Molecular cloning of partial cDNAs for rabbit liver apolipoprotein B and the regulation of its mRNA levels by dietary cholesterol

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ABSTRACT Apolipoprotein B (apoB) is the major protein of plasma very low density lipoprotein (VLDL) and low density lipoprotein (LDL). Here we report the molecular cloning of cDNAs for rabbit liver apoB, by use of the expression vector Agt11, and the use of these cDNAs to study the regulation of apoB mRNA levels by dietary cholesterol. The β-galactosidase-apoB fusion proteins expressed by recombinant clones were identified with guinea pig anti-rabbit LDL antibodies. The cloned cDNAs hybridized to an 18-kilobase mRNA that was present in liver and intestine. Slot blot analysis showed that this mRNA was not present in other tissues studied, with the possible exception of kidney. When rabbits are fed a high-cholesterol diet, they develop severe hypercholesterolemia. Most of the excess cholesterol is contained in β-VLDL, a cholesteryl ester-rich lipoprotein that contains apoB and apoE. We addressed the question of whether increased apoB mRNA levels, and by inference increased apoB synthetic rates, are responsible for the accumulation of β-VLDL. A comparison of apoB mRNA levels showed that cholesteryl-fed rabbits had lower liver apoB mRNA levels than control rabbits. We suggest that the accumulation of plasma β-VLDL in cholesteryl-fed rabbits is not due to an increased production of β-VLDL but solely due to a suppression of hepatic LDL receptors.

Apolipoprotein B (apoB) plays a central role in cholesterol metabolism. It is an obligatory constituent of chylomicrons, very low density lipoproteins (VLDL), and low density lipoproteins (LDL) (1). Circulating apoB exists in two forms which are immunologically related. ApoB-100 has a molecular weight of about 400,000 and is made in the liver, whereas apoB-48 has a molecular weight of about 200,000 and is made in the intestine (2, 3). The liver secretes apoB-100 into circulation as a structural protein of triacylglycerol-rich VLDL, which is converted to cholesteryl-rich LDL by the action of lipoprotein lipase (4). Despite the abundance of apoB in plasma, its structure and biosynthesis have not been well-characterized. This in large part is due to its size, its insolubility when delipidated, its tendency to aggregate, and its susceptibility to oxidation. Some progress has recently been made, however, with the identification and sequencing of partial cDNA clones for human and rat apoB (5–10).

The concentration of plasma LDL is strongly correlated with the risk of coronary heart disease. Two factors regulate the plasma concentration of LDL: (i) the rate of VLDL synthesis and (ii) the receptor-dependent removal of LDL from circulation. The importance of the LDL receptor in the regulation of plasma cholesterol levels has been demonstrated (11). However, the question of whether the production rate of apoB contributes to elevated LDL cholesterol levels remains unclear, although evidence from a number of studies suggests that alterations in the regulation of apoB synthesis may contribute to hypercholesterolemia and atherosclerosis (12–14).

To address the question of the regulation of apoB synthesis and its importance in maintaining cholesterol homeostasis, and to determine the amino acid sequence of apoB, we have cloned cDNAs for rabbit apoB by using the expression vector Agt11. We chose the rabbit for these studies because it has been used extensively as a model for hyperlipidemia and atherosclerosis (15–18) and has a known genetic counterpart (the Watanabe heritable hyperlipidemic) for human familial hypercholesterolemia. Also, a potential rabbit model for human familial combined hyperlipidemia has been identified.

The rabbit responds to dietary cholesterol with a drastic increase in its plasma cholesterol concentration. Much of the excess plasma cholesterol is contained in cholesteryl ester-rich VLDL, which contain apoB as well as apoE and are referred to as β-VLDL. The increase in plasma β-VLDL levels in cholesteryl-fed rabbit has been attributed to a diet-induced overproduction of β-VLDL and an impaired removal of β-VLDL as a result of saturation and suppression of hepatic LDL receptors (20). To determine the contribution of apoB and β-VLDL synthetic rates to cholesterol-induced hypercholesterolemia, we have used the cloned apoB cDNAs to compare apoB mRNA levels in livers of control and cholesteryl-fed rabbits. Our data show that cholesterol-fed rabbits have lower apoB mRNA levels than control rabbits and consequently have a lower capacity to synthesize and secrete apoB and VLDL. These results suggest that the accumulation of β-VLDL in plasma of cholesteryl-fed rabbits is due not to an overproduction of β-VLDL but solely to a suppression of hepatic LDL receptors.

