Regulation of hepatic apolipoprotein synthesis in the 17α-ethinyl estradiol-treated rat

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Abstract Regulatory mechanisms of hepatic apolipoprotein synthesis were studied in groups of male Sprague-Dawley rats made severely hypolipidemic by treatment with pharmacological doses of 17α-ethinyl estradiol. Treatment resulted in a marked reduction of plasma cholesterol and apolipoproteins B, A-I, and A-IV. Hepatic apoA-I mRNA and apoA-I synthesis were increased in the ethinyl estradiol-treated animals. Hepatic apoA-IV protein synthesis rates were unaltered; however, a reduction of the apoA-IV mRNA level was observed. Diet-control studies suggested the effects of 17α-ethinyl estradiol on apoA-I, unlike those on apoA-IV, appeared to be related to the steroid and not to reduced caloric intake. Livers of control and ethinyl estradiol-treated rats synthesized both $apoB_H$ and $apoB_L$. Total hepatic apoB ($apoB_L$ plus $apoB_H$) synthesis and apoB mRNA levels in the ethinyl estradiol-treated rats were similar to ad libitum fed or diet-controls. In ad libitum fed and diet-control rats, 21% and 32%, respectively, of newly synthesized hepatic apoB was $apoB_H$. In contrast, 47% of the newly synthesized apoB in the ethinyl estradiol-treated animal was $apoB_H$. Nucleotide sequence analysis of hepatic apoB mRNA confirmed a marked decrease in the proportion of the $apoB_L$ mRNA in ethinyl estradiol-treated animals. After cessation of 17α-ethinyl estradiol treatment, the hepatic apolipoprotein A-I synthesis rate, apolipoprotein A-I and A-IV mRNA levels, and the $apoB_H$ and $apoB_L$ synthesis rates, as well as plasma apolipoprotein and cholesterol levels, returned to normal. A major finding of the present study is that pharmacological doses of ethinyl estradiol do not affect total hepatic apoB synthesis, but increase the relative amount of $apoB_H$ synthesized. —**Seishima, M., C. L. Bisgaier, S. L. Davies, and R. M. Glickman.** Regulation of hepatic apolipoprotein synthesis in the 17α-ethinyl estradiol-treated rat. *J. Lipid Res.* 1991. **32:** 941–951.

Supplementary key words apoA-I • apoA-IV • apoB • mRNA

The liver and the intestine are the predominant sites of apolipoprotein synthesis in all species studied including humans (1, 2). Apolipoprotein synthetic rates in these organs may play a central role in determination of plasma lipoprotein levels. Therefore, an understanding of the mechanisms that regulate their synthesis is of importance. In rats, apolipoproteins (apo) B, A-I, and A-IV are synthesized at high rates in both organs (3–7). Rat intestinal apoB and apoA-I syntheses are not influenced by acute or chronic dietary lipid or cholesterol feeding (4, 5). Studies in nonhuman primates imply that dietary fatty acids or cholesterol do not increase the rate of hepatic apoB synthesis or mRNA levels (8). Thus, available data suggest that these genes appear to be constitutively expressed at high levels (3–8). It is unknown, however, whether intestinal or hepatic apolipoprotein synthesis can be regulated by levels of plasma lipoproteins. Since both liver and intestine have receptors for LDL (9, 10), the possibility exists that plasma levels of lipoproteins may influence apolipoprotein synthesis rates in both organs. We therefore studied the effects of 17α-ethinyl estradiol, a known hypolipidemic agent (11–21), on the synthesis of apoB, A-I, and A-IV in rat liver.

Recent advances have clarified the mechanism by which two forms of apoB are synthesized in liver and intestine. In human plasma, two immunologically similar forms of apoB exist (22). The larger form, apoB-100 (MW ~512 kDa) is a component of circulating VLDL, IDL, and

Abbreviations: apo, apolipoprotein; EE, 17α-ethinyl estradiol; IDL, intermediate density lipoprotein; LDL, low density lipoprotein; HDL, high density lipoprotein; VLDL, very low density lipoprotein; apoB-100, human high molecular weight apolipoprotein B (MW ~512 kDa); apoB-48, human low molecular weight apolipoprotein B (MW ~250 kDa); $apoB_H$, rat high molecular weight apolipoprotein B (MW ~335 kDa); $apoB_L$, rat low molecular weight apolipoprotein B (MW ~240 kDa); PBS, phosphate-buffered saline; PCR, polymerization chain reaction; Rat-AI, oligonucleotide probe for rat apoA-I mRNA; Rat-AIV, oligonucleotide probe for rat apoA-IV mRNA; Rat B-4, oligonucleotide probe for rat apoB mRNA; Rat-C1, oligonucleotide probe for rat apoB mRNA ~100 base pairs downstream of the $apoB_L$ stop codon; Rat-M2, oligonucleotide probe for rat apoB mRNA ~100 base pairs upstream of the $apoB_L$ stop codon; Rat-SEQ cDNA oligonucleotide probe complementary to one of the PCR amplified strands of the $apoB_L$ stop codon region; SD-SEQ, oligonucleotide probe for human apoB mRNA upstream of the substitution site.

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LDL, while the smaller form, apoB-48 (MW ~250 kDa) is a component of chylomicrons (2, 22). Similarly, rats produce two forms of apoB, a large molecular form, B_H (MW ~335 kDa), and a small molecular form, B_L (MW ~240 kDa) (23–25). In rats, the smaller apoB form is a chylomicron and VLDL constituent, and also appears to be a component of both intestinal and hepatic HDL (2, 23–27). The apoB-48 amino acid sequence is identical to the amino terminal apoB-100 sequence and does not contain the carboxyl terminal domain of apoB-100 which recognizes the LDL receptor (28–34). The liver and intestine transcribe a large ~14–14.5 kb mRNA for this protein (30, 35–38). In addition, a smaller ~7–7.5 kb mRNA has been found in the intestine (30, 35–38). In human liver, the 14–14.5 kb mRNA is translated into a 4563-amino acid preapolipoprotein B-100, followed by cleavage of a 27-amino acid signal peptide and glycosylation (28, 30, 33, 34, 39, 40). In contrast, human intestinal apoB mRNAs (both the 7–7.5 kb and 14–14.5 kb mRNA) contain a C to U substitution at nucleotide 6666. This change generates an in-frame stop codon at amino acid 2153 and results in synthesis of a 2152-amino acid product (apoB-48) (35, 37, 38). The C to U nucleotide substitution in the apoB mRNA is a co- or post-transcriptional event and is not present in the apoB gene (35). The mechanism responsible for the C→U substitution is not yet known, but appears to be developmentally regulated in human fetal intestine (41). One possible explanation could be a site-specific deamination of the cytosine residue. In rat liver, apoB mRNA has been reported to contain either C or U at the stop codon site, resulting in translation of both $apoB_H$ and B_L (42). In rat intestine, essentially complete C to U substitution of the primary apoB mRNA transcript occurs (42–45). The present study investigates the effect of ethinyl estradiol on the molecular forms of apoB synthesized by the rat liver. A striking finding of these studies was a marked increase in the synthesis of the larger form of hepatic apoB in the estrogen-treated rat.

