REPR and complementation factor(s) interact to modulate rat apolipoprotein B mRNA editing in response to alterations in cellular cholesterol flux

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Abstract Apolipoprotein B (apoB) mRNA editing is a posttranscriptional cytidine deamination involving several protein factor(s), one of which has recently been cloned. We have examined the effects of alterations in cellular cholesterol flux in the rat liver and small intestine as a means of dissecting the physiologic mechanisms regulating apoB mRNA editing, both in vivo and in isolated S-100 extracts. Hepatic cholesteryl ester accumulation was produced by feeding rats a high cholesterol diet, alone, or in combination with either ethinyl estradiol treatment, or after induction of hypothyroidism. Endogenous hepatic apoB mRNA editing decreased in parallel with the increase in cellular cholesteryl ester content ($r = -0.948$, $P < 0.001$). None of these conditions altered endogenous intestinal apoB mRNA editing. Hepatic S-100 extracts demonstrated decreased in vitro apoB RNA editing activity, in parallel with the changes observed in vivo. By contrast, the activity of intestinal S-100 extracts demonstrated a paradoxical increase in hypothyroid rats and a similar, paradoxical decrease in hyperthyroid rats, when compared to controls. Hepatic REPR mRNA, quantitated by RNase protection assay, showed a 25–50% decrease in cholesterol-fed rats. The editing activity of hepatic S-100 extracts prepared from cholesterol-fed, hypothyroid rats was restored to control levels with REPR supplementation but not with chicken intestinal S-100 extracts, suggesting that changes in REPR, but not complementation activity, may play a critical role in the regulation of apoB mRNA editing in rat liver. By contrast, the editing activity of intestinal S-100 extracts prepared from hyperthyroid animals was unaltered by supplementation with REPR, but was restored to control levels after the addition of chicken intestinal S-100 extracts. Taken together, the data suggest that tissue-specific factors regulate apoB mRNA editing in the rat and that the complex interplay of REPR and complementation factor(s) may be modulated in response to alterations in cholesterol flux, in vivo.—**Inui, Y., F. Giannoni, T. Funahashi, and N. O. Davidson.** REPR and complementation factor(s) interact to modulate rat apolipoprotein B mRNA editing in response to alterations in cellular cholesterol flux. *J. Lipid Res.* 1994. **35:** 1477–1489.

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Apolipoprotein B (apoB) is a major component of triglyceride-rich lipoproteins (chylomicrons and very low density lipoproteins) secreted by both mammalian small intestine and liver and plays a central role in the catabolism of circulating low density lipoproteins (reviewed in ref. 1). ApoB is synthesized in a tissue-specific manner, with the larger form, apoB-100, being produced in the liver from a full-length apoB mRNA, while apoB-48, which is colinear with the amino-terminus of apoB-100, is produced in the small intestine (2, 3). ApoB-48 production is linked to a posttranscriptional editing process in which a cytidine deamination alters a CAA codon in apoB-100 mRNA to a UAA and thereby changes a glutamine residue in apoB-100 to an in-frame stop codon, resulting in the translation of apoB-48 (2, 3). In humans, apoB-100 synthesis is essentially confined to the liver and hepatic apoB mRNA is virtually all unedited (2–4). By contrast, hepatic apoB mRNA is edited in some mammals, including the rat and mouse, and results in the synthesis and secretion of both apoB-100 and B-48 (5–8).

Several studies have demonstrated that rat hepatic apoB mRNA editing is regulated, both developmentally and also in response to nutritional and hormonal modulations (5, 8–10). An increase in hepatic apoB mRNA editing was found in rats treated with thyroid hormone and also in animals subjected to fasting and refeeding a high carbohydrate diet (5, 9). By contrast, animals treated with pharmacologic doses of estrogen were found to have a decrease in hepatic apoB mRNA editing (10). Although these conditions are recognized to have numerous effects on hepatic lipid metabolism, the common denominator responsible for the modulation of hepatic apoB mRNA editing is unknown. Additionally, these same manipulations failed to alter intestinal apoB mRNA editing, sug-

Abbreviations: apoB, apolipoprotein B; VLDL, very low density lipoproteins; LDL, low density lipoproteins; REPR, rat apolipoprotein B mRNA editing enzyme.

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gesting that the regulation of this process is dependent upon tissue-specific factors. Recent work has demonstrated that modulation of hepatic lipid metabolism in the rat is not necessarily accompanied by alterations in apoB mRNA editing, as animals treated with increasing doses of dexamethasone were found to have progressive, intrahepatic steatosis with no effects on apoB mRNA abundance or editing (11).

Other work has implicated the involvement of several distinct proteins in apoB mRNA editing, one of which, REPR, has recently been cloned (12). Differently sized proteins have been postulated to be involved in apoB mRNA editing by virtue of their ability to cross-link to apoB RNA and by their copurification with apoB RNA editing activity (13–15). The ability of REPR to edit a synthetic apoB RNA template in vitro requires the presence of an as yet uncharacterized complementation factor(s), suggesting that apoB mRNA editing is dependent upon the organization of a multicomponent complex (12). Evidence in support of this possibility comes from the observations that either chicken intestinal S-100 extracts or extracts prepared from human liver, neither of which alone is competent to edit an apoB RNA template, will both effectively complement REPR activity in vitro (16, 17).

We have examined the effects of altered cellular cholesterol flux in an attempt to dissect the molecular mechanisms linking alterations in lipoprotein assembly to the regulation of apoB mRNA editing. Animals were fed diets enriched in cholesterol, in some instances in the setting of simultaneous treatment with pharmacologic agents known to increase either cholesterol absorption or to increase the expression of hepatic low density lipoprotein receptors, maneuvers designed to further augment hepatic accumulation of cholesterol (18). ApoB mRNA editing was examined both in vivo and in isolated S-100 extracts prepared from the liver and small intestine. The results demonstrate a complex pattern of tissue-specific regulation and suggest that apoB mRNA editing is modulated through interactions between REPR and complementation factor(s) in the rat.

MATERIALS AND METHODS

Animal treatment protocols

Male Sprague-Dawley rats weighing 200–225 g were purchased from Charles River (Wilmington, MA). Animals were housed, five per cage, under standard conditions of temperature and light and allowed free access to water. Seven groups of animals were used in this study. (i) Control animals, which were fed the standard Purina rat chow, ad libitum. (ii) Cholesterol-fed animals, which were maintained for 2 weeks on regular chow supplemented with 2% (w/w) cholesterol (Sigma, St. Louis, MO). (iii) Estradiol-treated and (iv) cholesterol-fed/estradiol-treated rats, which were maintained for 2 weeks on either standard chow or cholesterol-rich (2% w/w) chow, then injected daily subcutaneously with 17α-ethinyl estradiol (Sigma, St. Louis, MO) dissolved in propylene glycol (1 mg/ml) at a dose of 5 mg/kg body weight for 3 days. This time point was chosen since longer treatments produced appetite loss and the animal failed to eat. (v) Hypothyroid rats, which were fed chow supplemented with 0.1% (w/w) propylthiouracil (2-thio-4-hydroxy-6-n-propylpyrimidine) (PTU) (Sigma, St. Louis, MO) for 3 weeks as previously described (19). This protocol has been established to produce marked reductions in the circulating levels of T_3 and T_4 in treated animals (19). (vi) Hypothyroid cholesterol-fed rats, which were fed 5% lard–1% cholesterol–0.3% taurocholic acid–0.1% PTU mixed with rat chow for three weeks (19). (vii) Hyperthyroid rats, which were prepared by intraperitoneal injection of T_3 (3,5,3'-triiodo-L-thyronine), 50 μg/100 g of body weight, on alternate days for 1 week (20). All animals were killed 16–20 h after the last dose.

