MOLECULAR PHARMACOLOGY, 27:394–398

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Phenobarbital Induces Rat Liver Apolipoprotein A-I mRNA

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Received June 25, 1984; Accepted December 13, 1984

SUMMARY

The effect of phenobarbital on the level of rat liver apolipoprotein A-I (apo-A-I) mRNA was studied. Poly(A$^+$)-RNA isolated from livers of control or phenobarbital-treated rats was translated *in vitro* in the rabbit reticulocyte lysate system and immunoprecipitated with rabbit antiserum against rat apo-A-I. The immunoprecipitate was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The translational activity of apo-A-I mRNA was estimated from the incorporation of [^{35}S]methionine into the apo-A-I band. It was found to be elevated 4-fold by 16 hr after rats received a single injection of phenobarbital. To study the effect of phenobarbital on the level of rat liver apo-A-I mRNA, a recombinant plasmid which contained a cDNA insert corresponding to rat liver apo-A-I mRNA was isolated and used to hybridize total liver poly(A$^+$)-RNA from control and phenobarbital-treated rats. There were 4.8- and 10-fold increases in the amount of hybridization to mRNAs from rats after they were treated with phenobarbital for 8 and 16 hr, respectively. Thus, phenobarbital increases the level of rat liver apo-A-I mRNA.

INTRODUCTION

Epidemiological evidence indicates that there is a strong predictive correlation between high levels of HDL[1] cholesterol and decreased incidence of coronary heart disease (1). The major constituent apolipoprotein of HDL is apo-A-I. One function of apo-A-I is as a cofactor of lecithin:cholesterol acyltransferase (2), the enzyme that catalyzes the esterification of cholesterol in the plasma to form cholesterol esters.

The mechanism(s) by which HDL affects coronary heart disease and how HDL levels are controlled have been the source of extensive recent investigations. Nevertheless, there is little definitive information. One possible clue has come from the observation that epileptics treated with anticonvulsant drugs such as phenobarbital or phenytoin have higher levels of HDL (3, 4) and a lower incidence of heart disease (5). Phenobarbital and other xenobiotics also increase HDL cholesterol and apo-A-I levels in rats. The mechanism by which xenobiotics increase plasma HDL levels is not understood. In the rat, apo-A-I is synthesized both in the intestine and in the liver (6, 7). Apo-A-I is initially synthesized as a preproprotein with 24-amino acid NH_2-terminal extension consisting of 18-amino acid presegment and a hexapeptide prosegment followed by the mature plasma protein (8, 9). Preapo-A-I undergoes post-translational cleavage to mature apo-A-I by an apparently specific protease (10). The level of liver apo-A-I mRNA is regulated by dietary cholesterol. When rabbits are fed a cholesterol-rich diet, their liver apo-A-I mRNA level is elevated 5-fold as measured by apo-A-I mRNA translational activity (11). In contrast, the intestinal apo-A-I mRNA level is not affected by dietary cholesterol.

In order to elucidate the mechanism by which various pharmacological agents and diets affect the expression of the apo-A-I gene, it is important to have specific probes to detect and quantitate the mRNA specific for this protein. Therefore, in this investigation, we report the identification of cDNA clones specific for apo-A-I and apo-E and the use of these clones to determine the effect of phenobarbital on the levels of liver apo-A-I and apo-E mRNAs.

MATERIALS AND METHODS

In vitro synthesis and quantitation of apo-A-I. Total poly(A$^+$)-RNA was isolated from rat liver and translated *in vitro* in the rabbit reticulocyte lysate system in the presence of [^{35}S]methionine as previously described (12). The translation mixtures were immunoprecipitated with polyclonal antiserum against rat apo-A-I. The immunoprecipitate was subjected to 10% polyacrylamide gel electrophoresis in the presence of 0.2% SDS. The level of liver apo-A-I mRNA was quantitated from the radioactivity associated with apo-A-I band on the gel (11).

Preparation of rat apo-E and apo-A-I antisera. Rat apo-E and apo-A-I were isolated from delipidated serum VLDL and HDL, respectively, using a column of Sephadex G-200 (1.2 × 300 cm) equilibrated with 4 M guanidine HCl, 10 mM Tris, pH 7.2. The isolated apolipoprotein, containing 0.5 mg of protein, was mixed with an equal volume of Freund's complete adjuvant and injected into multiple subcutaneous

[1] The abbreviations used are: HDL, high density lipoproteins; apo-A-I, apolipoprotein A-I; VLDL, very low density lipoproteins; SDS, sodium dodecyl sulfate.