MATERIALS AND METHODS

Ethinyl estradiol treatment

17α-Ethinyl estradiol (Sigma Chemical Co., St. Louis, MO) in propylene glycol (1 mg/ml) was subcutaneously administered to 200–280 g Sprague-Dawley male rats (5 mg/kg per day) for 5 days between 8:30 and 10:30 AM (19). Control animals were administered the vehicle alone. Animals were allowed free access to food and water during treatments. Ethinyl estradiol-treated animals consume less food, therefore diet-control groups were included in some studies. For the determination of hepatic apolipoprotein synthesis, on the morning of day 6, 8, 12, or 19 animals were anesthetized with sodium pentobarbital and then injected with 1.0 mCi of L-[4,5-^{3}H]leucine into the portal vein to determine in vivo hepatic apolipoprotein synthesis (42). After 15 min, a blood sample was taken from the left ventricle, and livers were immediately perfused in situ with approximately 200 ml of phosphate-buffered saline (PBS) containing 20 mM leucine. Blanched pieces of liver were removed for determination of apoA-I, A-IV, B_H, and B_L synthesis rates and extraction of total RNA to determine apolipoprotein mRNA content and nucleotide sequence analysis of apoB mRNA at the C→U substitution site (see below).

Apolipoprotein synthesis measurements

Apolipoprotein synthesis rates were determined in liver by immunoprecipitation as previously described (4, 5). Briefly, liver pieces were immediately homogenized at 4°C with a Polytron (Brinkmann Instruments, Westbury, NY) in 0.15 M NaCl, 5 mM Na_2EDTA, 1% Triton X-100, 65 mM Tris-HCl, 0.1% bovine serum albumin, 2 mM leucine, 0.02% NaN_3, pH 7.4, also containing 1 mM each of leupeptin, pepstatin, PMSF, soybean trypsin inhibitor, and benzamidine. Homogenates were centrifuged for 1 h at 4°C at 40,000 rpm in a 50.3 Ti rotor (Beckman, Fullerton, CA). The resulting 105,000 *g* supernatants were stored at −70°C until analysis. Total trichloroacetic acid precipitability was determined on aliquots of the 105,000 *g* supernatant, and trichloroacetic acid-precipitable material equivalent to 4×10^5 cpm was subjected to immunoprecipitation with either excess rabbit anti-rat apoA-I, A-IV, or B sera (4, 5). Apolipoproteins in washed immune complexes were released from staph A and immune complexes dissociated by heating pellets to 90°C for 3 min in 10% glycerol, 5% 2-mercaptoethanol, 2.5% sodium dodecyl sulfate in 65 mM Tris-HCl, pH 8.0 (harvest buffer). Heated harvest buffer-treated pellets were centrifuged and the supernatant fraction was separated by 4% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (46). The cylindrical gels were sliced into approximately thirty 2.0-mm slices. Gel slices were incubated overnight at 35°C in 6 ml of Econofluor-3% Protosol (New England Nuclear, Boston, MA) and their radioactive content was determined by liquid scintillation spectroscopy. The specificity of anti-apoA-I and anti-apoA-IV toward their respective antigens, and anti-apoB toward $apoB_L$, and conditions for immunoprecipitation with excess antibody have been previously described in detail (4, 5, 7, 47). When anti-apoB sera were used, only two radioactive bands were observed on all gels that comigrated with $apoB_H$ and B_L of rat VLDL. The addition of excess non-radioactive rat LDL to the 105,000 *g* hepatic supernatant completely displaced both band. Radiolabeled apolipoproteins were within 10% of each other for duplicate immunoprecipitations. Percent of total synthesis was determined by summing the background corrected cpm under the apolipoprotein peaks divided by trichloroacetic acid-

precipitable radioactivity. To compare synthesis rates of apoB_H to B_L on a molar basis, a correction was made that assumed 1.40 times more [^{3}H]leucine would be incorporated per apoB_H (335 kDa) than B_L (240 kDa) molecule (23–25), based on the known leucine content of each form (48).[4]

Messenger RNA measurements

Oligonucleotide probes. The oligonucleotides used in this study were synthesized on an Applied Biosystems 380A DNA synthesizer. Rat-AI (5'-TTCAAACTGGGACACA-TAGTCTCTGCCGCTGTC-3') recognizes a sequence 67-99 nucleotides downstream of the first base of rat apoA-I mRNA (49, 50). Rat-A-IV (5'C**G**GCCTCCTTGGCATT-GTTGCT(T/**C**)AGCTGG(G/**C**)TGAAGTAGTCCCACA-TCAC-3')[5] recognizes a nucleotide sequence 28–76 bases downstream from the first base of mature rat apoA-IV mRNA (51, 52). Rat-B4 (5'-TTGTCTCTCCCAGTCAG-ATGCATTCAGATA-3') recognizes a nucleotide sequence 135–164 bases upstream of the rat apoB_L stop codon (42). Rat-C1 (5'-CAATGATTCTATCAATAATCTG-3') and Rat-M2 (5'-TACTAATTGCCTTAGATAGTGCC-3') hybridize to sequences approximately 75 bases downstream (Rat-C1) and upstream (Rat-M2) of the apoB_L stop codon. These oligonucleotides were used in the polymerase chain reaction (PCR) amplification of a 153 bp fragment of apoB mRNA containing the site of the C→U substitution. Rat-SEQ (5'-GCCAAAATCAACTTGAATG-3') hybridizes to one strand of the 153 bp amplified region just downstream from Rat-M2. It was used to sequence through the region of the C→U substitution. SD-SEQ (5'-GCCAAAATCAACTTTAATG-3') hybridizes to both human apoB-48 and apoB-100 transcripts upstream of the substitution site, and was used to quantitate in vitro transcription products. Oligonucleotides were labeled with gamma ^{32}P-ATP (>5000 Ci/mmol, Amersham Corp., Arlington Heights, IL) using T_4 polynucleotide kinase (United States Biochemical, Cleveland, OH) as described (53), for use as hybridization probes or sequencing primers.

