Tissue-specific, developmental and nutritional regulation of the gene encoding the catalytic subunit of the rat apolipoprotein B mRNA editing enzyme: functional role in the modulation of apoB mRNA editing

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Abstract Apolipoprotein B (apoB) mRNA editing, a posttranscriptional site-specific cytidine deamination reaction, is mediated by a protein complex of which the catalytic component (REPR) has recently been cloned. REPR mRNA was demonstrated by RNase protection at highest abundance in small intestine and colon but the transcript was detectable in all tissues examined including kidney, spleen, lung, liver, and ovary. ApoB mRNA was found predominantly in the liver and small intestine but low levels were detected in all adult tissues examined and found to be variably (29–86% TAA) edited. In addition, S100 extracts prepared from spleen and kidney were competent to edit an apoB RNA template in vitro, suggesting that the entire apoB mRNA editing complex is present and functionally active in these tissues. In situ hybridization demonstrated REPR mRNA to be distributed along the entire villus-crypt axis, while apoB mRNA distribution did not extend into the crypts. In the liver, both apoB and REPR mRNA were detected in all cells of the hepatic lobule without an apparent gradient of expression. REPR mRNA was found in the red pulp of the spleen and in the superficial crypt cells of the colon. This distribution of REPR mRNA was recapitulated by immunocytochemical localization of the protein within these tissues. Finally, the developmental and nutritional modulation of REPR was examined in relation to endogenous apoB mRNA editing. Small intestinal apoB mRNA editing was found to undergo a developmentally regulated increase beginning at gestational day 20, preceding a developmental increase in REPR mRNA abundance. Additionally, hepatic and kidney apoB mRNA editing both revealed a temporal dissociation from alterations in REPR mRNA abundance. By contrast, adult rats subjected to fasting and refeeding a high carbohydrate diet, demonstrated concordant modulation of endogenous apoB mRNA editing and REPR mRNA abundance ($r = 0.92$, $P < 0.001$). Taken together, the data demonstrate that REPR and other components of the rat apoB mRNA editing complex are widely distributed and undergo distinct developmental and metabolic regulation that interact to regulate apoB mRNA editing in a tissue-specific manner. —**Funahashi, T., F. Giannoni, A. M. DePaoli, S. F. Skarosi, and N. O. Davidson.** Tissue-specific developmental and nutritional regulation of the gene encoding the catalytic subunit of the rat apolipoprotein B mRNA editing enzyme. *J. Lipid Res.* 1995. **36:** 414–428.

Supplementary key words REPR • cytidine deaminase • apoB • mRNA editing

Apolipoprotein B (apoB) is synthesized in the liver and small intestine of mammals as an integral component of triglyceride-rich lipoproteins and plays a crucial role in their secretion and subsequent catabolism. The mammalian small intestine secretes a form of apoB referred to as apoB-48 which is produced as a result of posttranscriptional editing of the nuclear transcript (1, 2). This reaction involves a site-specific cytidine deamination that changes a CAA codon, present in apoB-100, to a UAA stop codon in apoB-48. As a result of this reaction, the small intestine secretes a protein that is colinear with the amino terminal 48% of apoB-100. In humans, apoB-100 is produced in the liver from the unedited apoB mRNA, although the liver of other mammals, including the rat and mouse, demonstrates the capacity to edit at least a portion of the endogenous apoB mRNA (3).

ApoB mRNA editing is mediated by protein factor(s) that interact with a requisite RNA motif and result in a site-specific cytidine deamination (4, 5). This reaction can be reproduced in vitro using a synthetic apoB RNA template and has been attributed to an enzyme, most likely a complex, of which one component (REPR, rat apoB mRNA editing protein) has recently been cloned (6). The distribution of REPR mRNA was originally inferred from Northern analysis to be widespread, including tissues which were previously assumed to have little apoB mRNA (6). REPR will edit a synthetic apoB RNA tem-

Abbreviations: apoB, apolipoprotein B; REPR, catalytic subunit of the rat apolipoprotein B mRNA editing protein.

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plate in vitro but has an absolute requirement for additional complementation factor(s) in order to mediate this reaction (6–8). These complementation factor(s) were identified functionally in chicken intestinal S100 extracts and more recently in extracts from human liver, neither of which possess endogenous apoB RNA editing activity and secrete exclusively apoB-100 (7, 8). REPR demonstrates homology to other cytidine deaminases in a region containing a conserved motif that coordinates zinc binding; additionally, REPR contains cytidine deaminase activity, comparable to authentic *E. coli* cytidine deaminase (9). Taken together, the data strongly suggest that REPR is the catalytic component of the apoB mRNA editing complex, but the identity and composition of the complementation factor(s) remains unknown. The importance of these factor(s) in tissues such as the human liver is emphasized by the demonstration that transfection of REPR into HepG2 cells, a human hepatoma cell line that secretes only apoB-100, results in the appearance of edited apoB mRNA and the secretion of both apoB-100 and B-48 (7). Information concerning the tissue distribution and regulation of these factor(s) will thus be of importance to an understanding of the molecular mechanisms regulating apoB mRNA editing in vivo.

