: Total polyadenylated enriched mRNA was prepared from rat liver by guanidine-HCl extraction and oligo(dT)-cellulose chromatography. It was translated *in vitro* in an mRNA-dependent wheat germ system and rabbit reticulocyte lysate system, using radiolabeled leucine or methionine as amino acid precursor. A product, designated preapoE, was specifically precipitated by a rabbit anti-rat apoE serum and accounted for 1.5% of the total radioactive peptides. It migrated as a single band of radioactivity on SDS gels with an apparent molecular weight similar to that of mature plasma apoE. Inclusion of dog pancreatic microsomal membranes in the translation reaction resulted in a slightly smaller product (by 500 daltons). It also converted the preapoE from an endoglycosidase H-resistant to an enzyme-sensitive species. This suggests that processing of preapoE takes place by the cotranslational removal of a signal peptide and core glycosylation of the mature protein.

Apolipoprotein E (apoE) is an apoprotein found in chylomicrons, very low density lipoproteins (VLDL) and high density lipoproteins in man and in other mammalian species (1-6). Its plasma concentration is stimulated by high cholesterol-feeding and carbohydrate ingestion (5,7). In the human fibroblast, apoE binds specifically to the low density lipoprotein receptor and is at least as effective as apoB in suppression of 3-hydroxy-3-methylglutaryl coenzyme A reductase, the rate-limiting enzyme in the cellular production of cholesterol (8,9). Recently, it has been demonstrated that uptake of chylomicron remnants is controlled by a specific hepatic receptor for apolipoprotein E in the perfused rat liver (10). Therefore, the regulation of this apoprotein is of considerable interest in the understanding of cholesterol homeostasis.

Abbreviations used are: Gdn-HCl, guanidine-hydrochloride; SDS, sodium dodecyl sulfate; VLDL, very low density lipoproteins; LDL, low density lipoproteins; apo, apolipoproteins; TCA, trichloroacetic acid. This work has been supported by NIH Research Grant HL-23470 and American Heart Association grants 78-1102 and 80-875, and a grant from the Texas Affiliate of the American Heart Association.

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The liver is a major site of synthesis of apoE (11,12). However, very little is known about the biosynthetic pathway of this apoprotein. Because of its induction in diet-induced hypercholesterolemia, its importance in LDL receptor recognition and its involvement in chylomicron remnant removal, we have initiated studies involving the biosynthesis of apoE *in vitro*. In this communication, we describe the isolation of total hepatic mRNA from the rat, and the translation of apoE *in vitro* in a heterologous protein synthesis system from wheat germ and rabbit reticulocyte lysate. We found that newly translated apoE designated preapoE, is similar in size to mature plasma apoE, but that preapoE can be processed by the addition of dog pancreatic microsomal membranes in the translation reaction. Furthermore, the processing of preapoE appeared to go through an endoglycosidase-H-sensitive glycopeptide intermediate.

Materials and Methods

Materials and Animals: Dithiothreitol was purchased from Sigma; oligo(dT) cellulose (T3) was obtained from Collaborative Research. Wheat germ was a gift of General Mills. ^{35}S-Methionine (1260 Ci/mmol) and ^{3}H-leucine (60 Ci/mmol) were purchased from Amersham. Guanidine-HCl (Gdn-HCl) was from Bethesda Research Labs. All other chemicals were reagent grade and were purchased from Fisher Scientific Co. Male Sprague-Dawley rats, weighing 200 gm, were obtained from Holtzman Farm (Wisconsin). For isolation of plasma lipoproteins they were fed either a normal commercial laboratory chow or one supplemented with 5% lard, 1% cholesterol, 0.3% taurocholic acid and 0.1% propylthiouracil (6) for one month.

Isolation of apoE: VLDL were isolated from rat plasma by repeated (x2) ultracentrifugal flotation at d = 1.006. The purified VLDL were not contaminated with albumin as determined by SDS gels and by the absence of an immunoprecipitin line against rabbit anti-rat albumin serum. ApoE was purified from VLDL by the method of Weisgraber *et al.* (6) except that Sephacryl S-200 was used instead of Sephadex G-200 in the fractionation of apolipoproteins. Apolipoproteins were identified by SDS polyacrylamide gel electrophoresis (13) and urea polyacrylamide gel electrophoresis at pH 8.8 (14).