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sites of a rabbit. Two additional injections were made at 14-day intervals. Antiserum was obtained 10 days after the final injection.

Isolation of rat liver polysomes and purification of mRNAs specific for apo-A-I and apo-E. The procedures used for isolation and purification of rat liver apo-A-I and apo-E mRNAs were essentially identical to those described by Kraus and Rosenberg (13). Briefly, rat liver polysomes were isolated from livers of male, Sprague-Dawley rats. Polysomes synthesizing apo-A-I and apo-E were immunoselected with rabbit IgG raised against rat plasma apo-A-I. The immune IgG was isolated by passing rabbit antiserum over a protein A-Sepharose affinity column. The isolated IgG was reacted with rat liver polysomes for 1 hr at 4°. The polysome-IgG complexes were immobilized on the protein A-Sepharose column. Ribosome subunits along with apo-A-I mRNA were eluted from the column with 25 mM Tris, pH 7.5, 20 mM EDTA, and 0.2 mg/ml heparin. Poly(A^+)-RNA was isolated from the eluate of oligo(dT)-cellulose affinity chromatography and precipitated at −20° after the addition of 2 volumes of ethanol. The purified mRNAs were utilized to conduct cell-free protein synthesis in the rabbit reticulocyte lysate translation system. The synthesized apolipoproteins were immunoprecipitated and identified from SDS-polyacrylamide gels by fluorograms as previously described (11).

Preparation of cDNA. The preparation of cDNA probes using the purified mRNAs was identical to published methods (14). These cDNA probes (10^7 cpm/μg) were utilized to screen a cDNA library previously constructed (14).

Hydrid selection assay. Hybrid select translation was carried out according to the procedures described by Cleveland *et al.* (15) and modified by Pickett *et al.* (14). The hybrid select mRNAs were translated *in vitro* using the rabbit reticulocyte lysate translation system and [^{35}S]methionine as labeled precursor. The translation mixture was immunoprecipitated with rabbit antiserum against rat apo-A-I and the radiolabeled polypeptide was identified on SDS-polyacrylamide gels as previously described (11).

Gel electrophoresis and RNA blot hybridization. Total rat liver poly(A^+)-RNA was isolated by oligo(dT)-cellulose affinity chromatography according to the methods of Chirgwin *et al.* (16) and electrophoresed in 1.5% agarose gels containing 10 mM methylmercury hydroxide according to the procedure of Bailey and Davidson (17) and transferred to DBM paper as described by Alwine *et al.* (18). Prehybridization, hybridization with ^{32}P-labeled cDNA, and washing procedures were the same as previously described (14). The DBM paper was allowed to dry at room temperature, covered with Saran Wrap, and exposed to X-ray film using Lightening Plus intensifying screens (DuPont).

RESULTS

Effects of phenobarbital on the levels of rat liver translatable apo-A-I mRNA. Fig. 1 shows that the primary immunoprecipitate of the *in vitro* translation product of rat liver mRNA is apo-A-I with a molecular weight of 28,800 which is about 2,000 larger than the molecular weight of plasma apo-A-I. Another protein of molecular weight 46,000 which co-precipitates with apo-A-I from the translation system may represent apo-A-I which is not completely dissociated from the IgG or another polypeptide. The level of apo-A-I mRNA from normal rat liver as quantitated from the amount of radioactivity associated with the apo-A-I band (Fig. 1, lane 1), is approximately 0.1% of total liver mRNA. This level increased 1.8-, 4.0-, and 3.2-fold after rats received a single intraperitoneal injection of phenobarbital (80 mg/kg) at 8 (lane 2), 16 (lane 3), and 24 (lane 4) hr, respectively.

Purification of apolipoprotein mRNAs by polysome immunoabsorption. In order to identify cDNA clones complementary to rat liver mRNAs specific for apo-A-I and apo-E, we purified the mRNAs by polysome immunoabsorption. As shown in Fig. 2, rabbit antiserum against rat plasma apo-A-I immunoselected a mixture of two mRNAs which coded for apo-A-I and apo-E. The reason for this is probably because of the presence of trace amounts of apo-E in the original apo-A-I preparation which was used for antibody production. When the *in vitro* translation product was subjected to immunoprecipitation with antiserum against apo-A-I, more apo-A-I than apo-E was immunoprecipitated (Fig. 2, lane c).