Previous reports have demonstrated that apoB mRNA editing is regulated in a variety of settings and in a species-specific manner. ApoB mRNA editing was demonstrated to undergo developmental regulation in human, pig, and rodent small intestine and also the rat and mouse liver (10–12). These findings have recently been extended with the demonstration that Caco-2 cells, a human colon carcinoma cell line, also demonstrates a developmental increase in apoB mRNA editing (13). Additionally, rat liver apoB mRNA editing was shown to undergo modulation in response to thyroid hormone and estrogen administration in addition to nutritional modulation mediated either by carbohydrate or cholesterol feeding (14–17). To date, no common denominator has emerged to account for the regulation of apoB mRNA editing in these diverse settings and there is little information concerning the molecular mechanisms which might regulate this process. The present study was undertaken in order to address the role of REPR in the regulation of apoB mRNA editing in the rat, specifically with a view to determining the cell-specific, developmental and nutritional regulation of this gene in relation to alterations in apoB mRNA editing.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats (200–250 g) were purchased from Charles River, Wilmington, MA and consumed regular rodent chow until being killed. The indicated organs were excised and RNA was extracted as previously described, from five to six animals per pool of RNA examined (18). Other adult animals (four per group) were subjected to a regimen of 24 or 48 h fasting and subsequently 24 or 48 h refeeding a high carbohydrate diet, as previously described (16). Timed-pregnant females were purchased from Charles River and the indicated tissues were pooled from six to twelve animals per organ, beginning at gestational day 17, in order to prepare a representative RNA pool from fetal and neonatal rats. These studies were performed on animals fed ad libitum.

RNase protection assay

An Sma I-KpnI fragment of the REPR cDNA (423 bp, nucleotides 88–510) was subcloned into pGEM 3Zf (+) (Promega, WI), and a 471 bp apoB cDNA (nucleotide 6512–6982) was subcloned into the Sma I site of pGEM 4Z (pRBF-CAA) (7, 17). A plasmid containing a mouse β-actin cDNA fragment (nucleotide 660–906) was purchased from Ambion (Austin, TX) and was used as an internal standard. These plasmids were linearized and radiolabeled antisense cRNA was synthesized with [α-^{32}P]UTP (3,000 Ci/mmol, NEN) and T7 RNA polymerase, which gave specific activities in the range of $1.7–2.6 \times 10^9$ cpm/μg. RNA solution hybridization was performed as previously detailed (17). Briefly, total RNA (50 μg) was coprecipitated with 4×10^4 cpm of either the ^{32}P-labeled REPR or apoB cRNA probe, in each case along with the β-actin cRNA probe and then redissolved in 30 μl of 80% formamide, 40 mM PIPES (pH 6.4), 0.4 M NaCl, and 5 mM EDTA. After denaturation at 90°C for 5 min, the mixture was incubated at 42°C for 16 h. The annealed mixture was incubated at 37°C for 30 min with RNase[A/T1], and the protected fragments were precipitated and analyzed on a 6% polyacrylamide/urea gel. The results were analyzed by autoradiography and quantitated by laser densitometric scanning. Standard curves were run to establish the linearity of the assay using the sense cRNAs. In all cases the autoradiographs were scanned at multiple exposures to insure linearity of response. The results of the RNase protection assay are presented as arbitrary units (corresponding to densitometric area) and normalized using the β-actin values.

ApoB mRNA editing

i) Endogenous apoB mRNA editing was determined following reverse transcription and polymerase chain reaction (RT-PCR) amplification of total cellular RNA, as previously validated (13). RNA samples were treated with DNase to remove genomic DNA and subjected to RT-PCR using rTth with oligonucleotides ND1 [5'-ATCTGAC-TGGGAGAGACAAGTAG-3', nt 6512–6534], and ND3 [5'-CACGGATATGATACTGTTCGTCAAGC-3', nt 6786–6751] as previously detailed (13). This reaction produced

an amplicon of 275 bp flanking the edited base (14). ApoB cDNA was purified from the oligonucleotides using Qiagen columns and the products were annealed to an antisense ^{32}P-labeled oligonucleotide [5'-AGTCCTGTG-CATCATAATTATCTCTAATATACTGA-3'] at 70°C for 10 min. Primer extension was performed at 42°C using T7 DNA polymerase, as previously described (13). The reaction products were analyzed on an 8% polyacrylamide-urea gel and subjected to autoradiography. The ratio of edited to unedited apoB cDNA was determined by laser densitometric scanning. *ii)* ApoB RNA editing was also determined in S-100 extracts, prepared from the indicated tissues exactly as previously described (7). Ten fmol of a synthetic 361 nt apoB RNA (nucleotides 6512–6872) was incubated with the indicated amounts of S100 extract for 2 h at 30°C in 10 mM HEPES, pH 7.9, 100 mM KCl, 0.1 mM EDTA, 0.25 mM DTT, 10% glycerol, 250 ng tRNA, and 20 units of RNasin (Promega) in a final volume of 20 μl. Where indicated in the figure legend, 10 μg chicken intestinal S100 extract was added to the incubation mixture. The reaction was stopped with an equal volume of 100 mM Tris-HCl, pH 7.5, 10 mM EDTA, 0.4% SDS, 200 mM NaCl, 500 ng/μl tRNA, and 200 ng/μl proteinase K. The RNA was extracted and annealed to an end-labeled, downstream oligonucleotide as previously described (8). The annealed products were extended in the presence of 500 μM dATP, dCTP, dTTP, and dideoxy GTP using 10 units of Moloney murine leukemia virus reverse transcriptase at 42°C for 90 min. The reaction products were analyzed as above and the proportion of edited and unedited apoB RNA was determined by laser scanning densitometry.