Preparation of antibody: Purified rat apoE (0.04 mg/ml) was emulsified in an equal volume of Freund's complete adjuvant. Two male New Zealand white rabbits were injected at multiple subcutaneous sites on the back with 2.6 ml emulsion containing 104 ug of apoE. After the initial immunization, they received subcutaneous injections (35 ug each time) every 2 weeks. After two months sera were obtained and tested for anti-apoE activity by immunodiffusion and immunoelectrophoresis. An immunoglobulin fraction was prepared by ammonium sulfate precipitation of total serum as previously described (15).

Isolation of total liver mRNA: Total rat liver cellular RNA was purified by the Gdn-HCl method of Deeley *et al.* (16) Polyadenylate containing mRNA was isolated from the total RNA by affinity chromatography on oligo(dT)-cellulose as described by Aviv and Leder (17).

Preparation of wheat germ extract and translation in vitro: Nuclease-treated reticulocyte lysate translation system (19) was purchased from Bethesda Research Laboratories, Maryland. Wheat germ extract was prepared by the method of Davies et al. (18). A total incubation mixture of 50 ul contained 20 ul wheat germ extract and 30 ul of a mixture composed of 10 mM Hepes (pH 7.6), 3 mM dithiothreitol, 1.6 mM ATP, 0.033 mM GTP, 13 mM creatine phosphate, 2 ug creatine phosphokinase, 100 mM KCl, 0.16 mM magnesium acetate, 0.16 mM spermidine, 0.63 mM 19 L-amino acids (minus methionine or leucine), 8 uCi ^{35}S-methionine or 10 uCi ^{3}H-leucine and various amounts of mRNA; H_2O was added to adjust the volume of 30 ul. The reaction was carried out at 25°C for differing periods of time. At the end of an incubation period, 5 ul aliquots were precipitated in 1 ml 10% trichloroacetic acid, heated to 95°C for 10 min, cooled in ice, and filtered on glass-fiber filters. The remainder of the reaction mixture was used for quantitation of immunoprecipitable apoE synthesized in vitro.

Quantitation of immunoprecipitable apoE synthesized in vitro: Inactivated Staphylcoccus aureus Cowan I strain (Pansorbin, Calbiochem) were extensively washed and prepared according to the method of Kessler et al. (20). The wheat germ reaction products were first centrifuged at 105,000 x g for 60 min at 4°C. The supernatant fraction was then incubated with 30 ul of prewashed Pansorbin at room temperature for 5 min. Pansorbin was removed by low speed centrifugation. Newly synthesized ^{3}H- or ^{35}S-apoE was precipitated in a reaction mixture containing 0.5 ul rabbit anti-rat apoE IgG and 300 ul of TNENN buffer (50 mM Tris, pH 7.5, 0.15 M NaCl, 5 mM EDTA, 0.02% NaN_3, 0.5% NP-40, containing 10 mM leucine or 10 mM methionine) per 100 ul of the reaction product. The mixture was incubated at room temperature for 2 hr and then at 4°C overnight. Fifteen ul of Pansorbin was added and the mixture was gently shaken at 4°C for 40 min. The Pansorbin was collected by low-speed centrifugation. It was layered on top of a 1 M sucrose cushion in TNENN buffer and centrifuged at 2000 x g for 20 min. After another wash with TNENN buffer, immunoprecipitated product was released from the bacteria by incubation in 2.5% SDS, 10% glycerol, 5% mercaptoethanol and 65 mM Tris-HCl, pH 8, at 95°C for 5 min. It was either counted directly by scintillation spectrophotometry, or further analyzed by SDS gel electrophoresis.

Processing of putative apoE precursor: Dog pancreatic microsomal membrane fractions were isolated by the method of Shields and Blobel (21). They were added to the translation mixture at a concentration of 8 ul (15 A_{260} units/ml) per 30 ul reaction, prior to the initiation of translation. Immunoprecipitated products were analyzed by SDS-slab gel electrophoresis and visualized by fluorography as previously described (22-24).