Apolipoprotein mRNA levels. Total liver RNA was extracted as previously described (54). Briefly, pieces of liver were homogenized on ice in 6 M urea, 3 M LiCl for 1 min with a Polytron. RNA was precipitated overnight at 4°C, pelleted by centrifugation, and purified by phenol and chloroform extraction. Hepatic RNA (triplicate 10-μg aliquots for each sample) samples were applied to separate nitrocellulose filters (Amersham Corp., Arlington Heights, IL) using a Minifold II slot blotter (Schleicher and Schuell, Inc., Keene, NH). Filters were sequentially probed for apoA-I, apoA-IV, apoB, and polyadenylated RNA with ^{32}P-labeled oligonucleotide probes. Between hybridization steps, filters were washed twice at 75°C for 1 h in 5 mM Tris-HCl, pH 8.0, 2 mM Na_2EDTA, 0.1 × Denhardt's solution, and confirmed radioactivity-free prior to rehybridization with successive probes. Hybridizations and washings were at 55°C for ^{32}P-Rat-AI, 60°C for ^{32}P-Rat-AIV, 54°C for ^{32}P-Rat-B4, and room temperature (~21°C) for ^{32}P-oligo-dT_{15} (Boehringer Mannheim Biochemicals, Indianapolis, IN) (55) in conditions described by Church and Gilbert (56). Filters were exposed to Kodak X-AR5 film for up to 24 h with Kodak X-Omatic intensifying screens. The relative signal intensities for apolipoprotein and polyadenylated mRNA were measured by scanning of the autoradiograms with a Quick Scan Jr. densitometer (Helena Laboratories, Beaumont, TX).

Hepatic apoB mRNA sequence at the substitution site

Hepatic RNA from seven control and eight ethinyl estradiol-treated animals was subjected to selective amplification by PCR of a 153-bp region surrounding the site of the apoB_L stop codon as follows. Total liver RNA (5 μg) was denatured at 65°C in the presence of oligonucleotide Rat-C1, followed by annealing at 45°C for 20 min. cDNA was synthesized as described (57) except that magnesium chloride concentration was decreased to 3 mM and deoxynucleotides were decreased to 0.5 nM for compatibility with the subsequent amplification step.

The cDNA synthesis reaction was used directly in the amplification reaction. The final conditions for PCR were; 15 mM Tris-HCl, pH 8.3, 50 mM KCl, 2.5 mM $MgCl_2$, 0.15 mM each dNTP, 0.1% gelatin, 20 pmoles each of primer Rat-C1 and Rat-M2, and 1.7 units of Taq polymerase (Perkin-Elmer, Cetus, Norwalk, CT). The reaction mixture was overlayed with 50 μl mineral oil and underwent 25 cycles (94°C, 1 min; 55°C, 2 min; 72°C, 1 min) of amplification. Mineral oil was extracted with chloroform and unincorporated primers and deoxynucleotides were removed by centrifugation in a Centricon-30 microconcentrator (Amicon, Lexington, MA).

Direct sequence analysis of the amplified products was as described by Higuchi et al. (58) using ^{32}P-Rat-SEQ as primer. Samples were heat-denatured and electrophoresed in 7 M urea, 7% polyacrylamide sequencing gels. Gels were autoradiographed with Kodak X-AR5 film for 8–24 h. Levels of apoB_H and B_L mRNA were estimated by laser densitometry (300 Å computing densitometer, Molecular Dynamics, Sunnyvale, CA) of the C and T

[4]To our knowledge, the complete amino acid sequences of rat apoB_H and B_L are unknown, and the exact molecular weights of these proteins were estimated by prior analysis by SDS-polyacrylamide gel electrophoresis (23–25). Although we used a factor of 1.4 to correct synthesis on a mole basis (assumes apoB_H is 335 kDa), the studies of Sparks et al. (48) suggest that rat apoB_H is approximately 500 kDa (i.e., a correction factor of 2.1 should be used). When data are corrected by either factor, statistical significance does not change.

[5]This probe is used in studies to detect human apoA-IV mRNA and therefore was made to contain a human specific base at nucleotide 75 and equimolar mixtures of rat and human bases at nucleotides 47 and 54 (as indicated in bold and underlined) (51, 52).

bands corresponding to nucleotide 6666 of the human apoB mRNA sequence.

Verification of apoB mRNA substitution site assay

The verify the assay used to determine the ratio of apoB (non-stop) to apoB (stop) mRNA we analyzed RNA samples containing known amounts of apoB (non-stop) and apoB (stop) transcripts. Plasmids pHC-07 and pHT-AC contain 200 bp regions of human apoB-100 and B-48 cDNA sequence, respectively, inserted downstream from the T7 promoter in Bluescript KS+ (Strategene, San Diego, CA). Transcripts were synthesized in vitro from each plasmid. Concentrations of apoB-100 and B-48 transcripts were determined by hybridization analysis with radiolabeled SD-SEQ oligonucleotide, followed by densitometry of the autoradiogram. The in vitro transcription products were combined in different ratios, then subjected to enzymatic amplification and sequence analysis as described. For each sample, the ratio of apoB-100 to apoB-48 was determined by laser densitometry of the C and T bands corresponding to nucleotide 6666 in the sequencing autoradiogram.