In situ hybridization

Animals were anesthetized and perfused via transcardiac intubation with a solution of freshly prepared 4% paraformaldehyde. The indicated tissues were removed and placed in 15% sucrose–4% paraformaldehyde for 16 h at 4°C. Twelve-μm cryostat sections were prepared and mounted on gelatin–poly-L-lysine-coated slides. Each section was treated with a solution of 0.001% proteinase K in Tris-HCl, 50 mM EDTA, pH 8.0, for 30 min at 37°C, followed by 0.025% acetic anhydride for 10 min. The slides were rinsed extensively in 2× SSC and dehydrated through graded ethanols. ^{35}S-labeled sense and antisense cRNA probes were prepared by in vitro transcription of the REPR and apoB cDNAs used above for RNase protection. The tissue sections were hybridized for 16 h at 55°C in a solution of 50% formamide, 10% dextran sulfate, 0.3 M NaCl, 1× Denhardt's solution, 10 mM Tris-HCl, pH 8.0, 10 mM EDTA, and 1 × 10^7 cpm/ml of the respective sense or antisense cRNA. The slides were washed with 2× SSC for 30 min and 0.5× SSC for 10 min, both at room temperature. Unhybridized probe was removed by digestion with RNase A (20 μg/ml) for 30 min at 37°C. The slides were washed in 0.1× SSC at 60°C for 30 min and dehydrated through graded ethanols. The slides were initially exposed to Hyperfilm-βmax (Amersham) for 4 days to assess the signal intensity and then dipped in NTB-2 emulsion (Kodak). After 12 days exposure the slides were developed in D-19 developer, fixed with rapid fixer as described by the manufacturer (Kodak), and lightly counterstained with hematoxylin. The slides were photographed under dark-field illumination using an Olympus BH2 microscope with Kodak Tri-X Pan 400 film.

Immunocytochemical detection of REPR

Adult rat tissues were fixed in Bouins solution for 4–12 h at room temperature and embedded in paraffin blocks. Five-μm sections were prepared on Vectabond-coated slides, and immunocytochemical detection was carried out by one of two protocols. For tissues other than the spleen, slides were deparaffinized and aldehydes were quenched in 0.2 M glycine for 30 min at room temperature. The tissues were blocked for 15 min in 5% normal goat serum/3% (IgG-free) bovine serum albumin (BSA) and reacted with anti-REPR IgG (see below) diluted to 4 mg/100 ml in PBS/0.3% Triton X-100/1% BSA/2% normal goat serum for 30 min, both at room temperature. An anti-REPR antipeptide antibody was raised in rabbits against residues 17–36 (RIEPHEFEVFFDPRELRKET) of REPR (6) and serum was used to prepare an IgG fraction. Control incubations contained peptide-absorbed anti-IgG. The slides were washed with PBS/0.1% Tween-20 and in PBS alone (5 times each). After washing, the slides were incubated with biotinylated goat anti-rabbit IgG (Vectastain Elite, Vector Labs, Burlingame, CA) diluted in 0.3% Triton/1% BSA and washed as described above. After these washes the slides were treated for 15 min with 1% hydrogen peroxide in methanol to block endogenous peroxidases. Finally, the slides were reacted with avidin-conjugated horseradish peroxidase (Vector Labs) as previously described prior to color development and light counterstaining with hematoxylin. For immunocytochemical detection of REPR in the spleen, the protocol was modified to include an incubation for 1 min in Peroxo-Block® (Zymed laboratories) following the 0.2 M glycine incubation, as hydrogen peroxide alone was insufficient to block the endogenous peroxidases. In addition, the tissue sections were treated for 10 min at 37°C with 2% ficin (Zymed laboratories) prior to incubation in the primary antiserum in order to expose more reactive sites. Otherwise the incubation conditions were exactly as described for the other tissues. The slides were photographed on an Olympus BH2 microscope using Ektachrome 400 film.

RESULTS

REPR/apoB mRNA distribution, endogenous apoB mRNA editing, and in vitro editing activity

The tissue distribution of REPR mRNA, as demonstrated by RNase protection assay, reveals the presence of this transcript in every tissue examined (**Fig. 1**). These findings are consistent with and extend the previously published findings of Northern blot analysis that demonstrated REPR mRNA to be detectable in a number of rat tissues (6). REPR mRNA abundance, expressed as arbitrary units normalized to β-actin mRNA, appears most abundant in the small intestine, although readily detectable in colon, kidney, liver, and spleen.

Parallel analysis of apoB mRNA abundance is shown in **Fig. 2,** demonstrating the presence of this transcript at