After an overnight fast, the rats were exsanguinated and the liver and small intestine were removed. Portions were frozen in liquid nitrogen for subsequent RNA extraction and lipid analysis while aliquots of the liver and small intestine from hypothyroid, hypothyroid cholesterol-fed (hypo+chol), hyperthyroid (T_3-treated), and control groups were used for S-100 extract preparation (see below).

Analysis of serum, hepatic and intestinal lipids

Serum triglyceride and cholesterol levels were determined enzymatically using commercial kits (Sigma Diagnostics, Catalog No. 334-UV and 352). Hepatic lipids were extracted as described previously, separated by thin-layer chromatography, and triglycerides were recovered (11). Fatty acid methyl esters were prepared and analyzed by gas–liquid chromatography (11). Hepatic free cholesterol levels were determined using coprostanol as an internal standard (11). After saponification and extraction, total cholesterol was reassayed and cholesteryl ester mass was calculated as the difference between free and total cholesterol and normalized to protein concentrations (11).

RNA extraction and analysis

Total hepatic and intestinal RNA was extracted in 5 M guanidine thiocyanate as described (11). Quantitation of apoB mRNA abundance was determined by slot-blot analysis of serial dilutions (5, 2.5, 1 μg) applied directly to nylon membranes and probed with a 661 base pair rat apoB cDNA, labeled by random priming. Quantitation of apoA-I, A-IV, C-III, E, and L-FABP mRNA levels was determined by Northern analysis of 20 μg total RNA after transfer from denaturing formaldehyde agarose gels and cDNA hybridization as described (11). Autoradiograms at

comparable intensity were scanned with a laser densitometer (Ultroscan LX, LKB, Gaithersburg, MD). ApoB mRNA editing was assayed by direct primer extension as previously detailed, using 10 μg total RNA which was annealed to an end-labeled antisense 35-mer oligonucleotide primer and reverse-transcribed at 42°C for 90 min with 0.5 mM dATP, dCTP, dTTP, and dideoxy-GTP using 10 units Moloney murine leukemia virus reverse transcriptase (4, 9). The extension products were resolved by 8% polyacrylamide-urea electrophoresis and autoradiograms at comparable intensity scanned with a laser densitometer.

Preparation of cytosolic S-100 extracts

Rat and chicken small intestinal enterocytes were isolated as previously described (16). Rat livers were rinsed in cold PBS and finely minced. Cytosolic S-100 extracts were prepared as previously described (16, 17) with minor modifications. The intestinal cell pellets and minced livers were suspended in approximately 3 volumes of Dignam Buffer A (10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT) containing 10 μg/ml leupeptin, 10 μg/ml antipain, 0.5 mM benzamidine, and 0.5 mM PMSF. Cells were lysed in a glass Dounce homogenizer with a type A and then type B pestle, and the membrane fraction was pelleted by centrifugation at 750 *g* at 4°C for 10 min. The supernatant was recovered and equilibrated with 0.11 volumes of Dignam buffer B (300 mM HEPES, pH 7.9, 1.4 M KCl, 30 mM $MgCl_2$) and centrifuged for 60 min at 100,000 *g* at 4°C in an SW55Ti rotor (Beckman). The supernatant was then dialyzed for at least 8 h at 4°C against Dignam buffer D (20 mM HEPES, pH 7.9, 20% glycerol, 100 mM KCl, 0.2 mM Na_2EDTA, 0.5 mM DTT, 0.5 mM PMSF, and 0.5 mM benzamidine). All procedures were performed on ice and using ice cold solutions. The final cytosolic S-100 extract was stored in aliquots at −80°C. Xenopus oocyte homogenate expressing REPR was prepared as previously described (12).

In vitro apoB RNA editing assay

Five to 400 fmoles of a 361nt synthetic rat apoB RNA (nucleotides 6512–6872) prepared as described (16) was incubated for 120–150 min at 30°C with the indicated amounts of hepatic or intestinal S-100 extract in 10 mM HEPES, pH 7.9, 100 mM KCl, 0.1 mM Na_2 EDTA, 0.25 mM DTT, 10% glycerol, 250 ng tRNA, and 20 units of RNase inhibitor in a final volume of 20 μl. Where indicated in the figure legend, chicken intestinal S-100 extract or oocyte homogenate expressing REPR was added directly to the reaction mixture. The reaction was stopped by adding an equal volume of 100 mM Tris-HCl, pH 7.5, 10 mM Na_2EDTA, 0.4% SDS, 200 mM NaCl, 500 ng/μl tRNA, and 200 ng/μl proteinase K. After a 30-min incubation at 30°C, RNA was extracted with phenol–chloroform and analyzed by primer extension as described above.

REPR quantitation by RNase protection assay

A 423 bp Sma I-Kpn I fragment (nt 88-510) from REPR cDNA (12) was subcloned into pGEM 3Zf (+) (Promega, WI), linearized, and radiolabeled antisense cRNA was synthesized with T7 RNA polymerase using [α-^{32}P]UTP (3,000 Ci/mmol, New England Nuclear) to yield specific activities in the range of 1.7–2.6 × 10^9 cpm/μg. A 250 bp mouse β-actin cDNA (ffl7315-20, Ambion, TX) was used to generate an antisense cRNA with SP6 RNA polymerase, for use as an internal standard. RNA solution hybridization was performed using the RPA II kit from Ambion (ffl1410) with minor modifications to the manufacturer's protocol. Fifty μg total RNA was coprecipitated with 4 × 10^8 cpm freshly radiolabeled REPR and β-actin cRNA probes and redissolved in 30 μl 80% formamide, 40 mM PIPES, pH 6.4, 0.4 M NaCl, and 1 mM EDTA. The mixture was denatured at 90°C for 5 min and then annealed at 42°C for 16 h. After annealing, 300 μl buffer containing 2.5 U/ml RNase A and 100 U/ml RNase T1 was added and incubated at 37°C for 30 min to digest the unprotected riboprobes. Subsequently, 300 μl of RNase inactivation-precipitation buffer (Ambion) was added and after 20 min at −20°C, protected fragments were precipitated and resolved on a 6% polyacrylamide-urea gel. After exposure to XAR film, autoradiographs with comparable intensity of REPR and β-actin bands were scanned using a laser densitometer.

Data presentation and statistical analysis

All data are presented as mean ± SD. Comparisons between groups were made using unpaired, two-tailed *t*-tests with Welch's alternate modification for samples with unequal standard deviation. These comparisons were made using the InStat program (GraphPad software, San Diego, CA). Regression analysis was conducted using the Cricket Graph 1.3 program (Computer Associates, New York, NY).

RESULTS

Body weights, serum and hepatic lipid content

The various dietary and hormonal manipulations produced predictable alterations in serum and hepatic lipid concentrations as detailed in **Table 1.** Cholesterol feeding produced a 25-fold increase in hepatic cholesteryl ester content while hepatic cholesteryl ester content increased 7- and 60-fold in the estradiol and estradiol+cholesterol groups, respectively. Hyperthyroid rats showed undetectable hepatic cholesteryl ester levels, while hypothyroid animals consuming a high cholesterol diet demonstrated a 4.5-fold increase in total serum cholesterol and a 150-fold increase in hepatic cholesteryl ester content (Table 1).