Measurement of mRNA synthetic rates

Messenger RNA synthetic rates were measured by a nuclear run-on assay (59–61). In these preliminary studies, rat hepatic nuclei were isolated on day 6 from animals treated for 5 days with ethinyl estradiol or vehicle (ad libitum and diet control groups). Nuclei (3.5×10^7) were incubated with 180 μCi α-^{32}P-UTP, 1 mM each of ribo-GTP, ATP, and CTP, 350 U RNasin, 10 mM phosphocreatine in 300 mM $(NH_4)_2$ SO_4, 4.0 mM $MgCl_2$, 200 mM NaCl, 0.4 mM EDTA, 4.0 mM $MnCl_2$, 0.1 mM PMSF, 1.2 mM dithiothreitol in 40% glycerol, 100 mM Tris-HCl, pH 7.9 (total incubation volume 250 μl) for 30 min at 26°C with periodic agitation. Two hundred μg yeast tRNA (26 μl) and 92 U DNase I (4 μl) were added and incubation was continued for an additional 20 min at 26°C. After the addition of 280 μl of 2% sodium dodecyl sulfate, 20 mM EDTA, 40 mM Tris-HCl, pH 7.8, and 10 μl proteinase K (100 μg), incubation continued overnight at 42°C. RNA was extracted with guanidine thiocyanate. Centricon 30 tubes were used to concentrate RNA and remove unincorporated nucleotides. RNA was stored at −20°C in 100 μl 5 mM EDTA, 300 mM sodium acetate, 50 mM Tris-HCl, pH 7.2, and 1 ml ethanol.

HB 101 competent cells (BRL) were transformed with rat apoA-I cDNA (pAI-3) in pBR322 and rat apoA-IV cDNA in pSP65 (Promega Biotec, Madison, WI). These plasmids were a gift from Dr. Jeffrey I. Gordon (Washington University School of Medicine, St. Louis, MO). Transformations were confirmed by appropriate antibiotic resistance and the appropriate sized *Pst*I (apoA-I) and *EcoR*1 plus *Hind* III (apoA-IV) restriction fragments. A cDNA library was constructed in lambda gt10 from Sprague-Dawley rat liver mRNA. Clones containing apoB cDNA were identified by plaque hybridization with 5' end-labeled oligonucleotide Rat-Cl. An apoB fragment approximately 1.3 kb in size was subcloned into vector Bluescript KS+ yielding plasmid pRB7L2. Plasmids were used to detect apolipoprotein transcripts in the nuclear run-on experiments.

Just prior to use, aliquots of plasmid cDNA (60 μg) were dried on a Speed Vac, and pellets were boiled in 25 μl of 0.1 N NaOH for 8 min, followed by cooling on ice and addition of 1.2 ml of 2.5 M NaCl. Plasmid cDNA (5 μg/slot) was blotted on nitrocellulose filters, followed by baking (80°C/h h), and incubation in prehybridization buffer overnight at 42°C. Individual filters (containing apoA-I, apoA-IV and apoB cDNA) were incubated with $1.6–8.6 \times 10^6$ cpm RNA for 48 h at 42°C. Washed filters were exposed to Kodak X-Omat film for 44 h at −70°C. Exposed film was quantitated by densitometry. Relative percent synthesis is expressed as 100 × densitometric area(mm^2)/total RNA cpm.

Measurement of plasma cholesterol and apolipoproteins

Plasma cholesterol was determined by the enzymatic method of Allain et al. (62). Plasma apoA-I, apoA-IV, and apoB were determined by immunoelectrophoresis (63, 64). Electrophoresis plates (20 × 10 cm) contained 5%

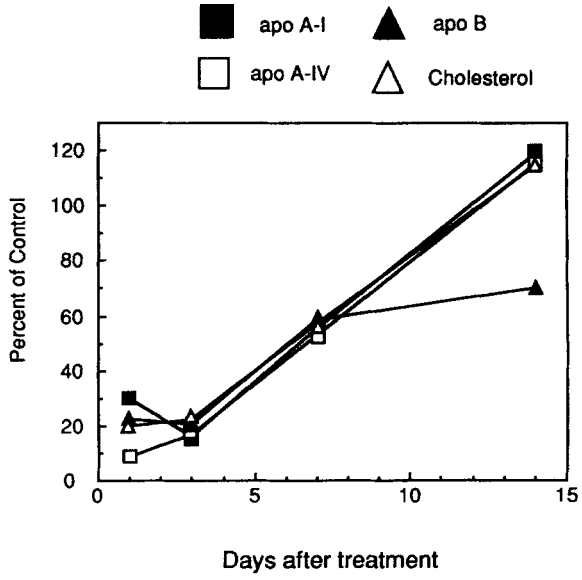

Fig. 1. Effect of ethinyl estradiol on plasma apolipoproteins and cholesterol. Rats were treated with propylene glycol vehicle or ethinyl estradiol for 5 days (5 mg/kg per day) and allowed to recover for up to 14 days. Plasma was assayed for apoA-I (■), apoA-IV (□), apoB (▲), and cholesterol (△). Each point represents the average of determinations from 5 to 13 animals.