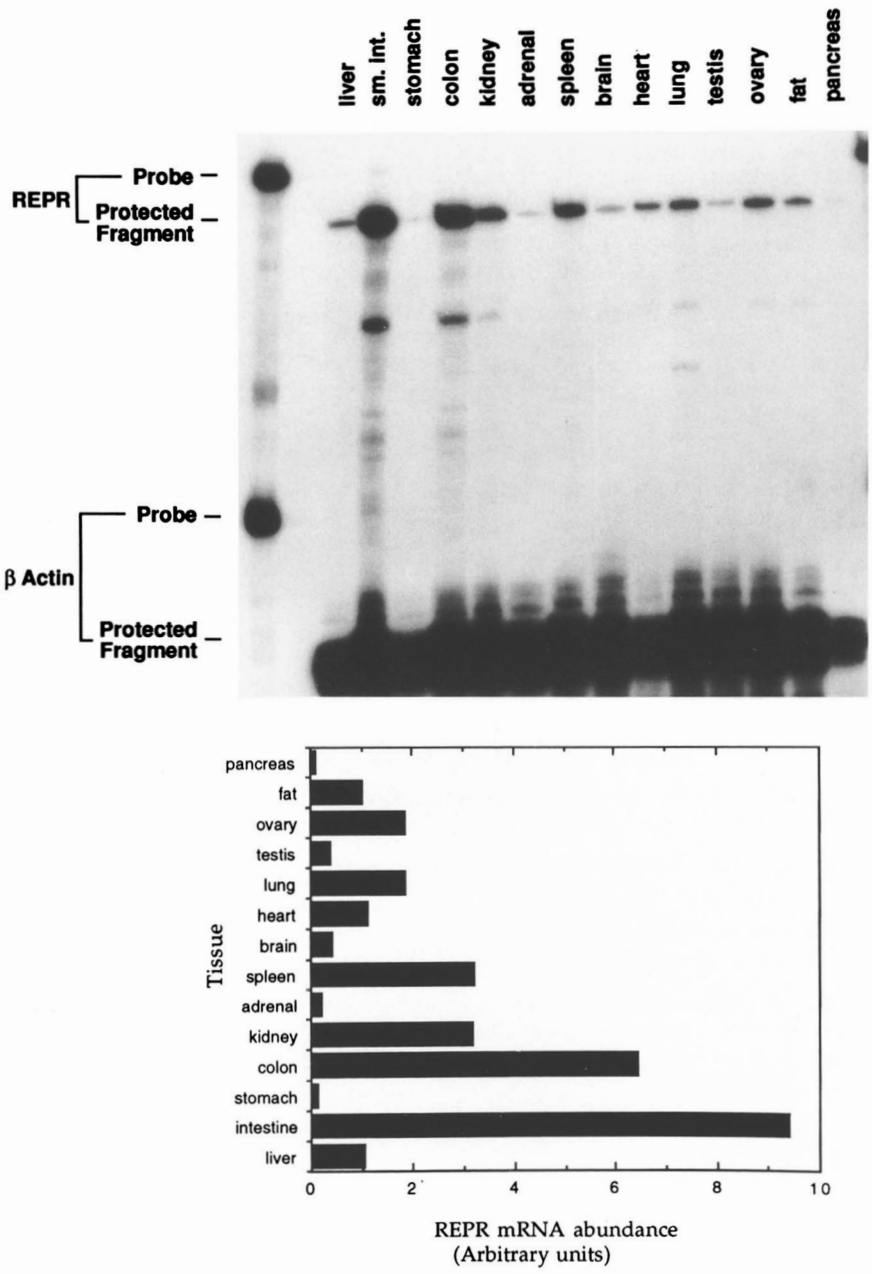


Fig. 1. Distribution of REPR mRNA in adult rat tissues. Upper panel: total RNA (50 μg) from a pool (n = 5 or 6 animals per pool) of adult rat tissue was hybridized with a 423 nt cRNA (nucleotides 88–510) transcribed from a Sma I-KpnI fragment of the REPR cDNA together with a 247 nt cRNA transcribed from a mouse β-actin cDNA (nucleotide 660–906). Following digestion of the unhybridized probe, the reaction products were analyzed on a denaturing 6% polyacrylamide gel and subjected to autoradiography. A representative analysis (14 h exposure) is illustrated. Lower panel: autoradiographs from multiple exposures within the linear range of film sensitivity were scanned with a laser densitometer to provide an estimate of the relative distribution of REPR mRNA. The data is expressed following normalization to the β-actin signal, and presented as arbitrary units.