Effects of endoglycosidase-H on translation products: Cell-free translation products were treated with endoglycodase-H (Miles Laboratories, Indiana) as described by M. Bielinska and I. Boime (25) with minor modifications. Immunoprecipitated products were suspended in 50 mM sodium acetate pH 5, 0.1 M mercaptoethanol, 0.5% Triton X-100, 0.8% SDS, and heated at 95°C for 5 min. The products released from the Pansorbin were then incubated with 0.05 units of endoglycosidase H, 20 ug of bovine albumin for 18 hr at 25°C. The reaction was stopped by addition of 10% trichloroacetic acid. The trichloroacetic acid precipitates were dissolved in 65 mM Tris-HCl, pH 8, 5% mercaptoethanol, 10% glycerol, 2.5% SDS, and analyzed on 10% SDS-polyacrylamide gel electrophoresis as described above.

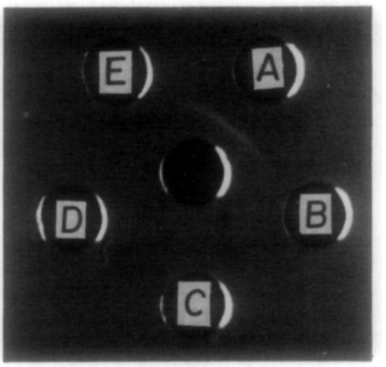


Fig. 1: Immunodiffusion of apoproteins against rabbit anti-rat apoE. Rabbit anti-rat apoE serum was prepared as described in Materials and Methods using purified apoE as antigen. The center well contained 7 ul of the total antiserum. Each of the wells contained 7 ul of the indicated antigens at 1.5 ug/ul: A. rat apoE; B. rat albumin; C. rat apoC proteins; D. rat apoA-I; E. human apoE.

Results and Discussion

ApoE purification and antisera production: ApoE was purified from total rat VLDL ($d \leq 1.006$ g/ml) by the procedure described in Methods. It was homogeneous by SDS and urea polyacrylamide gel electrophoresis, and had an apparent mol. wt. of 37,000. Amino acid composition of purified rat apoE was essentially identical to that reported by Weisgraber *et al.* (6) (data not shown). Anti-apoE sera were produced in rabbits. On Ouchterlony plates, they were found to be specific for apoE, and did not cross-react with rat albumin, apoC proteins, or apoA-I. There was also no reactivity against human apoE (Fig. 1). Immunoelectrophoresis of the antisera against apoE and delipidated rat plasma showed a single precipitin arc and confirms that we have generated a monospecific antiserum (data not shown).

Translation of rat apoE in vitro: When total rat hepatic polyA RNA was translated *in vitro* in a wheat germ system, there was a mRNA-dependent incorporation of ^{35}S-methionine into TCA-precipitable proteins (Fig. 2A). The translation was linear for 90 min. When immunoprecipitable ^{35}S-apoE synthesized *in vitro*, designated preapoE, was quantified by the procedures described in Methods, it was found to constitute about 1.5% of the newly synthesized pro-