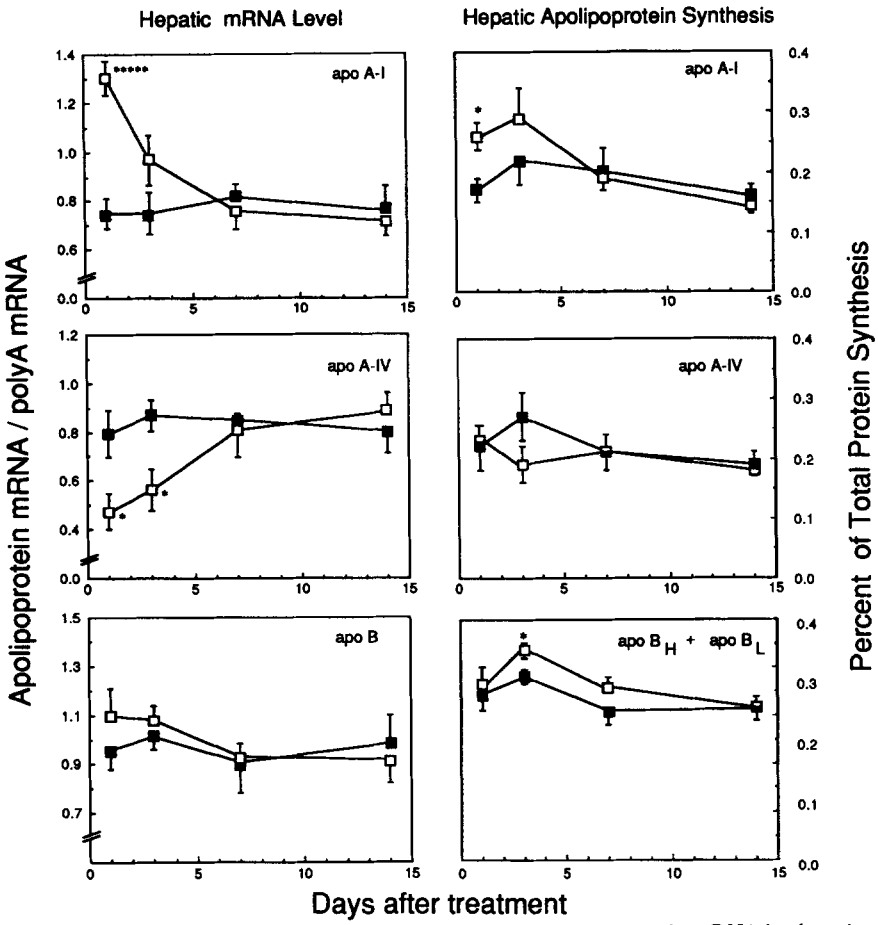

Fig. 2. Effect of recovery from ethinyl estradiol treatment on hepatic apolipoprotein mRNA levels and synthetic rates. Rats were treated with a propylene glycol vehicle (■) or 5 mg/kg per day ethinyl estradiol (□) for 5 days and allowed to recover for up to 14 days, as described in Materials and Methods. Total hepatic RNA was extracted and quantitated by slot blot analysis as described in Materials and Methods. Triplicate determinations were made from each rat. Data represent mean ± SEM of specific apolipoprotein mRNA/PolyA mRNA from 5 to 13 animals per group (three left side panels). In addition, livers were labeled with [^{3}H]leucine in vivo and after 15 min apoA-I, apoA-IV, and apoB de novo synthetic rates (three right side panels) were determined by immunoprecipitation as described in Materials and Methods. Duplicate determinations were made from each sample. Data represent mean ± SEM of apoA-I, apoA-IV, and apoB synthesis from 5 to 13 animals per group. Significance determined by unpaired two-sided *t*-test. *, ($P < 0.05$); ****, ($P < 0.001$).

rabbit anti-rat apoA-I, apoA-IV, or apoB sera in 1% agarose in 45 mM barbital buffer, pH 8.8. For all immunoelectrophoresis assays, plasmas were initially incubated in 45 mM barbital buffer, pH 8.8, containing either 1% Triton X-100 (apoB assays) or 4 M urea (apoA-I and apoA-IV assays) for 30 min at 37°C. Samples were then applied to wells and electrophoresed for 16 h at 1 mA/cm. For all assays, appropriate dilutions of unknown plasmas were made to fall within a range in which rocket height was linearally related to concentration.

RESULTS

Plasma levels of cholesterol, apolipoproteins B, A-I, and A-IV were significantly decreased by 82%, 78%, 70%, and 92%, respectively, in rats treated for 5 days with ethinyl estradiol (5 mg/kg per day). After prolonged cessation of drug treatment, plasma apolipoprotein and cholesterol levels approached normal levels (**Fig. 1**). The marked reduction in both plasma LDL and HDL is consistent with earlier studies on the pharmacological effects of this hormone in rats (11–13, 15–19). In this experiment, ethinyl estradiol treatment lowered but did not cause a significant difference in animal weight when assessed on day 6. However, after cessation of the drug, a significant difference in animal weights was observed on days 8, 12, and 16. Thus, in subsequent studies (see below) reduced-chow intake groups (diet-controls) were included that were weight matched.

Significant effects on apolipoprotein synthesis were observed in the rat liver after estrogen treatment (**Fig. 2**). Both hepatic apoA-I synthesis and mRNA levels were significantly elevated by estrogen treatment. After cessation

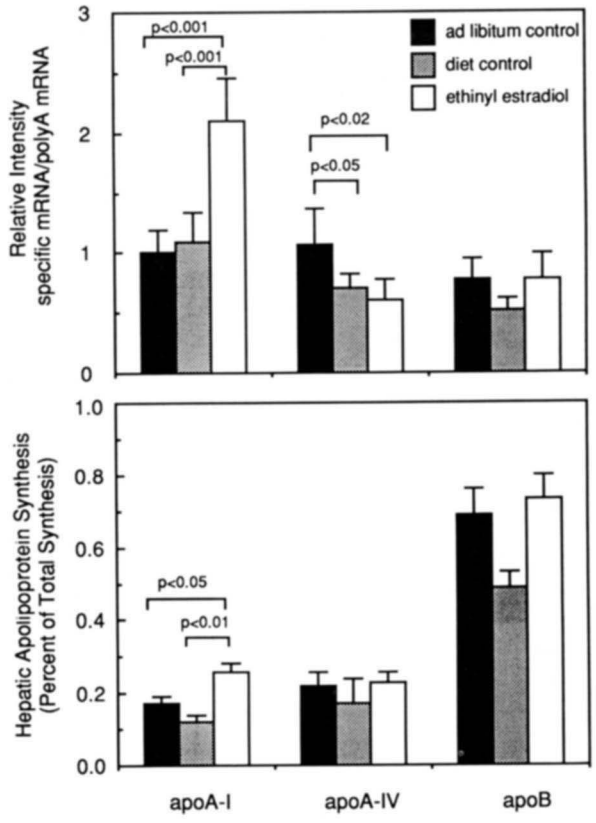

Fig. 3. Effect of ethinyl estradiol and caloric intake on hepatic mRNA levels and apolipoprotein synthesis rates. Rats were treated with a propylene glycol vehicle (■) or 5 mg/kg per day ethinyl estradiol (□) for 5 days and killed the following day. For diet control studies (▩), animals were individually housed and food consumption of the ethinyl estradiol group was determined daily. Diet-control animals were fed the following day the average amount of food consumed by the ethinyl estradiol-treated rats. Hepatic mRNA levels ± SEM (top panel) from ad libitum control (n = 7), diet-control (n = 5), and ethinyl estradiol-treated (n = 5) rats were quantitated as described in Fig. 2 and Material and Methods. All samples were analyzed on the same filters, which were subsequently blotted for polyA mRNA. Hepatic apolipoprotein synthesis rates ± SEM (bottom panel) were determined as described in Materials and Methods from ad libitum control (n = 7), diet-control (n = 5), and ethinyl estradiol-treated (n = 13) rats. Significance was determined by an unpaired two-sided *t*-test.