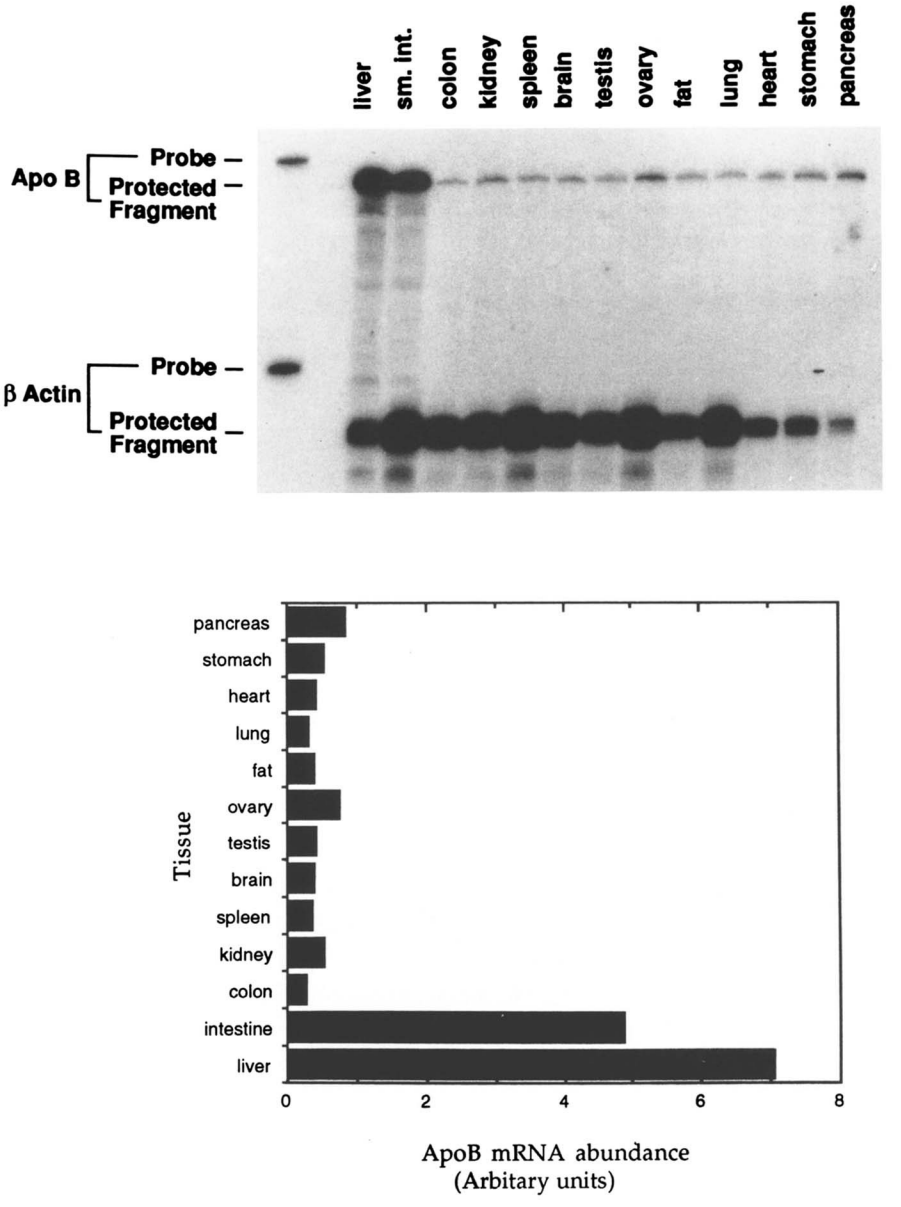


Fig. 2. Distribution of apoB mRNA in adult rat tissues. Upper panel: total RNA (50 μg) from a pool (n = 5 or 6 animals per pool) of adult rat tissue was hybridized with a 471 nt apoB cRNA (nucleotide 6512–6982) transcribed from pRBF-CAA, together with a 247 nt cRNA transcribed from a mouse β-actin cDNA (nucleotide 660–906). After digestion of the unhybridized probe, the reaction products were analyzed on a denaturing 6% polyacrylamide gel and subjected to autoradiography. A representative analysis (7 h exposure) is illustrated. Lower panel: autoradiographs from multiple exposures within the linear range of film sensitivity were scanned with a laser densitometer to provide an estimate of the relative distribution of apoB mRNA. The data is expressed following normalization to the β-actin signal, and presented as arbitrary units.

highest abundance in the small intestine, but with detectable transcript at lower levels in all tissues examined. ApoB mRNA was found in both edited and unedited forms, in tissues with relatively abundant REPR mRNA such as colon, kidney, liver, and spleen as well as in tissues with lower levels of REPR mRNA including brain, adrenal, and stomach, as shown in **Fig. 3.** A large range of apoB mRNA editing was encountered with 29% of colon apoB mRNA in the edited form while brain apoB mRNA was approximately 50% edited. Although some variability is noted in the extent of apoB mRNA editing in different preparations (see adrenal -1 and -2, Fig. 3) there appears to be no consistent relationship between the estimated relative REPR mRNA abundance and the extent of endogenous apoB mRNA editing, specifically in extrahepatic, extraintestinal tissues with comparable apoB mRNA abundance (Fig. 2).

S-100 extracts were prepared from kidney, spleen, and

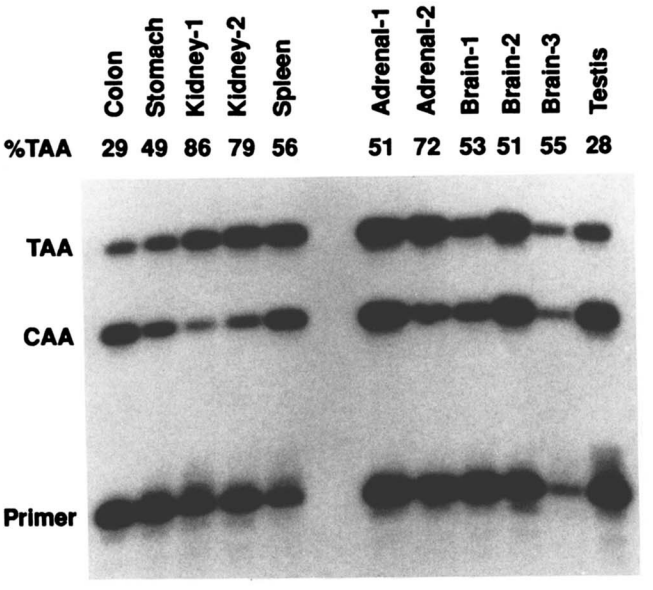


Fig. 3. Endogenous apoB mRNA editing in extrahepatic/extraintestinal adult rat tissue. Endogenous apoB mRNA flanking the edited base was amplified by RT-PCR from several peripheral tissues, as indicated. The products of RT-PCR were analyzed by primer extension to determine the proportion of edited (TAA) and unedited (CAA) apoB cDNA. A representative analysis is illustrated.

liver and in vitro apoB RNA editing was examined in order to demonstrate functional activity of REPR in these tissues. As shown in **Fig. 4,** left panel, S-100 extracts prepared from spleen edited only 2–3% of a synthetic apoB RNA template but showed effective apoB RNA editing (38%) when complemented by 10 μg chicken intestinal S-100 extract. This implies that REPR is functional in the rat spleen but that a relative deficiency of the complementation factor(s), provided by chicken intestinal S-100 extracts, limits the activity of spleen S-100 extracts when assayed alone. The apparent inhibition of apoB RNA editing activity when 50 μg spleen S-100 extracts were mixed with 10 μg chicken intestinal S-100 extracts (7% vs. 38%) is similar to that previously noted in assays using REPR expressed in Xenopus oocytes and reinforces the hypothesis that editing efficiency is determined in part by the stoichiometry between REPR and the complementation factors, excess of either component producing inhibition of the reaction (7). S-100 extracts prepared from kidney demonstrated effective apoB RNA editing when added alone and showed further enhancement after the addition of chicken intestinal S-100 extracts (Fig. 4, middle panel). Liver S-100 extracts demonstrated a modest increase in apoB RNA editing after the addition of chicken intestinal S-100 extracts, with inhibition at the highest amounts used (Fig. 4, right panel), supporting the hypothesis advanced above with respect to the stoichiometry of the reaction components.