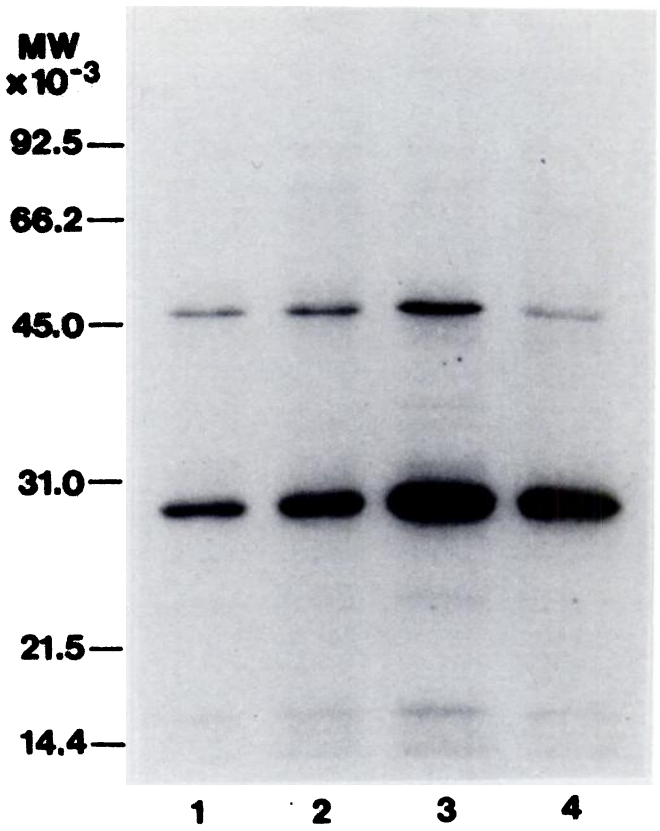

FIG. 1. *Fluorogram of SDS-polyacrylamide gel electrophoresis of liver apo-A-I immunoprecipitates obtained from in vitro translations programmed with poly(A^+)-RNA isolated from control and phenobarbital rats*

All immunoprecipitations were performed using equal amounts of radioactivity from each translation assay. Equal aliquots of the immunoprecipitates were then layered on the 10% SDS-polyacrylamide gel. Lane 1 represents control rats. Lanes 2, 3, and 4 represent rats who received phenobarbital for 8, 16, and 24 hr, respectively. Molecular weight markers are phosphorylase *b* (92,500), bovine serum albumin (66,200), ovalbumin (45,000), carbonic anhydrase (31,000), soybean trypsin inhibitor (21,500), and lysozyme (14,400).

Identification of apo-A-I and apo-E cDNA clones by hybrid select translation. The purified mRNAs were used to identify cDNA clones complementary to apo-A-I and apo-E. High specific activity cDNAs were synthesized from the purified mRNAs using reverse transcriptase. After base hydrolysis of the mRNA template, the cDNA probe was used to screen a rat liver cDNA library (14). From 200 cDNA clones, two clones (p121 and p164) gave strong hybridization signals. Plasmid DNAs from these two cDNA clones were isolated and used in hybrid select translation studies to establish the specificity of these

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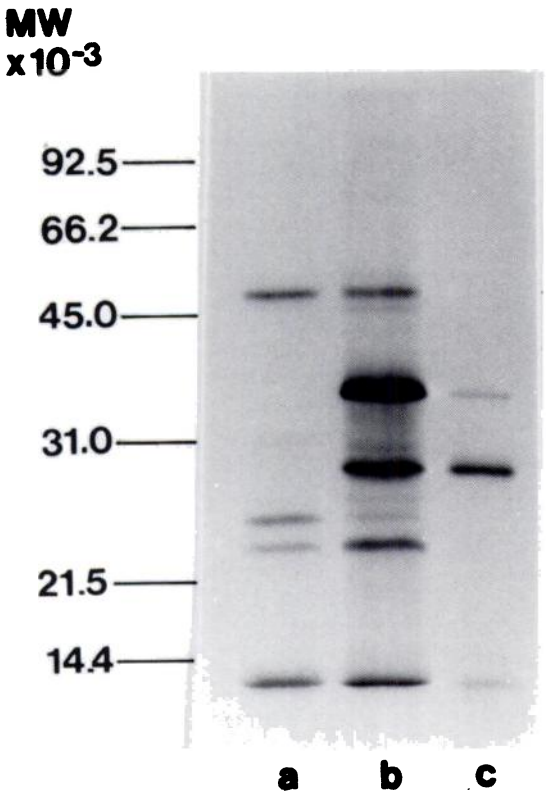