of ethinyl estradiol treatment, hepatic apoA-I synthetic rates and mRNA levels returned to normal. Additional studies suggest the effects on apoA-I were drug-specific and were not related to reduced dietary intake in the ethinyl estradiol-treated rat (**Fig. 3**). Nuclear run-on experiments measured on day 1 after 5 days of ethinyl estradiol treatment suggest an approximate doubling of the apoA-I mRNA synthetic rate. These preliminary data (mean ± SEM) for apoA-I mRNA synthesis (relative percent synthesis) were: vehicle-treated, 0.99 and 0.95 (n = 2), vehicle-treated diet-control, 0.86 ± 0.29 (n = 3), and ethinyl estradiol-treated, 1.75 ± 0.36 (n = 3). Hepatic apoA-IV synthesis was unchanged (Fig. 2) despite a significant decrease of apoA-IV mRNA levels. Caloric restriction to that level normally utilized by ethinyl estradiol-treated rats similarly resulted in reduced hepatic apoA-IV mRNA levels (Fig. 3). Our preliminary nuclear run-on data for apoA-IV mRNA synthesis rates suggest no difference between groups; vehicle-treated, 0.45 and 0.57 (n = 2), vehicle-treated diet-control, 0.44 ± 0.12 (n = 3), and ethinyl estradiol-treated, 0.24 and 0.40 (n = 2). Total hepatic newly synthesized apoB ($apoB_H$ plus $apoB_L$) and apoB mRNA levels were unaffected by estrogen (Fig. 2) or caloric intake (Fig. 3). Our preliminary nuclear run-on studies suggest apoB mRNA synthesis was unaffected by estrogen or caloric restriction; vehicle-treated, 3.06 and 2.91 (n = 2), vehicle-treated diet-control, 2.64 ± 0.63 (n = 3), and ethinyl estradiol-treated, 3.15 ± 0.16 (n = 3). However, examination of the molecular forms of hepatic apoB in the ethinyl estradiol-treated rat revealed striking alterations in the synthetic rates. Typically, in control rats, [^{3}H]leucine is incorporated into both $apoB_H$ and $apoB_L$; however, $apoB_L$ is the predominant form synthesized (**Fig. 4**). In contrast, livers from ethinyl estradiol-treated rats synthesize about equal amounts of $apoB_H$ and $apoB_L$ (Fig. 4). $ApoB_H$ and $apoB_L$ synthesis rates from ad libitum control (n = 7), diet-control (n = 5) and ethinyl estradiol-treated (n = 13) rats are shown in Fig. 4. Compared to ad libitum control rats, ethinyl estradiol treatment significantly increased $apoB_H$ synthesis, while $apoB_L$ synthesis was significantly de-

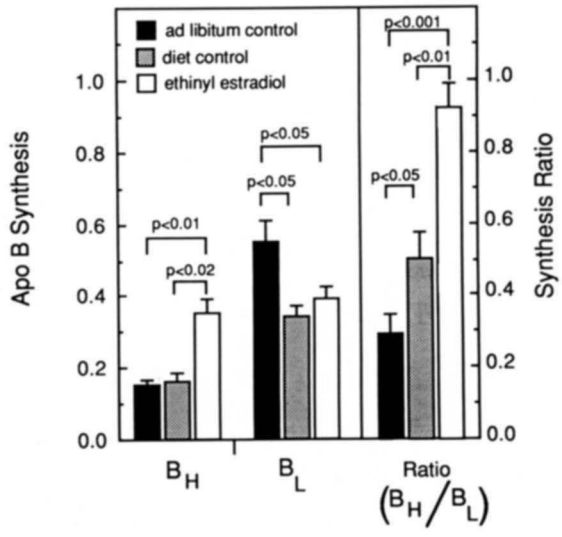

Fig. 4. Effect of ethinyl estradiol and caloric intake on hepatic molecular forms of apoB. Hepatic $apoB_H$ and $apoB_L$ de novo synthesis rates and the molar ratio of $apoB_H$ to $apoB_L$ synthesis rates were determined in vivo in ad libitum control (■) (n = 7), diet-control (▩) (n = 5) or ethinyl estradiol-treated (□) (n = 13) rats as described in Materials and Methods. To directly compare synthesis rates of $apoB_H$ to $apoB_L$ on a molar basis, $apoB_H$ values were divided by 1.4 to correct for the increased number of leucines per $apoB_H$ molecule as described in Materials and Methods. Values represent mean ± SEM of duplicate immunoprecipitations from each liver. Significance was determined by an unpaired two-sided *t*-test.

creased. This is also evident by the increased ratio of newly synthesized apoB$_H$ to apoB$_L$ (Fig. 4). In the caloric restricted control group, we observed a decreased apoB$_L$ synthesis, but no change in the synthesis of apoB$_H$ (Fig. 4). During the recovery phase, hepatic apoB synthesis rates returned to normal in the ethinyl estradiol-pretreated rats (**Fig. 5**). Using an assay that accurately