In situ hybridization and immunocytochemical localization of REPR

In situ hybridization detection of REPR mRNA in the adult, proximal small intestine demonstrates the transcript in all cells along the villus-crypt axis (**Fig. 5,** top panel) while apoB mRNA was restricted to the villus cells and demonstrated little signal in the crypts (Fig. 5, middle panel). All cells within the hepatic lobule appeared to contain both apoB mRNA and, at lower abundance, REPR mRNA (**Fig. 6**). Analysis of REPR mRNA within the spleen revealed a restricted distribution, predominantly within red pulp (**Fig. 7,** top panel), while REPR mRNA was expressed in the superficial crypt cells of the adult colon (Fig. 7, lower panel). ApoB mRNA was not detectable by in situ hybridization in tissues other than the small intestine and liver (data not shown).

Immunocytochemical localization of REPR (**Fig. 8**) demonstrates a predominant distribution in the superficial cells of the colonic crypts (Fig. 8A, B). REPR was demonstrated in the adult liver with an intense perinuclear staining pattern (Fig. 8C arrows). In the small intestine, REPR was shown in all cells along the villus-crypt axis (Fig. 8E) and demonstrated a diffuse cytoplasmic staining pattern. The distribution of REPR protein in the spleen recapitulated the distribution suggested by in situ hybridization with immunoreactivity confined to the red

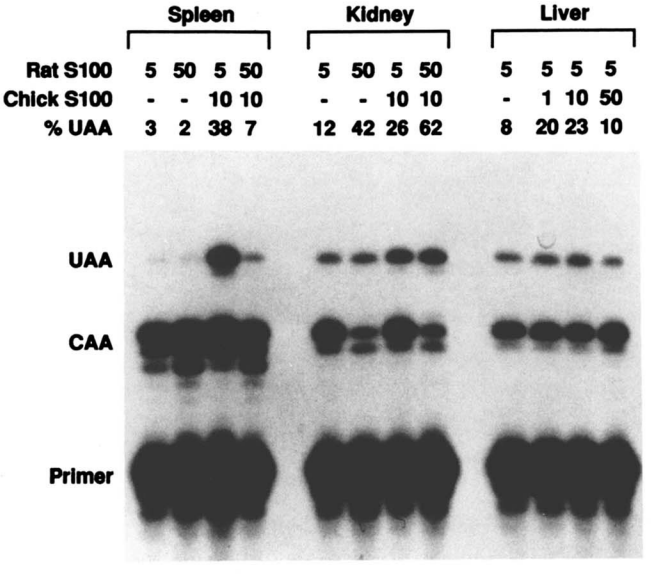


Fig. 4. ApoB RNA editing activity of tissue S-100 extracts from adult kidney, spleen, and liver. Cytosolic S-100 extracts were prepared from adult rat spleen, kidney, or liver and 5 or 50 μg was incubated with a synthetic apoB RNA template, either in the absence (–) or presence of 1, 10, or 50 μg chicken intestinal S-100 extracts (chick S100). After incubation, the RNA was extracted and primer extension analysis was undertaken to determine the proportion of edited (UAA) and unedited (CAA) substrate. Similar results were obtained from three different preparations of spleen and kidney S100 extracts.