MATERIALS AND METHODS

Reagents. DNA polymerase I, restriction endonucleases, EcoRI methylase, bacteriophage T4 ligase, and EcoRI linkers [d(CGCAATTCGG)] were obtained from New England Biolabs; calf intestinal alkaline phosphatase, from Boehringer Mannheim; and avian myeloblastosis virus reverse transcriptase, from Seikagaku America (St. Petersburg, FL). In vitro packaging reagents were from Amersham. Oligo(dT)-cellulose type III was obtained from Collaborative Research. Ultragel AcA 34 was from LKB.

Bacteriophage and Host Strains. Escherichia coli strains Y1088, Y1089, and Y1090 and bacteriophage Agt11 were obtained from the American Type Culture Collection.

Animals. Rabbits were maintained on two separate diets: (a) a control Purina rabbit chow and (b) a cholesterol-rich diet.

Abbreviations: apo, apolipoprotein; LDL, low density lipoprotein(s); VLDL, very low density lipoprotein(s); kb, kilobase(s).

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[0.5% (wt/wt) cholesterol and 10% (vol/wt) corn oil in Purina rabbit chow]. Rabbits were killed between 9:00 and 11:00 a.m. and were not fasted for these experiments.

Poly(A)+ RNA. Liver, brain, adrenal, and spleen RNAs were isolated by the guanidinium isothiocyanate method of Chirgwin et al. (21). Kidney and intestinal RNAs were prepared by proteinase K digestion followed by centrifugation through CsCl (22). Intestinal epithelial cells were scraped from the first 50 cm of small intestine. Poly(A)+ RNA was prepared by chromatography on oligo(dT)-cellulose (23).

Agt11 cDNA Library Construction. Standard procedures were followed for enzyme reactions (23). Poly(A)+ RNA was prepared from the liver of a rabbit fed a wheat starch/casein diet (18). Double-stranded cDNA was synthesized by the method of Okayama and Berg (24) as modified by Gubler and Hoffman (25), in which second-strand synthesis is mediated by RNase H and DNA polymerase I. First-strand synthesis was primed with (dT)12-18. The cDNA was methylated with EcoRI methylase and made blunt-ended with T4 polymerase. Phosphorylated EcoRI linkers were ligated to the cDNA with T4 ligase. The cDNA was digested with EcoRI restriction endonuclease and separated from free linkers by chromatography on ACA 34 Ultragel. Complementary DNAs were then ligated to EcoRI-digested, alkaline phosphatase-treated Agt11 vector DNA (26), and packaged in vitro (23).

Preparation of IgG and Screening of the cDNA Library. Rabbit LDL was obtained from hyperlipidemic, wheat starch/casein-fed rabbits by ultracentrifugation of plasma (27). Rabbit apoE was purified as described (28). Polyvalent antibodies to native and denatured rabbit LDL and rabbit apoE were raised in guinea pigs. Guinea pig IgG was isolated by chromatography on protein A-Sepharose. Antibodies that bind to E. coli or Agt11 antigens were removed from the IgG by absorption with a lysate from E. coli Y1090 and C6 (Agt11) (29). IgG was diluted in 5% dried milk in 16.1 mM Na2HPO4/1.5 mM KH2PO4/137 mM NaCl/2.7 mM KCl (pH 7.4) with 0.1% Nonidet P-40 (blocking solution), so that 1 ng of apoB could be detected with 125I-labeled protein A on a dot blot.

The Agt11 library was screened as described (26, 30), using 125I-labeled protein A to detect antigen–antibody complexes. Phage were plaque-purified until a homogeneous phage population was obtained.

Immunoblot Analysis. E. coli Y1089 was lysogenized with Agt11 and recombinant phage (30). Freeze-thaw lysates were prepared as described (30). The lysates were treated with DNase I (5 µg/ml) for 15 min at room temperature and were subjected to electrophoresis in NaDodSO4/5% polyacrylamide gels. Proteins were stained with Coomassie brilliant blue or transferred electrophoretically to nitrocellulose. Fusion proteins were detected as described for screening the cDNA library.

Analyses of mRNA. After EcoRI digestion of the recombinant Agt11, the cDNA insert was purified by electrophoresis from a 2% agarose gel. Poly(A)+ RNA was size-fractionated by electrophoresis in a formaldehyde-containing denaturing gel (15) and blotted onto a nitrocellulose membrane for hybridization analysis (23). For slot blot analysis, poly(A)+ RNA in 50 µl of 6× SSC (1X SSC = 150 mM NaCl/15 mM trisodium citrate) was heated at 65°C for 10 min and was applied to a nitrocellulose filter, using a slot blot apparatus (Schleicher & Schuell). Blots were probed with 32P-labeled nick-translated cDNA insert (23).