TABLE 1. Body and liver weights, and lipid concentrations in serum and liver

Groups	(n)	Weight			Serum Lipid		Hepatic Lipid		
		Body	Liver	Liver/Body	TG	TC	TG	FC	CE
		g			*mg/dl*		*μg/mg protein*		
Control	(7)	293 ± 11	9.3 ± 0.1	3.1 ± 0.3	52 ± 12	86 ± 8	1.7 ± 0.3	15.7 ± 2.3	1.1 ± 0.6
Cholesterol	(5)	293 ± 9	11.3 ± 0.4[a]	3.9 ± 0.1	80 ± 4[a]	91 ± 10	4.2 ± 2.1	19.8 ± 2.8	25.0 ± 13.9[a]
Estradiol	(5)	288 ± 8	12.6 ± 0.7[a]	4.4 ± 0.2	24 ± 5[a]	8 ± 1[a]	2.4 ± 0.1	18.1 ± 2.1	7.7 ± 1.9
Estradiol + cholesterol	(7)	281 ± 10	13.5 ± 0.9[a]	4.8 ± 0.2	17 ± 3[a]	8 ± 1[a]	8.5 ± 2.7[a,c]	21.3 ± 2.6[d]	61.8 ± 7.6[a,c]
Hyperthyroid	(5)	288 ± 12	9.8 ± 0.7	3.4 ± 0.2	93 ± 22[b]	74 ± 10	2.8 ± 0.4	17.3 ± 3.8	n.d.
Hypothyroid	(5)	294 ± 4	9.2 ± 0.5	3.1 ± 0.2	44 ± 12	96 ± 4	2.4 ± 0.6	16.3 ± 1.1	1.3 ± 1.5
Hypothyroid + cholesterol	(7)	253 ± 10[a]	9.7 ± 1.0	3.8 ± 0.4	32 ± 13[b]	391 ± 80[a]	6.0 ± 2.5[c]	23.6 ± 2.4[f]	146.8 ± 58.0[a,f]

Data are presented as means ± SD; n, number; TG, triglyceride; FC, free cholesterol; TC, total cholesterol; CE, cholesteryl ester; n.d., not detectable.
[a] $P < 0.001$ versus control.
[b] $P < 0.05$ versus control.
[c] $P < 0.001$; [d] $P < 0.05$ versus estradiol-treated animals.
[e] $P < 0.01$; [f] $P < 0.001$ versus hypothyroid animals.

Apolipoprotein and liver fatty acid-binding protein mRNA abundance

Determination of mRNA levels by slot-blot and Northern analysis showed that apoB, E, and C-III mRNA abundance in the liver remained unchanged in all groups (data not shown), while ethinyl estradiol and PTU treatment decreased hepatic apoA-IV mRNA levels by 50%, as previously shown (10). Additionally, estradiol-treated cholesterol-fed animals showed a 50% decrease in hepatic L-FABP mRNA abundance (data not shown). Hyperthyroid animals demonstrated increased hepatic apolipoprotein A-I and A-IV mRNA abundance and unchanged L-FABP mRNA abundance, as previously reported (20).

Endogenous apolipoprotein B mRNA editing

Figure 1A shows hepatic apoB mRNA editing in representative samples of the seven groups examined, as determined by primer extension. Control animals contained 59.8 ± 3.3% of edited transcript (n = 7). As previously demonstrated (5), T_3-treated rats showed an increase in apoB mRNA editing (85.8 ± 2.7%, n = 5, $P < 0.0005$ compared to control), while in hypothyroid and estradiol-treated rats, apoB mRNA editing decreased to 55.6 ± 2.3%, n = 5 and 44.6 ± 1.6%, n = 5, $P < 0.04$ and $P < 0.0001$, respectively. Cholesterol feeding alone decreased hepatic apoB mRNA editing to 45.5 ± 2.4% UAA, n = 5, ($P < 0.0005$ compared to control). Dietary cholesterol supplementation of either estradiol-treated or hypothyroid rats produced a further, significant decrease in apoB mRNA editing (37.1 ± 3.5%, n = 5 and 28.0 ± 5.4%, n = 7 respectively, $P < 0.0005$) when compared to either estradiol-treated or hypothyroid animals. By contrast, endogenous apoB mRNA editing was unaltered in the small intestine of the groups examined (Fig. 1B).

Endogenous hepatic apoB mRNA editing was correlated with lipid accumulation, revealing a striking, inverse correlation with cholesteryl ester content, (**Fig. 2C,** $P < 0.001$). ApoB mRNA editing also demonstrated an inverse correlation with hepatic free cholesterol content (Fig. 2B, $P < 0.01$), and to a lesser extent with triglyceride content (Fig. 2A, $P < 0.05$).

Taken together, these results demonstrate a reduction in endogenous hepatic apoB mRNA editing in the rat liver following modulations that increase hepatic cholesterol ester accumulation.

Determination of in vitro apoB mRNA editing activity of hepatic and intestinal S-100 extracts

To determine whether these changes in endogenous apoB mRNA editing were associated with parallel changes in editing activity in vitro, S-100 extracts were prepared from liver and small intestine from animals with different levels of endogenous hepatic apoB mRNA editing. These S-100 extracts were then used in an in vitro conversion assay with a synthetic rat apoB RNA template. Initial experiments, using either hepatic or small intestinal extracts, confirmed (16) that apoB RNA editing was linear with increasing S-100 protein over 60 min, with a gradual plateau reached by 2–3 h (data not shown). Longer incubations (120–150 min) were chosen for the experiments presented for two reasons. First, apoB RNA editing efficiency, particularly with hepatic S-100 extracts, was generally less than 10% of the input when using shorter incubation times (data not shown), in comparison to the data presented in Tables 2 and 3 where virtually all the values are greater than 10%. This was considered important since others have proposed that differences between apoB RNA editing values lower than 10% are difficult to quantitate precisely (8). Secondly, there was greater varia-