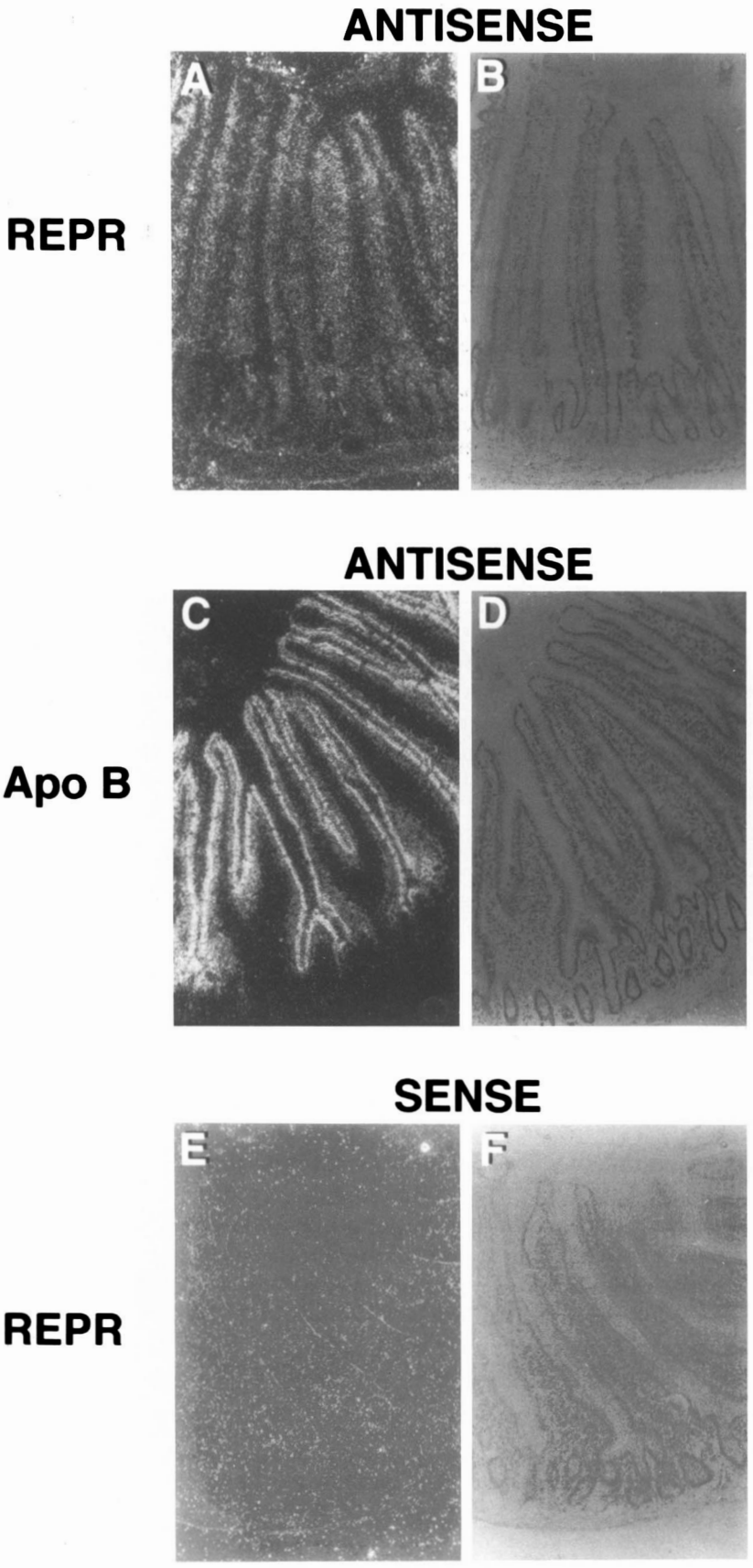


Fig. 5. In situ hybridization of REPR and apoB mRNA in adult rat proximal small intestine. ^{35}S-labeled [REPR/apoB] antisense or sense cRNA probes were prepared by in vitro transcription and hybridized with cryostat sections as described in Methods. The sections were counterstained with hematoxylin and photographed under either dark-field (panels A, C, E) or bright-field (B, D, F) illumination.