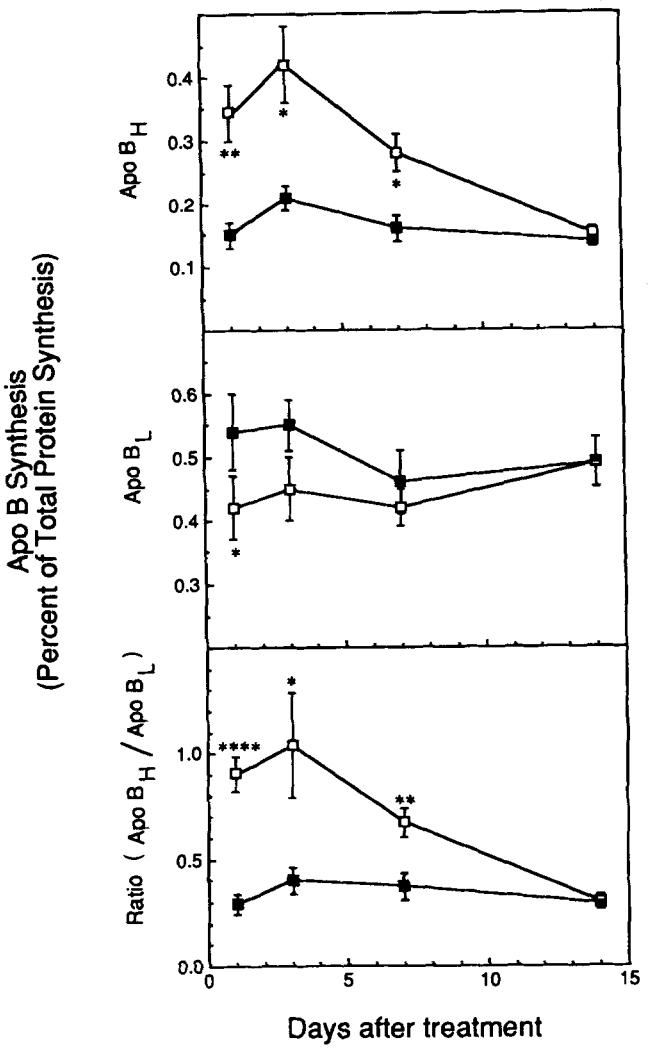

Fig. 5. Effect of recovery from ethinyl estradiol treatment on hepatic apoB synthetic rates. Rats were treated with a propylene glycol vehicle (■) or 5 mg/kg per day ethinyl estradiol (□) for 5 days and allowed to recover for up to 14 days, as described in Materials and Methods. Livers from control and ethinyl estradiol-treated rats were labeled with [^{3}H]leucine in vivo and after 15-min apoB$_H$ and apoB$_L$ de novo synthetic rates were determined by immunoprecipitation as described in Materials and Methods. Duplicate determinations were made from each sample. To compare synthesis rates of apoB$_H$ to apoB$_L$ on a molar basis, a correction was made that assumed 1.40 times more [^{3}H]leucine would be incorporated per apoB$_H$ (335 kDa) than B$_L$ (240 kDa) molecule (23–25), based on the known leucine content of each form (48). Data represent mean ± SEM of apoB$_H$ synthesis (top panel), apoB$_L$ synthesis (middle panel) and the ratio of apoB$_H$ to apoB$_L$ synthesis (bottom panel) from 5 to 13 animals per group. Significance was determined by unpaired two-sided *t*-test. *, ($P < 0.05$); **, ($P < 0.01$); ****, ($P < 0.001$). Total apoB synthesis (apoB$_H$ plus apoB$_L$) is shown in Fig. 2.

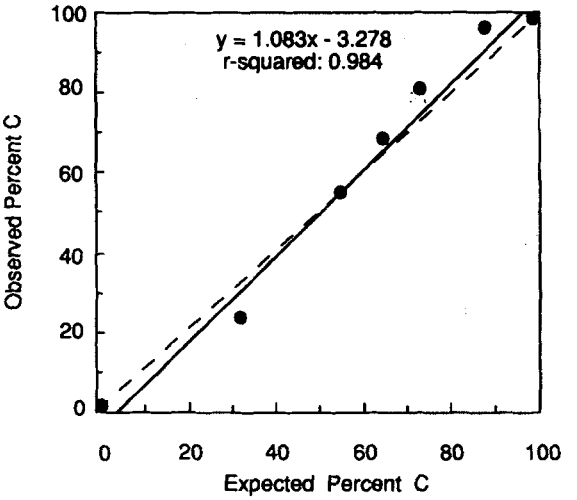

Fig. 6. Verification of apoB mRNA substitution site assay. The substitution site assay was verified using known mixtures of 100% human apoB-100 (non-stop) to 100% apoB-48 (stop) mRNA. These mixtures (n = 7) were amplified by the polymerase chain reaction (25 cycles) and subjected to sequence analysis as described in Materials and Methods. The regression line relating the observed and expected values (solid line) was compared to the line of identity (dashed line) using an F-test. No significant differences were observed.

determines the ratio of C to U at the apoB substitution site (**Fig. 6**), we analyzed the frequency of the stop codon, in ad libitum control and ethinyl estradiol-treated animals (**Fig. 7**). A decreased amount of C to U substitution (increased amount of apoB$_H$ mRNA) was found in the ethinyl estradiol-treated group. This result is correlated with the increase in apoB$_H$ synthesis observed in the ethinyl estradiol-treated rat (Fig. 7).

DISCUSSION

In the present study we investigated the effects of ethinyl estradiol on hepatic apolipoprotein synthesis. The rationale for these studies was to determine whether severely reduced plasma levels of lipids and apolipoproteins affected rates of apolipoprotein synthesis in rat liver. The liver is the major catabolic site for LDL. Chylomicron remnants and possibly whole HDL particles, by virtue of their apoE content, are primarily cleared by liver-LDL and possibly remnant-specific (i.e., apoE) receptors (1, 9, 65).

The studies of Davis and Roheim (12) demonstrated that pharmacological doses of ethinyl estradiol (5 mg/kg per day) administered to rats result in a rapid and near complete removal of plasma LDL and HDL. This hypolipidemic effect was primarily due to an increased number of hepatic LDL-receptors that recognize both LDL and apoE-containing HDL (11, 17–21). In these studies (19, 20), LDL-receptor number remained high despite continuous hepatic cholesterol delivery. In the present studies