RESULTS

Construction of the cDNA Library and Isolation of an ApoB Clone. A rabbit liver Agt11 cDNA library was constructed for immunological screening with antibodies directed against apoB and contained ~8 X 108 independent recombinants. Of these, 85% contained inserts, judging from the inactivation of β-galactosidase (26).

Approximately 108 phage from the rabbit liver cDNA library were screened with guinea pig anti-rabbit LDL IgG. Guinea pigs were used for antibody production because their IgG binds protein A with high affinity (31). Out of numerous putative clones in the primary screen, two, λB-1 and λB-2, were positive on repeated plaque-purification. The size of the λB-1 cDNA insert was approximately 170 base pairs, while the length of the λB-2 cDNA insert was 1.8 kilobases (kb). These two clones did not cross-hybridize.

Characterization of Fusion Protein. Clone λB-1 was characterized in more detail. To demonstrate that λB-1 did indeed encode a β-galactosidase fusion protein recognized specifically by antibodies to apoB, the fusion protein was examined by gel electrophoresis and immunoblot analysis. For this, the recombinant phage was used to lysogenize E. coli Y1089. Lysates from isopropyl β-D-thiogalactopyranoside-induced lysogens Y1089(λB-1) and Y1089(λB11) were subjected to NaDodSO4/polyacrylamide gel electrophoresis and immunoblot analysis. The β-galactosidase band present in the Y1089(λB11) lysate (Fig. 1, lane 1) was absent in the Y1089(λB-1) lysate (lane 2), which instead produced a protein with a molecular weight of ~130,000. On blots, the fusion protein reacted with anti-LDL IgG (lane 4), whereas β-galactosidase (lane 3) did not.

Since rabbit LDL contains small amounts of apoE, anti-LDL IgG also recognizes apoE, although with 1/20th the sensitivity. To confirm that the fusion protein expressed by λB-1 contained only antigenic apoB epitopes, a blot of Y1089(λB-1) proteins was probed with anti-apoE IgG. The absence of a labeled band (Fig. 1, lane 5) shows that the antigenic determinant on the fusion protein is not recognized by anti-apoE IgG. The fusion protein therefore contains antigenically responsive apoB epitopes.

Identification and Tissue Distribution of ApoB mRNA. To provide further evidence that λB-1 encodes a region of apoB, two additional criteria were used. First, the mRNA detected by blot-hybridization analysis should be large enough to

![Fig. 1. Analysis of the fusion protein encoded by λB-1. Lysates from a lysogenic E. coli Y1089 carrying agt11 (lanes 1 and 3) or λB-1 (lanes 2, 4, and 5) were analyzed by NaDodSO4/PAGE followed by staining with Coomassie blue (lanes 1 and 2) or immunoblotting with anti-LDL IgG (lanes 3 and 4) or anti-apoE IgG (lane 5). The position of β-galactosidase (β-gal, M, 116,000) is indicated.](image)
encode a protein the size of rabbit apoB ($M_r = 320,000$) (32), and second, this mRNA should be present in the liver and intestine and should be greatly diminished or absent in tissues not actively involved in lipoprotein synthesis and secretion.

Fig. 2A shows a blot of electrophoretically separated rabbit liver poly(A)⁺ RNA that was probed with $^{32}$P-labeled λB-1 cDNA. The rabbit liver mRNA recognized by the probe is about 18 kb long (lanes 1 and 2).

The intestine is the only other tissue that is known to secrete lipoproteins that contain apoB. A blot of fractionated liver poly(A)⁺ RNA from a normal rabbit and of intestinal poly(A)⁺ RNA from normal and cholesterol-fed rabbits is shown in Fig. 2B. The liver poly(A)⁺ RNA was included as a size marker. The blot was first probed with $^{32}$P-labeled λB-1 cDNA (lanes 1–3). λB-1 cDNA hybridized to a high molecular weight intestinal mRNA from both normal and cholesterol-fed rabbits, although the labeling was very faint for the normal rabbits. The same blot was probed with nick-translated λB-2 cDNA (lanes 4–6), which hybridized to a mRNA that is identical in size to the mRNA recognized by λB-1 cDNA. Fig. 2B shows the presence of the 18-kb intestinal mRNA more clearly, although the labeling was still faint for the intestinal mRNA from the normal rabbit (lane 5).

Hybridization of λB-1 cDNA to poly(A)⁺ RNA from adrenal, brain, kidney, spleen, and liver was compared on a slot blot (Fig. 3). As expected, λB-1 hybridized to liver poly(A)⁺ RNA. No appreciable hybridization was detected to brain, spleen, or adrenal poly(A)⁺ RNA, although a small amount of hybridization was observed for kidney poly(A)⁺ RNA. The low intensity of the hybridization signal for kidney poly(A)⁺ RNA precluded analysis of electrophoretically fractionated RNA to determine whether λB-1 cDNA hybridized to an 18-kb kidney mRNA.