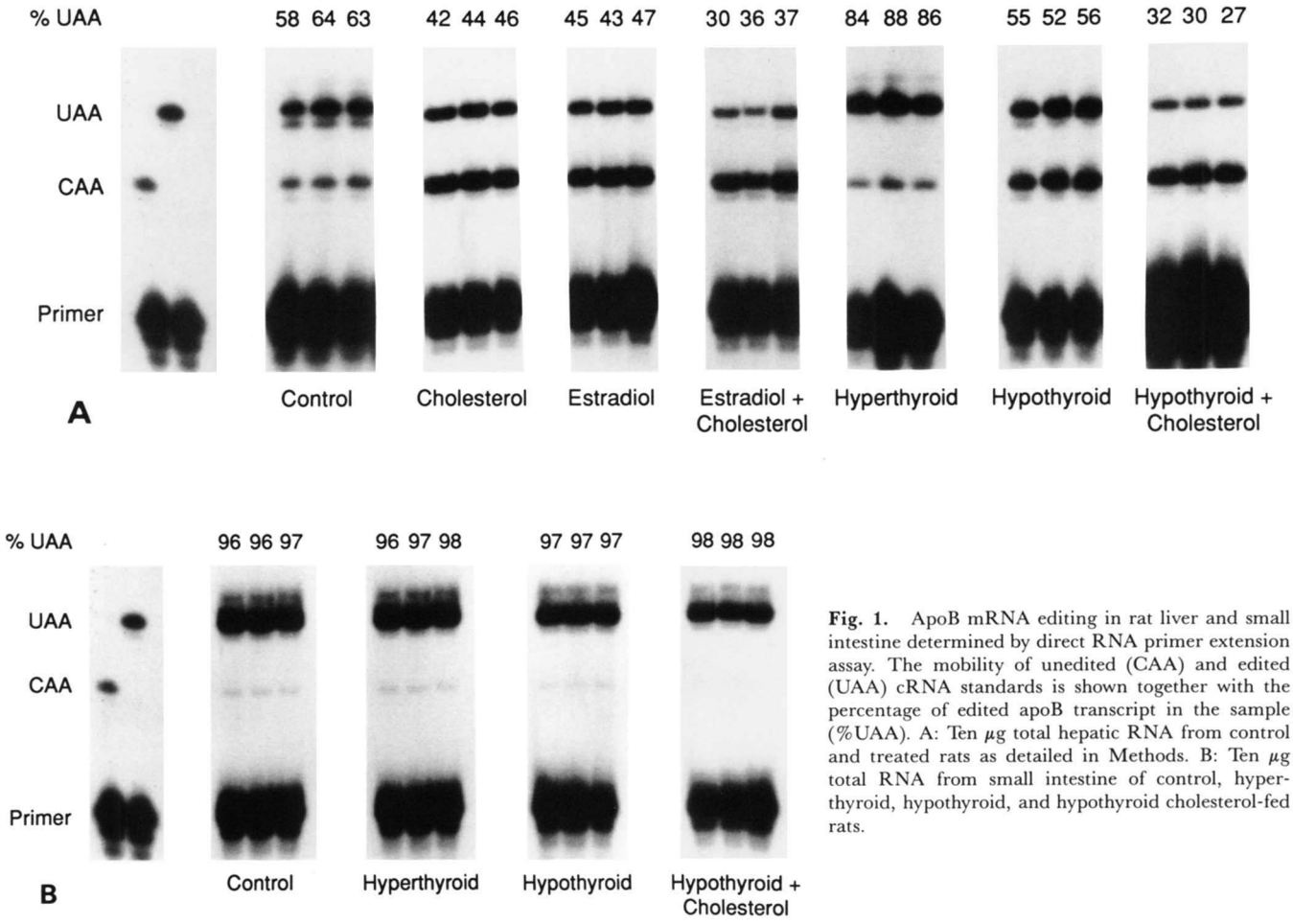

Fig. 1. ApoB mRNA editing in rat liver and small intestine determined by direct RNA primer extension assay. The mobility of unedited (CAA) and edited (UAA) cRNA standards is shown together with the percentage of edited apoB transcript in the sample (%UAA). A: Ten μg total hepatic RNA from control and treated rats as detailed in Methods. B: Ten μg total RNA from small intestine of control, hyperthyroid, hypothyroid, and hypothyroid cholesterol-fed rats.

bility between samples when shorter incubation times were used and, consequently, differences between the groups were obscured (data not shown).

Table 2 (upper panel) shows the incremental activity, with increasing protein concentration (2.5–50 μg), in hepatic S-100 extracts from the four groups, using 10 fmol synthetic apoB RNA. ApoB RNA editing activity was increased in S-100 extracts from the T_3-treated and decreased in S-100 extracts from cholesterol fed+hypothyroid animals, while the activity of S-100 extracts from hypothyroid animals was no different from control liver. Experiments were also conducted using constant amounts (5 μg) of hepatic S-100 extract in incubations with increasing concentrations (0.25–20 nM) of synthetic RNA (Table 2, lower panel). These data confirm the patterns observed above, with increased editing activity in the T_3-treated and decreased activity in the cholesterol fed+hypothyroid liver S-100 extracts. The results are also internally consistent with the changes in endogenous apoB mRNA editing demonstrated above. Analysis of double reciprocal plots of the hepatic apoB RNA editing data failed to exhibit Michaelis-Menten kinetics (data not shown), an issue discussed below in greater detail.

Different findings were encountered using intestinal S-100 extracts from the four groups. There was a discrepancy between endogenous intestinal apoB mRNA editing, which was unaltered in the different conditions, and the editing activity of S-100 extracts on a synthetic rat apoB RNA template, which was paradoxically increased in the hypothyroid and decreased in the hyperthyroid animals (**Table 3**). Additionally, and in contrast with the data presented above, intestinal apoB RNA editing exhibited Michaelis-Menten kinetics as evidenced by the high correlation coefficients obtained in double reciprocal (Lineweaver-Burk) plots (r^2 = 0.986, 0.997, 0.994, 0.982 for control, hypothyroid, hypothyroid+cholesterol-fed and hyperthyroid animals, respectively). Values for "apparent K_m" were comparable to previous values (2.86 nM, ref. 16), with the present results for control [3.56 nM], hypothyroid [3.18 nM], T_3-treated [3.39 nM], and hypothyroid+cholesterol fed animals [2.05 nM] being similar. The calculated "apparent V_{max}" for each group