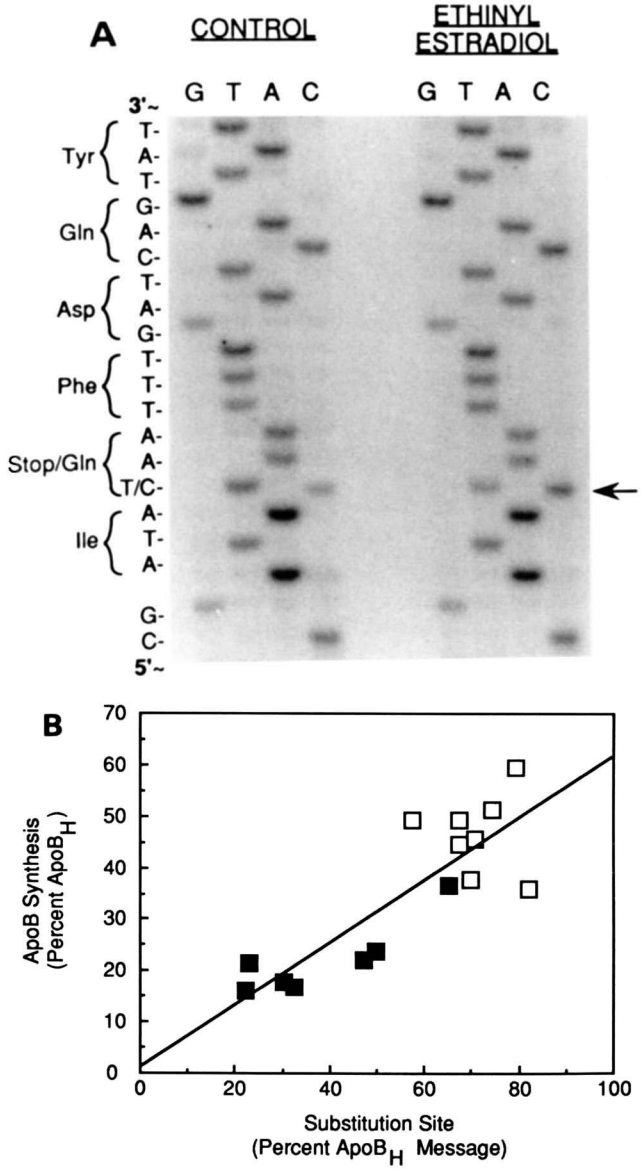

Fig. 7. Nucleotide sequence analysis of the apoB mRNA C→U substitution site. Rats (ad libitum fed) were treated with a propylene glycol vehicle (■) (n = 7) or 5 mg/kg per day ethinyl estradiol (□) (n = 8) for 5 days and killed the following day. Hepatic RNA at the region of the substitution site was amplified by the polymerase chain reaction (25 cycles) and subjected to sequence determination as described in Materials and Methods. Shown are typical sequence gels from a control and an ethinyl estradiol-treated rat (A). The relative proportion of C to T in the amplified cDNA at the substitution site was determined by laser densitometry. Values for all animals are shown as the relationship between percent apoB$_H$ mRNA and percent apoB$_H$ synthesis (B). Correlation coefficient and significance of correlation (by analysis of variance) were $r^2 = 0.73$ and $P < 0.0001$ for the regression line $y = 0.6046x + 1.1398$.

we investigated the effects this hypolipidemic agent has on regulation of hepatic apolipoprotein synthesis.

Ethinyl estradiol showed variable effects on hepatic apolipoprotein synthesis (Fig. 2–5). Specifically, we observed increased hepatic apoA-I synthesis rates and mRNA levels. Preliminary nuclear run-on analysis suggests hepatic apoA-I mRNA synthesis was elevated by 80.4% in the ethinyl estradiol-treated compared to the ad libitum control rats. Reducing caloric intake to the level consumed by ethinyl estradiol animals did not effect the observed elevation of apoA-I mRNA synthesis, apoA-I levels (Fig. 3) or protein synthesis (Fig. 3). Although ethinyl estradiol caused the hepatic apoA-IV mRNA to decrease, the apoA-IV synthesis rate was unaltered (Fig. 3). However, hepatic apoA-IV mRNA levels were also reduced in diet control rats (Fig. 3), suggesting that factors other than ethinyl estradiol were responsible for the observed effect. Despite reduced hepatic apoA-IV mRNA levels, mRNA synthesis (preliminary data) and apoA-IV protein synthesis were unaltered (Fig. 3). The exact mechanism for this finding is unclear. Further studies are needed to determine the mechanisms involved. The organ explant and polysome runoff studies in rats chronically fed atherogenic diets suggest that intestine and hepatic apoA-I and apoA-IV are translationally regulated (66). In the present studies using the estrogen-treated rat, hepatic apoA-I transcription, mRNA levels, and protein synthesis were all elevated, and suggest apoA-I can also be pre-translationally regulated.

A major finding of this study was that the estrogen-treated rat exhibited marked changes in the form of apoB synthesized by the liver. While total rates of apoB mRNA synthesis, apoB synthesis, and total apoB mRNA levels were unchanged by estrogen treatment (Fig. 2), a striking increase in the synthesis of the larger form of apoB (apoB$_H$) and a marked decrease in apoB$_L$ synthesis were observed (Figs. 4–7). Decreased caloric intake resulted in a reduction in the amount of apoB$_L$ synthesized and no change in apoB$_H$ synthesis (Fig. 3). Recent studies in the propylthiouracil-induced hypothyroid rat have demonstrated that pharmacological doses of thyroxine increased the relative amount of hepatic apoB$_L$ to apoB$_H$ mRNA (42). Thyroid hormone replenishment in these rats stimulated formation of the stop codon in the rat liver apoB mRNA. Whether this was a direct effect of thyroxine is unknown; it was the first demonstration of a possible hormonal regulation of apoB mRNA stop codon. The present study demonstrates that pharmacological concentrations of ethinyl estradiol also regulate the form of apoB synthesized by the rat liver. However, with ethinyl estradiol, unlike thyroxine, we found a marked increase in apoB$_H$ synthesis. This result could occur in several ways. Estrogen has been shown to stabilize apolipoprotein mRNA in the avian liver (67) and could selectively stabilize apoB$_H$ mRNA. It is also possible that estrogen inhibits the mechanism by which a stop codon is inserted into apoB mRNA. Perhaps estrogen inhibits the putative deaminase thought to be responsible for this effect, and results in a greater synthesis of apoB$_H$ as observed in the present studies. An additional mechanism altering the form of

apoB synthesized by rat liver could result from the enhanced catabolism of lipoproteins resulting from estrogen treatment. We were unable to demonstrate a direct effect of estrogen or lipoprotein depletion on enhancing the level $apoB_H$ mRNA in primary cultures of rat hepatocytes or rat McArdle 7777 hepatoma cells.[6] Other studies are currently in progress to elucidate the mechanisms involved.

The present studies using an estrogen-treated rat demonstrate that, within the liver, variable effects on apolipoprotein synthesis are also observed. Significantly, the ability to influence the molecular form of apoB synthesized by rat liver may provide a useful model to explore the mechanisms involved in apoB biosynthesis.

This work was supported by Grants AM-21367 and HL-21006 (Specialized Center of Research in Arteriosclerosis) from the National Institutes of Health. The authors thank Mary V. Siebenkas for technical assistance with parts of these studies. The authors are grateful to Dr. Mary Sorci-Thomas for providing us with a protocol used in our nuclear-run on assays and to Dr. Jeffrey Gordon for providing us with rat apoA-I and apoA-IV plasmids.

Manuscript received 19 September 1990 and in revised form 20 February 1991.

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