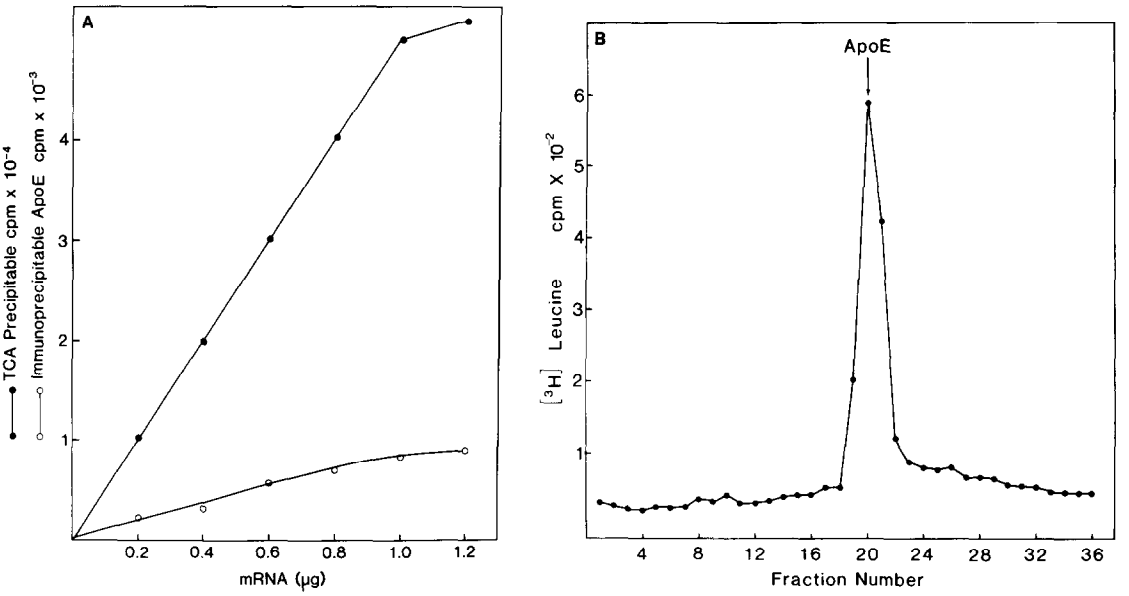


Fig. 2A: Translation of rat liver poly(A)RNA in wheat germ extract in vitro. TCA-precipitable and immunoprecipitable counts were determined as described in Materials and Methods. ^{35}S-Methionine (8 uCi per assay) was the radioactive amino acid precursor used. Incubation was at 25°C for 60 min. Background counts (without mRNA) of 1,700 (TCA) and 100 (immunoprecipitable) were subtracted.

Fig. 2B: Sodium dodecyl sulfate acrylamide gel electrophoresis of apoE synthesized in vitro. The immunoprecipitable apoE in vitro product was analyzed on 7.5% polyacrylamide gel containing 0.1% SDS (13). At the end of electrophoresis, 2 mm gel slices were cut, dissolved in 30% H_2O_2 and counted in ScintiVerse (Fisher Scientific Co.) as described previously (15). The arrow indicates the migration of plasma apoE loaded simultaneously on another gel.

teins (Fig. 2A). When the immunoprecipitated product was analyzed on a 7.5% acrylamide gel in SDS, only a single band of radioactive products, approximately the same size as plasma apoE, was identified on the gel (Fig. 2B).

To further confirm the specificity of the preapoE translated in vitro, a number of competition experiments were performed (Fig. 3). The addition of unlabeled rat apoE to the immunoprecipitation procedure was found to abolish the radioactive band. In contrast, the addition of another protein, ovalbumin, did not result in any detectable inhibition. Furthermore, when nonimmune serum was substituted for anti-apoE serum, the radioactive band was absent.

Processing and endoglycosidase H sensitivity of preapoE: Even though prepoE had the same mobility as plasma apoE on SDS gels, since apoE is a secretory

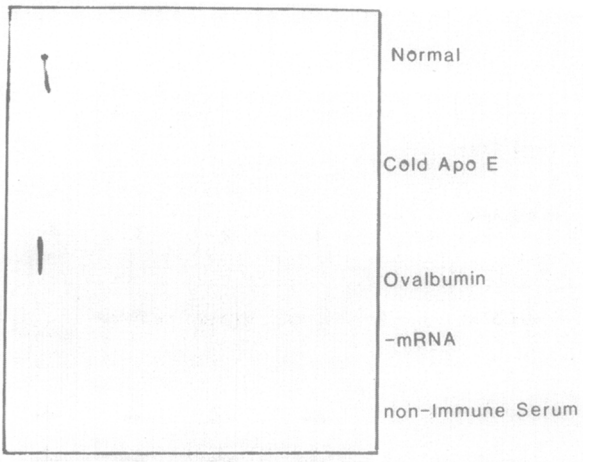
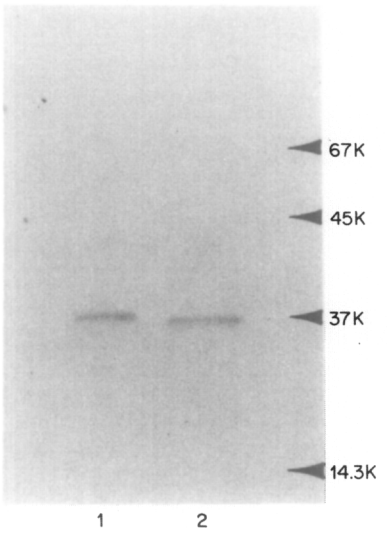
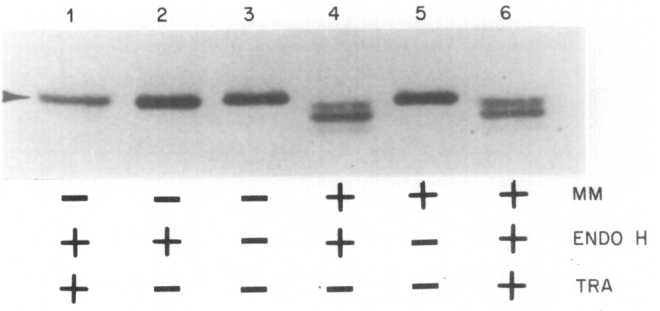