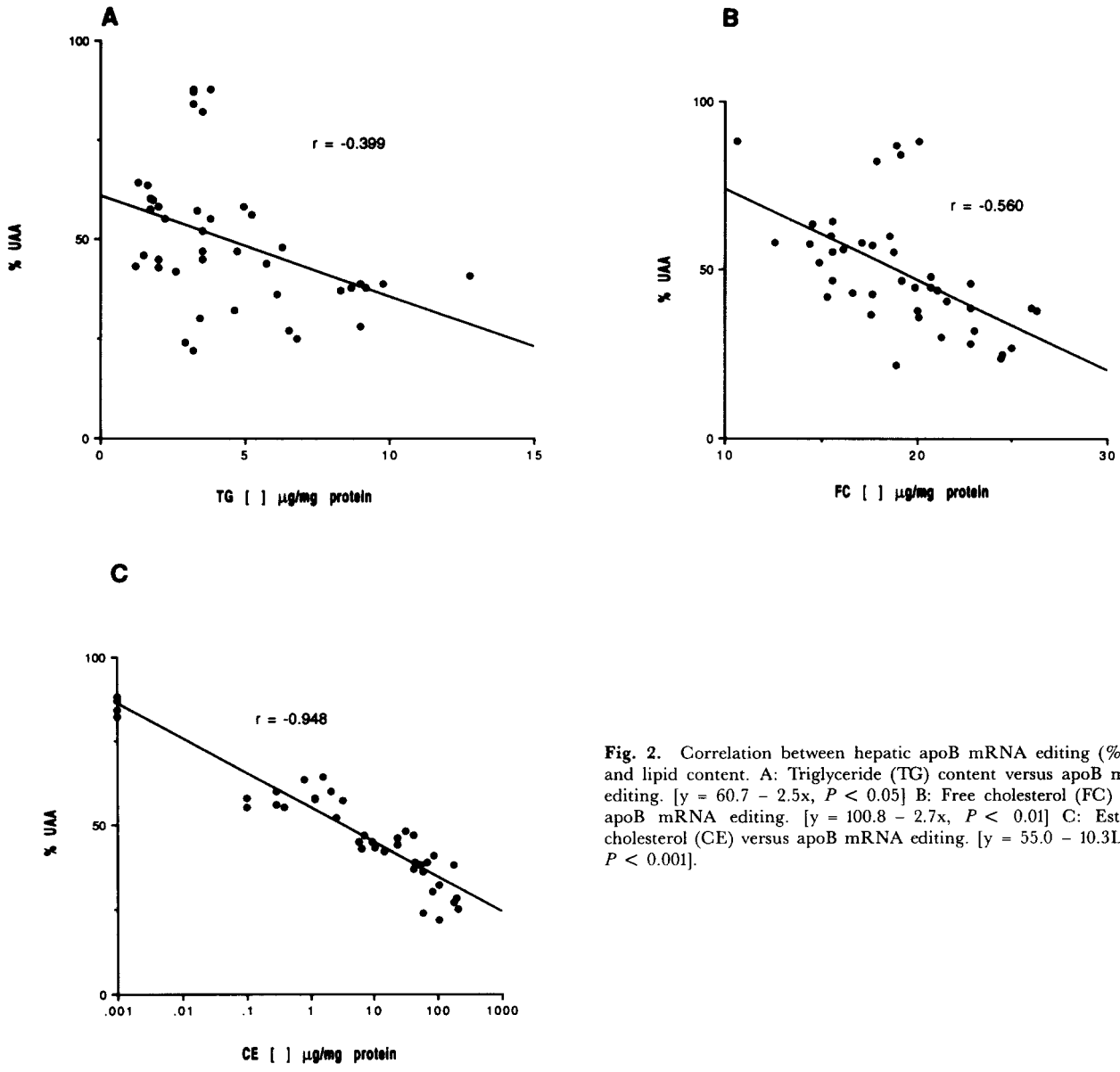

Fig. 2. Correlation between hepatic apoB mRNA editing (%UAA) and lipid content. A: Triglyceride (TG) content versus apoB mRNA editing. [y = 60.7 – 2.5x, $P < 0.05$] B: Free cholesterol (FC) versus apoB mRNA editing. [y = 100.8 – 2.7x, $P < 0.01$] C: Esterified cholesterol (CE) versus apoB mRNA editing. [y = 55.0 – 10.3Log(x), $P < 0.001$].

ranged from 4.51 fmol/µg per h in hyperthyroid animals to 11.10 fmol/µg per h in hypothyroid animals with control (7.78 fmol/µg per h) and hypothyroid+cholesterol fed animals (7.55 fmol/µg per h) demonstrating intermediate values. It bears emphasis, however, that these values are presented by way of contrast with the results with hepatic S-100 extracts and cannot strictly be used to demonstrate the presence of "noncompetitive enzyme inhibition" in the small intestine since initial enzyme kinetics were not measured. The findings do, however, reinforce the importance of tissue-specific factors in the regulation of apoB mRNA editing and, as discussed below, several potential mechanisms were considered further.

REPR mRNA quantitation by RNase protection assay

An RNase protection assay was developed to quantitate REPR mRNA, with the relative abundance normalized to β-actin as a housekeeping control. Hepatic REPR mRNA levels were unaltered in hypothyroid and T_3-treated animals when compared to controls (**Fig. 3A**). By contrast, there was a 45% decrease in hepatic REPR mRNA abundance in estradiol+cholesterol-treated animals, compared to estradiol alone ($P < 0.005$) and a 68% decrease in the hypothyroid+cholesterol-fed animals, compared to hypothyroid animals ($P < 0.005$). When compared to untreated chow-fed controls, each of the

TABLE 2. In vitro apoB RNA editing using hepatic S-100 extracts

	ApoB RNA Editing (%UAA)			
[S-100] μg	Control	T3 Treated	Hypothyroid	Hypo + Chol
2.5	14.2 ± 2.0	42.0 ± 8.2[a]	13.8 ± 3.0	8.7 ± 1.0[a]
5.0	26.7 ± 4.7	48.1 ± 6.3[a]	24.3 ± 3.9	9.3 ± 5.1[a]
10	29.6 ± 6.3	54.9 ± 7.3[a]	27.3 ± 4.0	17.0 ± 6.4[b]
25	36.9 ± 3.3	61.1 ± 3.0[a]	33.3 ± 6.0	24.9 ± 4.4[a]
50	40.8 ± 3.6	62.1 ± 4.0[a]	35.6 ± 6.9	21.8 ± 3.9[a]
[cRNA]nM	ApoB RNA Editing (fmol/μg/h)			
0.25	0.07 ± 0.02	0.24 ± 0.03[a]	0.06 ± 0.02	0.04 ± 0.005[c]
0.5	0.24 ± 0.05	0.44 ± 0.08[a]	0.18 ± 0.08	0.07 ± 0.02[a]
1.0	0.35 ± 0.10	0.76 ± 0.14[a]	0.34 ± 0.14	0.19 ± 0.17
2.0	0.95 ± 0.18	1.32 ± 0.39	0.82 ± 0.32	0.61 ± 0.17[b]
4.0	1.98 ± 0.45	2.89 ± 0.65[c]	1.82 ± 0.33	1.37 ± 0.09[a]
10	2.08 ± 0.35	3.03 ± 0.21[a]	1.95 ± 0.33	1.47 ± 0.10[b]
20	2.15 ± 0.35	3.20 ± 0.22[a]	1.97 ± 0.33	1.45 ± 0.08[b]

S-100 extracts (n = 5 per group) were prepared from livers of animals subjected to the dietary and hormonal manipulations described in Methods. These extracts were then used in an in vitro RNA editing assay with apoB RNA transcribed from a rat cDNA template flanking the edited base and editing determined by primer extension. Data are presented as mean ± SD. Upper panel: 10 fmoles of a synthetic rat apoB cRNA was incubated with increasing amounts (2, 5, 10, 25, 50 μg protein) of liver S-100 extract (n = 5 per group). Primer extension was performed as detailed in Methods and percent conversion (%UAA) was determined by laser densitometry. Lower panel: 5 μg hepatic S-100 extract from the same groups was incubated with increasing concentrations of apoB cRNA (0.25, 0.5, 1, 2, 4, 10, 20 nM) and the data are presented as fmoles of converted cRNA per μg of protein per hour. Differences were determined by unpaired *t*-test.

[a] $P < 0.002$; [b] $P < 0.02$; [c] $P < 0.04$, compared to control animals, respectively.

cholesterol-fed groups demonstrated a 25–50% decrease in hepatic REPR mRNA abundance (data not shown). These results suggest that alterations in hepatic cholesterol content may produce small but significant alterations in REPR mRNA abundance. Reagents are currently being developed to undertake a parallel analysis of REPR protein abundance in these tissue extracts but, as yet, no information exists concerning this crucial issue. REPR mRNA abundance remained unchanged in the small intestine under conditions shown above to be as-

TABLE 3. In vitro apoB RNA editing using small intestinal S-100 extracts

	ApoB RNA Editing (%UAA)			
[S-100] μg	Control	T3 Treated	Hypothyroid	Hypo + Chol
0.2	9.7 ± 3.0	7.3 ± 1.77	14.9 ± 7.3	17.6 ± 10.3
0.5	19.6 ± 12.8	7.8 ± 1.6	43.2 ± 23.5	42.6 ± 15.2
1.0	40.6 ± 14.1	16.1 ± 8.0[a]	52.4 ± 19.3	66.4 ± 12.1
2.5	58.2 ± 14.4	25.6 ± 11.5[a]	78.9 ± 6.7[b]	69.5 ± 7.5
5.0	56.1 ± 17.6	39.1 ± 21.9	78.7 ± 9.3[b]	69.9 ± 18
[cRNA]nM	ApoB RNA Editing (fmol/μg/h)			
0.25	0.54 ± 0.14	0.32 ± 0.14[b]	0.78 ± 0.07[c]	0.79 ± 0.13
0.5	0.86 ± 0.29	0.51 ± 0.23	1.41 ± 0.27[c]	1.39 ± 0.20
1.0	1.50 ± 0.36	0.82 ± 0.40[b]	2.44 ± 0.50[c]	2.20 ± 0.21
2.0	2.90 ± 0.73	1.62 ± 0.65[b]	4.12 ± 0.38[c]	3.63 ± 0.41
4.0	4.74 ± 1.25	3.15 ± 1.81	7.62 ± 0.98[c]	6.72 ± 0.51
10	5.80	3.94	10.10	6.72
20	6.36	3.32	7.52	5.60

S-100 extracts (n = 5 per group, except for 10 and 20 nM values which are the average of two animals per group) were prepared from isolated small intestinal cells of animals subjected to the dietary and hormonal manipulations described in Methods. These extracts were then used in an in vitro RNA editing assay with apoB RNA transcribed from a rat cDNA template flanking the edited base and editing determined by primer extension. Data are presented as mean ± SD. Upper panel: 10 fmoles of a synthetic rat apoB cRNA was incubated with increasing amounts (0.2, 0.5, 1, 2.5, 5, 10 μg protein) of intestinal S-100 extract (n = 5 per group). Primer extension was performed as detailed in Methods and percent conversion (%UAA) was determined by laser densitometry. Lower panel: 2.5 μg intestinal S-100 extract from the same groups was incubated with increasing concentrations of apoB cRNA (0.25, 0.5, 1, 2, 4, 10, 20 nM) and the data are presented as fmoles of converted cRNA per μg of protein per hour. Differences were determined by unpaired *t*-test.