The high concentration of the 18-kb mRNA in liver and intestine relative to other tissues is consistent with its identity as apoB mRNA.

**Regulation of the 18-kb ApoB mRNA Concentration.** To determine whether the hypercholesterolemia induced by a high-cholesterol diet is due in part to an increase in the rate of synthesis and secretion of apoB, we compared apoB mRNA levels in livers from rabbits fed a control or a high-cholesterol diet. Plasma cholesterol concentrations increased from 40–60 mg/dl for chow-fed (control) rabbits, to 300–500 mg/dl for rabbits fed the high-cholesterol diet for 2 weeks, to >1000 mg/dl for rabbits fed the high-cholesterol diet for a month or more. Contrary to expectations, blot analysis (Fig. 2A) showed a reduction rather than an increase in apoB mRNA content for cholesterol-fed rabbits. This observation was confirmed with slot blots of poly(A)⁺ RNA (Fig. 4), which demonstrated a decrease in the amount of liver apoB mRNA both after short-term (2 weeks) cholesterol feeding (column 2) and after long-term (4 months) cholesterol feeding (column 3), as compared to a control chow diet (column 1). Slot blots of liver poly(A)⁺ RNA from four additional cholesterol-fed rabbits and control rabbits gave the same results.

In contrast to the liver apoB mRNA, intestinal apoB mRNA levels are higher for cholesterol-fed rabbits (Fig. 2B). However, subsequent slot blot analyses of intestinal poly(A)⁺ RNA from four more rabbits have shown this increase to be variable, ranging from the increase seen in Fig. 2 to a modest 20% increase.

**DISCUSSION**

We have described the construction of a rabbit liver cDNA expression library in λgt11, the immunological identification of cDNA clones that encode a region of apoB, and the use of these cDNAs to demonstrate that liver apoB mRNA levels decrease in rabbits fed a high-cholesterol diet.

Clones λB-1 and λB-2 were found by screening the λgt11 library with an antibody made against LDL. The identification of λB-1 as a cDNA clone that encodes a region of apoB is based on three lines of evidence. First, the fusion protein encoded by λB-1 contains epitopes that are recognized by an antibody to apoB. Second, λB-1 cDNA hybridizes to a rabbit liver mRNA that is large enough to code for a protein the size

![Fig. 2. Blot-hybridization analysis of apoB RNA. (A) Liver poly(A)⁺ RNA (10 µg) from a chow-fed (lane 1) and a cholesterol-fed rabbit (lane 2) probed with $^{32}$P-labeled λB-1 cDNA. Molecular size markers shown correspond to fragments of HindIII-digested λ DNA. (B) Lanes 1–3 and 4–6 are autoradiographs of the same blot, hybridized with $^{32}$P-labeled λB-1 cDNA (lanes 1–3) and λB-2 cDNA (lanes 4–6), of liver poly(A)⁺ RNA (10 µg) from a chow-fed rabbit (lanes 1 and 4) and of intestinal poly(A)⁺ RNA (10 µg) from a chow-fed rabbit (lanes 2 and 5) and from a cholesterol-fed rabbit (lanes 3 and 6).](image)

![Fig. 3. Slot blot analysis of poly(A)⁺ RNA from liver, kidney, spleen, adrenal, and brain. The blot was probed with $^{32}$P-labeled λB-1 cDNA.](image)

![Fig. 4. Slot blot analysis of liver poly(A)⁺ RNA from a chow-fed rabbit (column 1), a rabbit fed a high-cholesterol diet for 2 weeks (column 2), and a rabbit fed a high-cholesterol diet for 4 months (column 3). The blot was probed with $^{32}$P-labeled λB-1 cDNA.](image)
of apoB. Based on an estimated molecular weight of 320,000 for rabbit apoB (32), a coding region of about 9 kb is needed for this protein. The 18-kb mRNA detected clearly fulfills this requirement and is consistent with the size of human and rat apoB mRNA (5–8). The third line of evidence that supports the identification of αβ-1 is that the 18-kb mRNA to which it hybridizes is present in high concentrations in liver and intestine relative to adrenal, brain, kidney, and spleen, consistent with the fact that liver and intestine are the only two tissues known to be actively involved in the synthesis and secretion of lipoproteins that contain apoB (1). αβ-1 hybridized weakly to kidney poly(A)+ RNA. Whether this represents the presence of apoB mRNA remains to be determined. However, in avian species, apoB synthesis has been reported in kidney (33). Finally, preliminary data (not shown) indicate that αβ-1 shares substantial sequence homology with the N-terminal region of the partial human apoB sequence reported by Knott et al. (7) and with the partial sequence reported by Mehrabian et al. (10).