Fig. 3: Antibody specificity of apoE synthesized in vitro. Total mRNA was translated in vitro using ^{35}S-methionine as the labeled amino acid precursor as described in Methods. The figure shows the products, visualized by fluorography, separated on a slab gel (7.5% acrylamide, 0.1% SDS). Lanes (from left to right) contain: (normal) immunoprecipitation product using rabbit anti-rat apoE serum; (cold apoE) same as normal, except that 50 ug of rat apoE was included in the immunoprecipitation mixture; (ovalbumin) same as normal, except that 500 ug ovalbumin was included; (-mRNA) the immunoprecipitation was performed on a wheat germ assay without the addition of exogenous rat liver; mRNA (nonimmune serum) same as normal, except that a nonimmune serum was used for the immunoprecipitation instead of the rabbit anti-rat apoE antiserum.

protein, we were interested in whether a signal peptide-containing precursor is the initial translation product (26). When dog microsomal membranes were included in the translation reaction, preapoE was cleaved into a smaller peptide (Fig. 4A). However, the change in size of the product was minimal (500 daltons). This could be explained either by a very short signal peptide of 3-5 amino acids, which would be unprecedented (27), or by some further modification of the processed product resulting in an aberrant mobility on SDS gels. To test whether some form of core glycosylation might be involved in the post-translational modification of apoE, we incubated the various translation products in the presence of endoglycosidase H, which cleaves between the two N-acetylglucosamine units of a mannose-rich core region in serum-type glycoproteins (28) (Fig. 4B). Indeed, endoglycosidase H specifically cleaved microsomal membrane processed apoE into smaller products, but did not affect unprocessed preapoE. (The minimal change in mol. wt. on incubating preapoE with endoglyco-



(A)



(B)

Fig. 4A: Processing of ^{35}S-preapoE. Translation in vitro was performed as described in Methods. Translation products were immunoprecipitated, separated on 10% acrylamide gel in 0.1% SDS, and visualized by fluorography. Lane 1: Translation in the absence of dog microsomal membranes. Lane 2: Translation in the presence of dog pancreatic microsomal membranes. The mol. wt. standards, are from top to bottom: Bovine serum albumin (67,000), ovalbumin (45,000), rat apoE (37,000), lysozyme (14,300).

Fig. 4B: Endoglycosidase H sensitivity of translation products. Samples ± prior microsomal membrane treatment were incubated for 18 hr at 25°C with the various components as described in Methods. They were analyzed by 10% acrylamide slab gel in 0.1% SDS and visualized by fluorography. Position of rat plasma apoE is indicated by the arrow. MM, dog microsomal membranes added during translation; ENDO H, endoglycosidase H added; TRA, trasylol added.

sidase H, apparent in Figure 4B [lane 2] is due to minor protease contamination of Miles endoglycosidase H, and is completely inhibited by the inclusion of the protease inhibitor Trasylol [10 KIU, Mobay Chemical Co., N.Y.] in the incubation mixture.) There are two major and one minor endoglycosidase H products from processed apoE which are quite reproducible. This suggests that there may be different sites of enzyme-sensitivity in the glycosylated protein.