[a] $P < 0.005$; [b] $P < 0.04$; [c] $P < 0.02$, compared to control animals, respectively.

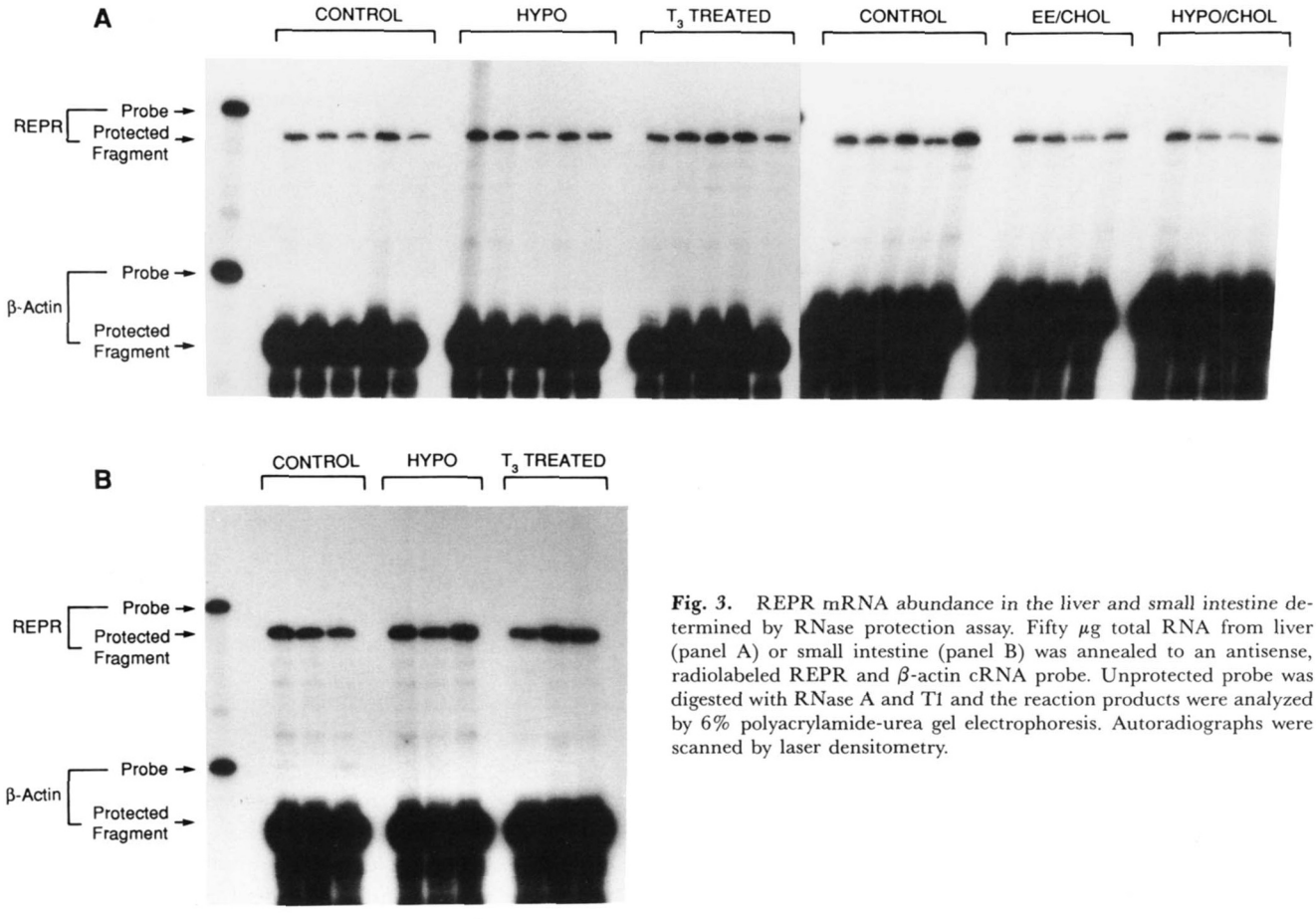

Fig. 3. REPR mRNA abundance in the liver and small intestine determined by RNase protection assay. Fifty μg total RNA from liver (panel A) or small intestine (panel B) was annealed to an antisense, radiolabeled REPR and β-actin cRNA probe. Unprotected probe was digested with RNase A and T1 and the reaction products were analyzed by 6% polyacrylamide-urea gel electrophoresis. Autoradiographs were scanned by laser densitometry.

sociated with large differences in the editing activity of S-100 extracts (Fig. 3B).

Supplementation experiments with REPR and chick intestinal S-100 extracts

To further investigate the importance of REPR in the regulation of apoB RNA editing activity, in vitro supplementation reactions were performed with REPR, using either hepatic or intestinal S-100 extracts. It has been recently demonstrated that apoB RNA editing occurs in vitro after the interaction of REPR, expressed in xenopus oocytes, and complementation factor(s) present in chick intestinal S-100 extracts (12). Additionally, chick intestinal S-100 extracts have been previously shown to enhance the editing activity of rat intestinal S-100 extracts in vitro (16). **Figure 4** shows a representative supplementation experiment, illustrating the effects on the editing activity of hepatic S-100 extracts from control, hypothyroid + cholesterol and T_3-treated animals. In these experiments, apoB RNA editing was determined alone and after the addition of increasing amounts of either oocyte homogenate (expressing REPR) or chick intestinal S-100 extracts. REPR supplementation produced a dose-dependent increase in apoB RNA editing in all groups (Fig. 4, lanes 2–5). Of relevance to the in vivo studies described above, supplementation of liver S-100 extracts from hypothyroid + cholesterol-fed animals with REPR yielded an editing activity undistinguishable from that of controls (Fig. 4, middle panel, lanes 2–4). The inhibition of editing activity with the highest quantities of REPR in hypothyroid + cholesterol-fed animals is unexplained (Fig. 4, middle panel, lane 5) but may reflect the importance of stoichiometric considerations in the editing complex, as recently demonstrated (17). By contrast, supplementation of any of the hepatic S-100 extracts with chicken intestinal S-100 extracts (as a source of complementation activity) produced no effect on in vitro apoB RNA editing efficiency (Fig. 4, lanes 6–8). Taken together, these data suggest that REPR is the limiting component of the editing complex in rat liver and confirms the importance of its potential physiological regulation in cholesterol-fed rats.