FIG. 2. *Purification of rat liver apo-A-I and apo-E mRNAs by polysome immunoabsorption*

The mRNAs were purified by polysome immunoabsorption and translated *in vitro*. Lane a, endogenous translation products synthesized by the rabbit reticulocyte lysate system in the absence of added mRNA. Lane b, *in vitro* translation products directed by mRNA isolated from polysomes immunoabsorbed by antibody raised against apo-A-I. Lane c, immunoprecipitation of the total translation products displayed in lane b with antibody raised against apo-A-I. The radioactive band in lane b with molecular weight of 28,800 is apo-A-I, and with a molecular weight of 34,000 is apo-E. The molecular weight markers are the same as presented in Fig. 1.

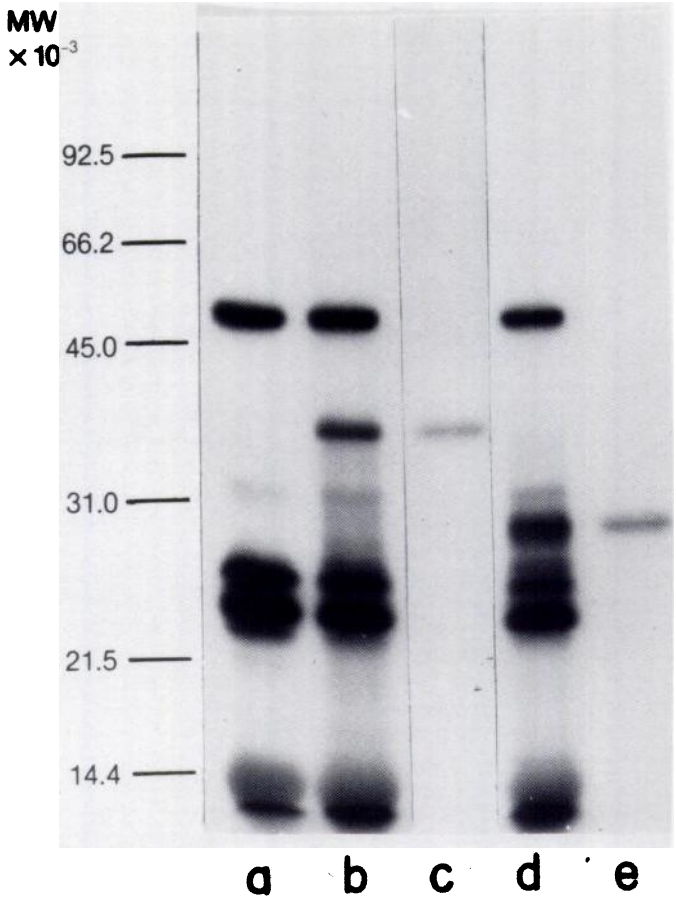

FIG. 3. *Hybrid select translation analyses utilizing rat liver apo-E and apo-A-I clones*

Lane a, endogenous *in vitro* translation products directed by the rabbit reticulocyte lysate in the absence of added mRNA. Lane b, total translation products of mRNA hybrid-selected by clone p121. Lane c, immunoprecipitate of translation products of lane b by antiserum against rat apo-A-I. Lane d, total translation products of mRNA hybrid-selected by clone p164. Lane e, immunoprecipitate of translation products of lane d by antiserum against rat apo-A-I. The fluorogram was obtained after overnight (lanes a, b, d, and e) and 1-week exposure (lane c). The molecular weight markers are the same as presented in Fig. 1.

clones. Fig. 3, lane b, shows that recombinant clone p121 hybrid-selected a mRNA species which is translated *in vitro*, to produce a polypeptide with a molecular weight of 34,000. This polypeptide can be precipitated with either the antiserum against apo-A-I (Fig. 3, lane c) or antiserum against apo-E (Fig. 4, lane c). Recombinant clone p164 hybrid-selected a mRNA species which is translated *in vitro* to produce a polypeptide with a molecular weight of 28,800 (Fig. 3, lane d). This polypeptide can be precipitated with the antiserum against apo-A-I (Fig. 3, lane e).