Thus, we have demonstrated that rat apoE is synthesized via an endoglycosidase H-sensitive intermediate. Since the mature plasma protein is known to be a glycoprotein (6), our findings are compatible with a mannose-rich inter-

mediate(s) in the biosynthesis of apoE. It is interesting that under similar conditions, plasma apoE is not cleaved by endoglycosidase H (data not shown). This is probably due to the addition of peripheral sugars, like fucose, or of sialic acid (6), presumably in the Golgi membranes (28), which results in an endoglycosidase H-resistant glycoprotein. Such subsequent modification of preapoE also results in further changes in the mobility of the protein on SDS gels so that it assumes the apparent size of the mature plasma protein.

References

1. Koga, S., Bolis, L. and Scanu, A.M. (1971) Biochim. Biophys. Acta 236, 416-430.
2. Shore, V.G. and Shore, B. (1973) Biochemistry 12, 502-507.
3. Utermann, G. (1975) Hoppe-Seyler's Z. Physiol. Chem. Biol. 356, 1113-1121.
4. Herbert, N.F., Windmueller, H.G., Bersot, T.P. and Shulman, R.S. (1974) J. Biol. Chem. 249, 5718-5724.
5. Swaney, J.B., Braithwaite, F. and Eder, H.G. (1977) Biochemistry 16, 271-278.
6. Weisgraber, H.K., Mahley, R.W. and Assmann, G. (1977) Atherosclerosis 28, 121-140.
7. Mahley, R.W. and Holcombe, K.S. (1977) J. Lipid Res. 18, 314-324.
8. Mahley, R.W. and Innerarity, T.L. (1977) J. Biol. Chem. 252, 3980-3986.
9. Pitas, R.E., Innerarity, T.L. and Mahley, R.W. (1980) J. Biol. Chem. 225, 5454-5460.
10. Sherrill, C.B., Innerarity, T.L. and Mahley, R.W. (1980) J. Biol. Chem. 255, 1804-1807.
11. Lo, C.-H. and Marsh, J.B. (1970) J. Biol. Chem. 245, 5001-5006.
12. Felker, E.T., Fainaru, M., Hamilton, R.L. and Havel, R.J. (1977) J. Lipid Res. 18, 465-472.
13. Weber, K. and Osborn, M. (1969) J. Biol. Chem. 244, 4406-4412.
14. Kane, J.P. (1973) Anal. Biochem. 53, 350-354.
15. Chan, L., Jackson, R.L., O'Malley, B.W. and Means, A.R. (1976) J. Clin. Invest. 58, 368-379.
16. Deeley, R.G., Gordon, J.I., Burns, A.T.H., Mullinix, K.P., Bina-Stein, M. and Goldberger, R.F. (1977) J. Biol. Chem. 252, 8310-8319.
17. Aviv, H. and Leder, P. (1972) Proc. Natl. Acad. Sci. USA 69, 1408-1412.
18. Davies, J.W., Albers, A.J.M., Struks, E.J. and Kammew, A.V. (1977) FEBS Lett. 77, 265-269.
19. Pelham, H.R.B. and Jackson, R.J. (1976) Eur. J. Biochem. 67, 247-256.
20. Kessler, S.W. (1975) J. Immunol. 115, 1617-1624.
21. Shields, D. and Blobel, G. (1978) J. Biol. Chem. 253, 3753-3756.
22. Laemmli, U.K. (1970) Nature 227, 680-685.
23. Laskey, R.A. and Mills, A.D. (1975) Eur. J. Biochem. 56, 335-341.
24. Chan, L., Bradley, W.A., Jackson, R.L. and Means, A.R. (1980) Endocrinology 106, 275-283.
25. Bielinska, M. and Boime, I. (1979) Proc. Natl. Acad. Sci. USA 76, 1208-1212.
26. Blobel, G. and Dobberstein, B. (1975) J. Cell Biol. 67, 835-851.
27. Blobel, G. (1980) Proc. Natl. Acad. Sci. USA 77, 1496-1500.
28. Tarentino, A.L. and Maley, F. (1974) J. Biol. Chem. 2498, 811-817.
29. Schachter, H. (1974) Biochem. Soc. Symp. 40, 57-71.