Supplementation experiments were also undertaken with small intestinal S-100 extracts, as illustrated in **Fig. 5.** REPR supplementation failed to enhance the editing activity of intestinal extracts from any group and, at the highest doses tested (10 μg), inhibited editing ac-

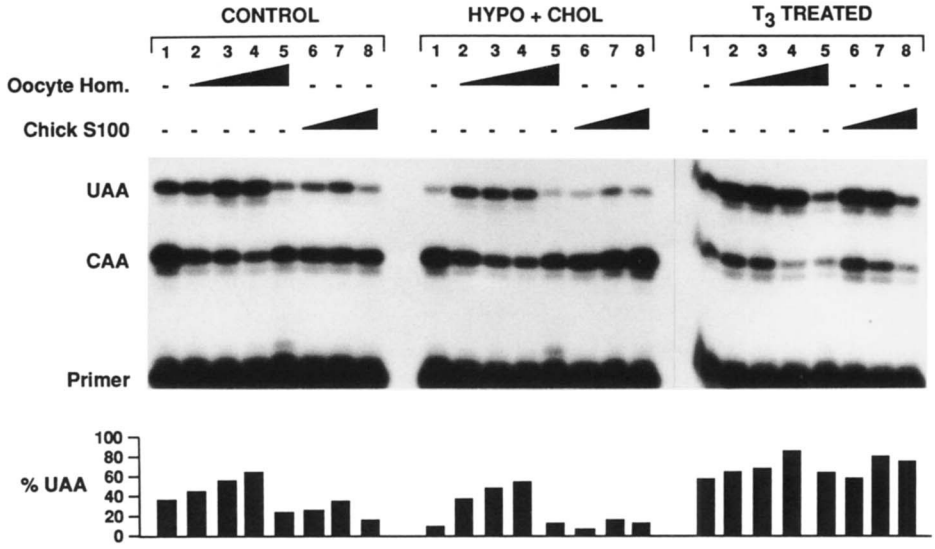

Fig. 4. In vitro editing activity of hepatic S-100 extracts: effects of supplementation with either REPR or chick intestinal S-100 extracts. S-100 extracts were prepared from control (left panel), hypothyroid+cholesterol fed (hypo+chol, central panel), and T_3-treated (right panel) rats. S-100 extracts (5 μg) were incubated alone (lane 1), or with either increasing amounts (0.2, 0.5, 2, 10 μg) of oocyte homogenates expressing REPR (lanes 2 to 5) or increasing amounts (2, 10, 50 μg) of chick intestinal extracts (lanes 6, 7, 8). The mobility of primer, unedited (CAA), and edited (UAA) apoB RNA is shown. Bar graph shows the percent conversion (%UAA) in each lane. Similar results were obtained in two independent experiments using S-100 extracts from the groups indicated.

tivity (lane 5, left, middle and right panels). This observation suggests that the stoichiometry of REPR and complementation factor(s) plays an important role in the regulation of apoB RNA editing, as alluded to above in the context of liver S-100 extracts prepared from hypothyroid+cholesterol-fed animals. By contrast, supplementation of intestinal S-100 extracts with chick intestinal S-100 enhanced editing activity, particularly in the control and T_3-treated animals (Fig. 5, left and right panels, lanes 6 and 7). Taken together, these data suggest that REPR

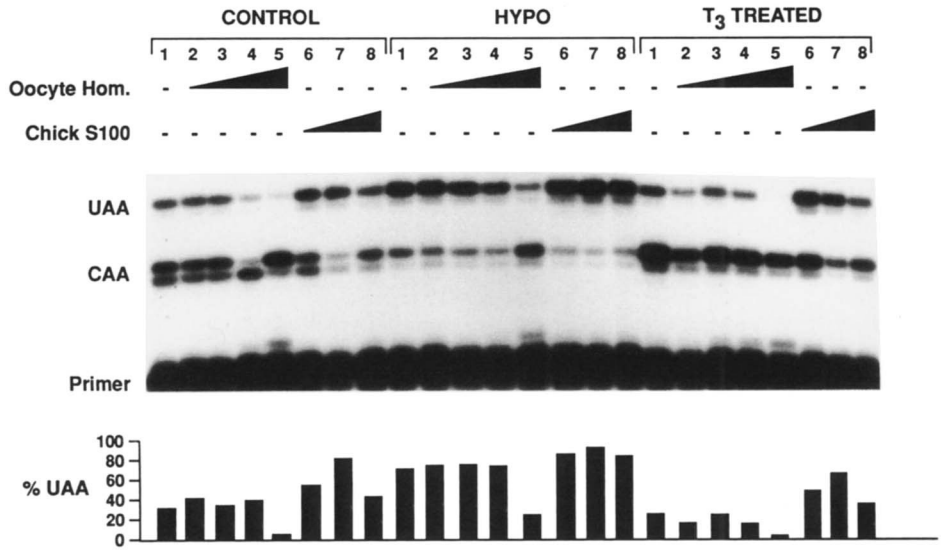

Fig. 5. In vitro editing activity of small intestinal S-100 extracts: effects of supplementation with either REPR or chick intestinal S-100 extracts. S-100 extracts were prepared from control (left panel), hypothyroid (central panel), and T_3-treated (right panel) rats. S-100 extracts (2.5 μg) were incubated alone (lane 1), or with either increasing amounts (0.2, 0.5, 2, 10 μg) of oocyte homogenates expressing REPR (lanes 2 to 5) or increasing amounts (2, 10, 50 μg) of chick intestinal extracts (lanes 6, 7, 8). The mobility of primer, unedited (CAA), and edited (UAA) apoB RNA is shown. Bar graph shows the percent conversion (%UAA) in each lane. Similar results were obtained in two independent experiments using S-100 extracts from the groups indicated.

is not limiting in the small intestine, in contrast to the liver, and that changes in the abundance and/or composition of the complementation factor(s) may explain the differences in editing activity observed in vitro.

DISCUSSION

The present studies were undertaken in order to begin a systematic evaluation of the regulation of apoB mRNA editing by cellular lipid flux. Several important observations emerged from these studies, among them, that endogenous hepatic apoB mRNA editing is decreased in response to cholesterol accumulation and in a manner that was recapitulated using isolated S-100 extracts and a synthetic apoB RNA template. Furthermore, decreased in vitro apoB RNA editing was reversible upon supplementation of hepatic S-100 extracts with REPR expressed in Xenopus oocytes, suggesting that this protein may be of importance in the physiologic regulation of hepatic apoB mRNA editing in the rat. These findings contrast with the results of parallel studies examining small intestinal apoB mRNA editing, in which there was a divergence between endogenous apoB mRNA editing and in vitro editing of a synthetic apoB RNA template.