The identification of clone λβ-2 as a cDNA that encodes a region of apoB is based on the recognition of the protein it encodes by antibodies to LDL, the hybridization of both αβ-1 and λβ-2 to 18-kb mRNA, and the finding that the relative levels of apoB mRNA detected in intestine (Fig. 2B) and liver (data not shown) of control and cholesterol-fed rabbits were the same when measured by hybridization with αβ-1 or λβ-2.

Both αβ-1 and λβ-2 cDNAs hybridize with an 18-kb mRNA in intestine and liver. Similar findings have been reported for rat (5) and human (7) intestine. Since the principal form of apoB synthesized and secreted by the intestine is apoB-48 (32), which has a molecular weight that is about one-half that of apoB-100, this raises the possibility that a separate mRNA exists for apoB-48, formed by differential splicing of the same apoB gene. In separate experiments (unpublished data), we have found that an antibody made to the fusion protein encoded by λβ-1 recognizes apoB-100 but not apoB-48, indicating that our clone corresponds to a region of apoB-100 that is not shared by apoB-48. In view of this, αβ-1 would not be expected to hybridize to an apoB-48 mRNA if it existed. We do not know the location of the λβ-2 DNA sequence in apoB. Alternatively, apoB-48 may be formed from apoB-100 by posttranslational processing. In support of this view is recent evidence that rat intestinal apoB-48 is formed from apoB-100 by proteolytic processing (34). This is also consistent with our finding that chylomicrons from cholesterol-fed rabbits contain only apoB-48 (32).

The regulation of apoB synthesis and its role in the development of hypercholesterolemia are not understood. The availability of apoB cDNAs allows us to address this question in terms of apoB mRNA levels. In this paper, we have addressed the question of whether the apoB mRNA level, and hence the capacity to synthesize apoB-containing lipoproteins, increases with cholesterol feeding. Contrary to expectations, our data show that the liver apoB mRNA concentration in cholesterol-fed rabbits is lower than in chow-fed rabbits. The clear implication is that cholesterol feeding does not result in an overproduction of apoB by the liver. Further, since β-VLDL in the cholesterol-fed rabbit is secreted by the liver (32), we conclude that there is no overproduction of β-VLDL in the cholesterol-fed rabbit.

MacKinnon et al. (35) have shown that cholesterol feeding increases the amount of VLDL protein that accumulates during a 2-hr perfusion, from 1 ± 1.5 mg per liver to 11.7 ± 4.8 mg per liver. Apart from a possible increase in the rate of secretion of apoB by livers from cholesterol-fed rabbits, two other factors may contribute to this modest increase in accumulation of VLDL total protein: (i) more VLDL is expected to accumulate in perfusates from cholesterol-fed rabbits, even if the rates of secretion of VLDL were the same, because very few of the secreted lipoproteins would be taken up by the suppressed hepatic LDL receptors in cholesterol-fed rabbits (20, 36), and (ii) the accumulated protein represents newly synthesized protein as well as lipoproteins already present at the beginning of the perfusion, which are subsequently washed out. In view of the high plasma β-VLDL concentration found in cholesterol-fed rabbits, the contribution of washed-out β-VLDL to the total perfusate VLDL could be substantial. Thus, while these data do not yield a direct comparison of the relative amounts of apoB secreted by livers from control and cholesterol-fed rabbits, they do give an upper limit and demonstrate that, at the very least, there is no appreciable overproduction of apoB by livers from cholesterol-fed rabbits.

The plasma concentration of β-VLDL is determined by its rate of synthesis and secretion into circulation and its receptor-dependent removal from circulation. In view of the fact that apoB mRNA levels decrease, we conclude that the suppression of hepatic LDL receptors is primarily responsible for the hypercholesterolemia in cholesterol-fed rabbits. This suppression has been demonstrated by membrane binding (20) and by lipoprotein blotting experiments (36). Considering the above, together with the fact that chylomicron clearance is not impaired in cholesterol-fed rabbits (19), we suggest that when the rabbit consumes a high-cholesterol diet, the influx of dietary, cholesteryl ester-rich chylomicron remnants into the liver provides a source of cholesterol that can both suppress hepatic LDL receptors and be secreted as cholesteryl ester-rich VLDL. In the face of the decreased number of LDL receptors, the resulting plasma β-VLDL accumulate, even though their rate of synthesis is not increased.

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