Induction of the rat liver mRNA specific for apo-A-I by phenobarbital. Total liver poly(A^+)-RNA was isolated from control or phenobarbital-treated rats. The RNA was denatured with methylmercury hydroxide, electrophoresed on agarose gels, blotted to DBM paper, and hybridized to a nick-translated probe from either p121 or p164. Recombinant p164 hybridized to mRNAs whose average size is approximately 1200 nucleotides (Fig. 5, lane 1). Total poly(A^+)-RNA isolated from livers of rats after treatment with phenobarbital for 8 or 16 hr showed approximately 4.8- and 10-fold increases, respectively, in mRNAs hybridizing to p164 as revealed by densitometric scan of the autoradiograph of the RNA gel blot (Fig. 5). Using cDNA probe p121 for hybridization of liver poly(A^+)-RNA, no induction of liver apo-E mRNA was observed after rats were treated with phenobarbital (data not shown).

DISCUSSION

There is an inverse correlation between plasma levels of apo-A-I and HDL cholesterol and risk of development of coronary heart disease (1). The recent Lipid Clinics Coronary Primary Prevention Trial report (19) has shown that small increases in HDL cholesterol levels by cholestyramine treatment accounted for a 2% reduction in the risk of coronary heart disease. The mechanism by which apo-A-I and HDL prevent the development of coronary heart disease is not clear. It has been proposed

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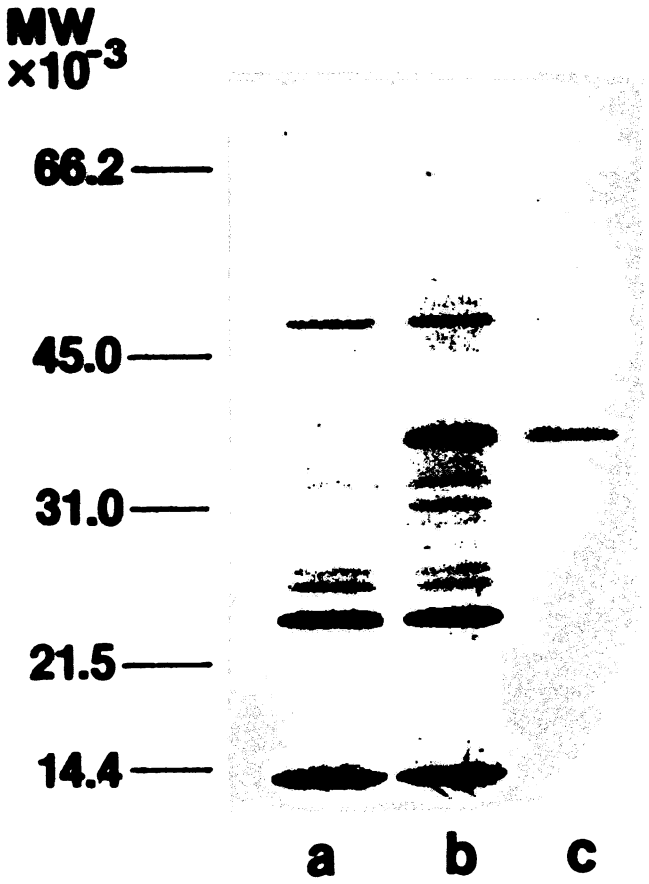

FIG. 4. *Hybrid select translation analyses utilizing clone p121*

Recombinant clone p121 was utilized for hybrid select translation. Lane a, endogenous *in vitro* translation products directed by the rabbit reticulocyte lysate in the absence of added mRNA. Lane b, total translation products of mRNA hybrid-selected by clone p121. Lane c, immunoprecipitate of translation products of lane b by antiserum against rat apo-E. The fluorogram was obtained after overnight exposure.