Several studies have addressed the regulation of hepatic lipoprotein assembly by alterations in cholesterol flux and the findings are germane to the present work. Work by Fungwe et al. (21) has demonstrated that the hyperlipidemia accompanying cholesterol feeding in the rat reflects increased hepatic secretion of VLDL and apoB. These workers demonstrated increased apoB secretion in hepatic perfusates with increasing dietary cholesterol supplementation, although no distinction was made between apoB-100 and B-48 secretion and no direct measurements of apoB synthesis were undertaken (21). Earlier work from this group demonstrated that hepatic cholesteryl ester content was highly correlated with VLDL secretion and these findings lend direct support to the hypothesis that cholesteryl ester synthesis may be a critical determinant of hepatic apoB secretion (22, 23). The present studies extend these findings by demonstrating a significant inverse correlation between hepatic cholesteryl ester content and apoB mRNA editing. The speculation, based upon previous studies in which hepatic apoB mRNA editing and apoB synthesis have been simultaneously examined, would be a predictive ratio of edited and unedited apoB mRNA and newly synthesized apoB-100 and B-48 (5, 9, 20). However, other studies have demonstrated comparable secretion rates of newly synthesized apoB-100 and apoB-48 from isolated rat hepatocytes prepared from either control, chow-fed, or cholesterol-fed animals (24). These workers concluded that, despite massive accumulation of cholesteryl ester, there was no effect on apolipoprotein synthesis (24). These studies, however, did not report data on apoB mass secretion, nor was intracellular synthesis and turnover of apoB isoforms accounted for. In order to resolve these discrepancies, it will be necessary to determine whether additional, posttranslational events regulate intracellular apoB-100 and B-48 stability after cholesteryl ester accumulation and these studies will be the focus of future investigation. The current findings, demonstrating a decrease in endogenous hepatic apoB mRNA editing after expansion of cholesteryl ester stores, would be anticipated to result in a greater proportion of apoB-100 versus apoB-48 as the newly synthesized isoform. Demonstration of this outcome will await further study, but a fundamental issue to be resolved remains the presumed metabolic advantage to the cell of maintaining flexibility in the elaboration of a specific isoform of apoB.

Studies in HepG2 cells and both human and rabbit primary hepatocytes have suggested that cholesteryl ester synthesis or the size of a critical regulatory pool, is a key determinant of apoB secretion (25–30, and reviewed in reference 31, 32). Specifically, Cianflone et al. (25, 26), reporting studies in HepG2 cells, have argued that cholesteryl ester and not triglyceride synthesis is an immediate regulator of apoB secretion. This issue is somewhat controversial as some workers have found a decrease in apoB secretion after LDL administration to HepG2 cells (33), while others, in agreement with Cianflone et al. (25, 26) report an increase (27, 34–37). The route by which cholesterol is presented to the hepatocyte may be of importance in this regard as independent reports have demonstrated large increases in apoB secretion from HepG2 cells after uptake of chylomicron remnants (28, 38). These findings are relevant to the present studies, particularly as several different models of hepatic cholesterol accumulation were compared. The net result, namely that apoB mRNA editing is correlated with hepatic cholesteryl ester content per se and not with any particular feature of the model used to achieve hepatic cholesterol enrichment, for example increased dietary cholesterol intake alone or ethinyl estradiol treatment, suggests that hepatic apoB mRNA editing in the rat is less influenced by the cellular route of cholesterol delivery than by the absolute quantities presented for storage. In addition, indirect support for this suggestion is the lack of correlation between hepatic apoB mRNA editing and serum cholesterol levels (data not shown). A further point of comparison between the present studies and those alluded to above is that the alterations found in apoB secretion from HepG2 cells and rabbit hepatocytes, after increases or decreases in cholesteryl ester content, are thought to be largely mediated by alterations in posttranslational stability of apoB-100 (30, 33, 36). Previous work has demonstrated that hepatic apoB synthesis and secretion from rat hepatocytes is also subject to posttranslational regulation in the setting of a number of different hormonal and nutritional manipulations (39–41). By con-

trast, regulation of apoB gene expression through alterations in apoB mRNA editing does not occur in either HepG2 cells or rabbit hepatocytes (2, 4, 7). Thus, the present findings require interpretation in the context of work that suggests that apoB mRNA editing may be an important, additional level of control of hepatic apoB gene expression in those species in which the liver is competent to edit (7).

The results of studies examining in vitro editing of a synthetic rat apoB RNA template suggest that the alterations observed in endogenous hepatic apoB mRNA editing are reflected in cell-free extracts. Such internal consistency is reminiscent of the fasting-refeeding model of altered hepatic lipogenesis where earlier findings of increased apoB mRNA editing (9) were confirmed and demonstrated to be accompanied by increased editing efficiency using in vitro S-100 extracts (42). In vitro apoB RNA editing by hepatic S-100 extracts failed to exhibit Michaelis-Menten kinetics (data not shown), in part because the reaction conditions were not strictly within the linear range, but also, as suggested by the complementation experiments, because hepatic apoB RNA editing reflects the net activity of at least two different factors. Furthermore, the results suggest that the modulations observed may, in some situations, be attributable to alterations in the abundance or activity of REPR. Specifically, the decrease in apoB mRNA editing after cholesteryl ester accumulation was accompanied by a decrease in REPR mRNA abundance and was subsequently shown to be reversible after supplementation with oocyte homogenates expressing REPR. It remains to be demonstrated, however, whether these changes are associated with parallel alterations in endogenous REPR protein abundance or activity. By contrast, there was no alteration in hepatic REPR mRNA abundance after T_3 treatment, suggesting that additional factors are of importance in the regulation of hepatic apoB mRNA editing. Other workers have demonstrated the presence of proteins in rat liver S-100 extracts that bind with high specificity to apoB RNA and the possibility that these may play an important regulatory role is worthy of further consideration (13–15).

No changes were detectable in endogenous intestinal apoB mRNA editing in any of the experimental groups. Previous studies have demonstrated decreased small intestinal apoB-48 synthesis in hypothyroid rats consuming a high cholesterol, high fat diet (19). Along these lines, earlier studies demonstrated that rats subjected to prolonged biliary diversion demonstrated a 50% reduction in small intestinal apoB-48 synthesis rates (43). In neither situation, however, was endogenous apoB mRNA editing found to be altered (Inui, Y. and Davidson, N. O., unpublished observations). These findings, in conjunction with the present data, suggest that rat small intestinal apoB synthesis is regulated by mechanisms independent of alterations in endogenous apoB mRNA editing. These findings are in agreement with very recent findings in postconfluent Caco-2 cells where, following different perturbations of cellular fatty acid and cholesterol flux, no alterations were found in endogenous apoB mRNA editing compared to controls (44). Nevertheless, changes in apoB secretion have been demonstrated in Caco-2 cells following fatty acid challenges, accompanied, in some cases, by alterations in apoB mRNA abundance and also changes in posttranslational stability (45, 46). Taken together, however, the data suggest that endogenous apoB mRNA editing is not regulated in the adult rat small intestine, nor in postconfluent Caco-2 cells in response to alterations in cellular lipid flux. The situation with regard to in vitro editing of apoB RNA by rat small intestinal S-100 extracts is less clear. The conclusion, that in vitro editing activity demonstrated apparent Michaelis-Menten kinetics, must be qualified by the fact that these assays were conducted over a time frame beyond strict linearity. Nevertheless, the values for apparent K_m were comparable to previous results from our own and other groups (16, 47), and other workers have demonstrated a similar pattern (but different K_m) of enzyme kinetics for rabbit intestinal S-100 extracts (48).

The demonstration that chicken intestinal S-100 extracts, which alone are not competent to edit a synthetic mammalian apoB RNA template, but which complement the ability of REPR and which enhance in vitro editing by mammalian intestinal S-100 extracts, suggests that other factor(s) are required for the assembly of an editing complex (12, 16). The present findings suggest that elements of this complex may either be selectively depleted or inactive in intestinal S-100 extracts after T_3 treatment and, conversely, stabilized or induced in extracts from hypothyroid animals. An important issue to emerge from the present studies therefore is the nature of the T_3-dependent modulation of apoB RNA editing by small intestinal S-100 extracts. These effects appear to be independent of alterations in cholesterol flux in the small intestine, although further resolution of this issue awaits definition of the composition of the complementation factor(s).

These studies illustrate the complex, tissue-specific interactions that regulate apoB mRNA editing in the adult rat. Further investigation of the molecular basis for these interactions will be of immense importance in understanding the developmental regulation and restricted pattern of expression of this novel process and these will be the focus of future reports.

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