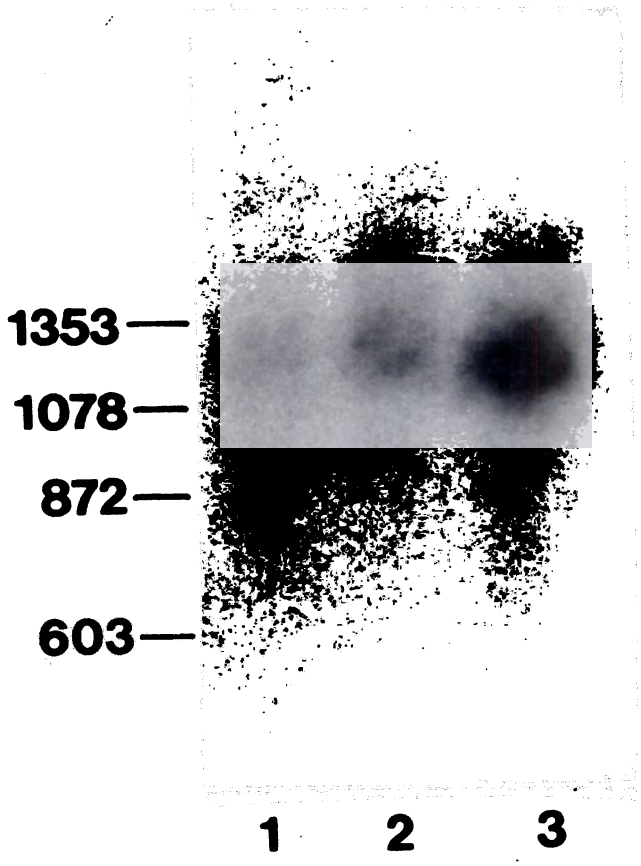

FIG. 5. *RNA gel blot hybridization of p164 to liver mRNA isolated from control and phenobarbital-treated rats*

Rat liver poly(A⁺)-RNA was isolated from control rats (lane 1) or from rats which received phenobarbital for 8 (lane 2) or 16 (lane 3) hr. The poly(A⁺)-RNA was electrophoresed on 1.5% agarose slab gels containing 10 mM methylmercury hydroxide and transferred to DBM paper following electrophoresis. ^{32}P-labeled clone p164 was hybridized to the RNA and specific hybridization was visualized by audioradiography.

that HDL is important in the process of reverse cholesterol transport by which cholesterol is removed from peripheral cells and transported to the liver for removal from the body (20). Thus, an increased level of plasma apo-A-I is beneficial. Several pharmacological agents are known to increase the plasma apo-A-I level: alcohol, hydrocarbon pesticides, and phenobarbital (4, 21, 22). Clinically, patients with transient ischemic attack or with epilepsy have higher plasma apo-A-I levels after they are treated with phenobarbital (23). Furthermore, patients with epilepsy under phenobarbital treatment had lower incidence of coronary heart disease (3, 4). Phenobarbital also increases plasma HDL-cholesterol levels in rats (24).

In order to study the mechanism responsible for induction of plasma levels of apo-A-I in rats at the molecular level, we have isolated two recombinant clones by screening a cDNA library using a cDNA probe which is synthesized from mRNA purified by the immunoabsorption method. These two clones correspond to apo-E (p121) and apo-A-I (p164) as identified by hybrid selection and immunoprecipitation methods. Using these clones, we demonstrated that phenobarbital induces the amount of liver apo-A-I mRNA in rats. The increased level of apo-A-I mRNA resulted in an increased *in vitro* translational activity of apo-A-I mRNA. This induction is specific for apo-A-I; no change was observed in the level of liver apo-E mRNA in rats treated with phenobarbital. The induction of liver apo-A-I mRNA explains at least in part the increased plasma level of apo-A-I in rats treated with phenobarbital. Because apo-A-I is also synthesized in other tissues (25), we cannot rule out the possibility that phenobarbital also affects the apo-A-I synthesis in these tissues nor do we know the effects of phenobarbital on the catabolism of apo-A-I.

Phenobarbital also increases the levels of several other liver enzymes in rats, e.g., cytochrome P-450, epoxide hydrolase, NADH-cytochrome P-450 reductase, UDP-glucuronyltransferase, and glutathione *S*-transferase (26–30). Recent studies (12, 14, 31–33) have demonstrated that the mechanism of the increase of their levels is the induction of liver mRNA encoding these enzymes in rats. There is also evidence (3) which shows that there is a direct correlation of levels of plasma apo-A-I, HDL

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cholesterol, and liver cytochrome P-450 in man with or without phenobarbital treatment. Whether there is a direct correlation of induction of liver mRNAs encoding apo-A-I and other enzymes, either linked by function or by structure, remains to be determined.

In conclusion, using the *in vitro* translation assay and recombinant DNA techniques, we have demonstrated that phenobarbital induces the level of rat liver apo-A-I mRNA. With the availability of cDNA clones, it is now possible to study the regulation of metabolism of apo-E and apo-A-I at the molecular level.

ACKNOWLEDGMENT

The authors would like to thank Joan Kiliyanski for her assistance in typing this manuscript.

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