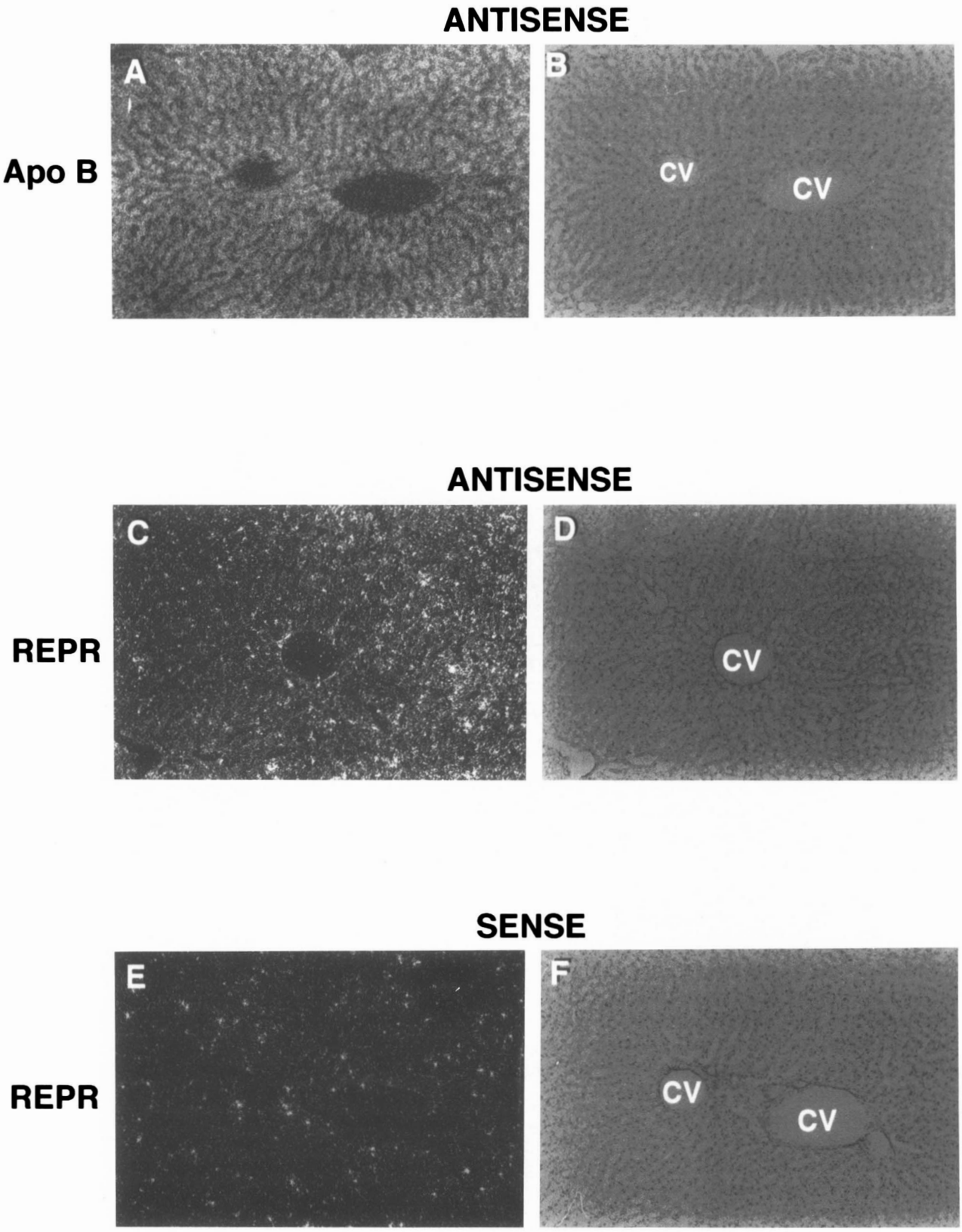


Fig. 6. In situ hybridization of REPR and apoB mRNA in adult rat liver. ^{35}S-labeled [REPR/apoB] antisense or sense cRNA probes were prepared by in vitro transcription and hybridized with cryostat sections as described in Methods. The sections were counterstained with hematoxylin and photographed under either dark-field (panels A, C, E) or bright-field (B, D, F) illumination; CV, central vein.

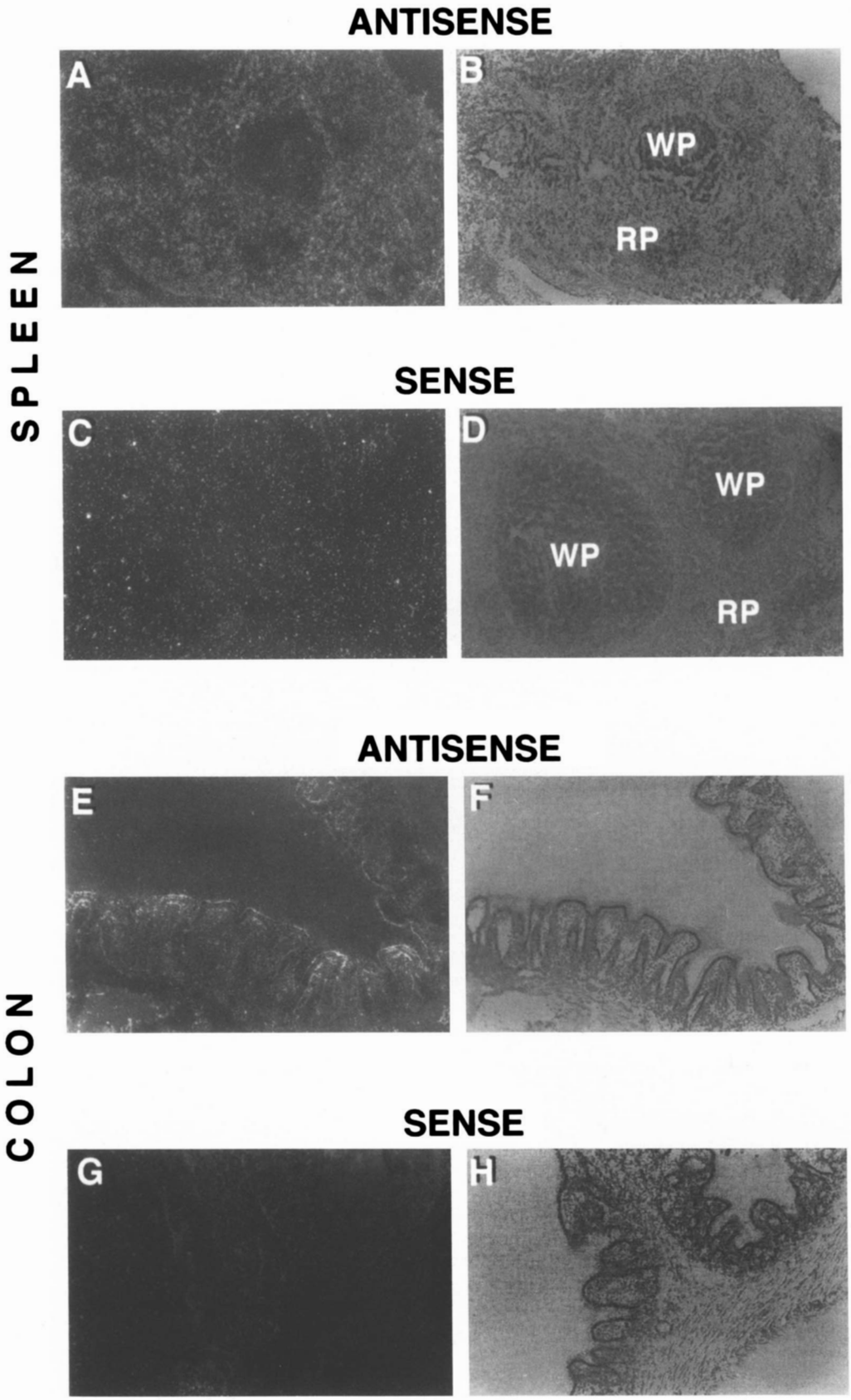


Fig. 7. In situ hybridization of REPR in adult rat spleen (panels A–D) and colon (panels E–H). ^{35}S-labeled REPR antisense or sense cRNA probes were prepared by in vitro transcription and hybridized with cryostat sections as described in Methods. The sections were counterstained with hematoxylin and photographed under either dark-field (A, C, E, G) or bright-field (B, D, F, H) illumination; RP, red pulp; WP, white pulp.

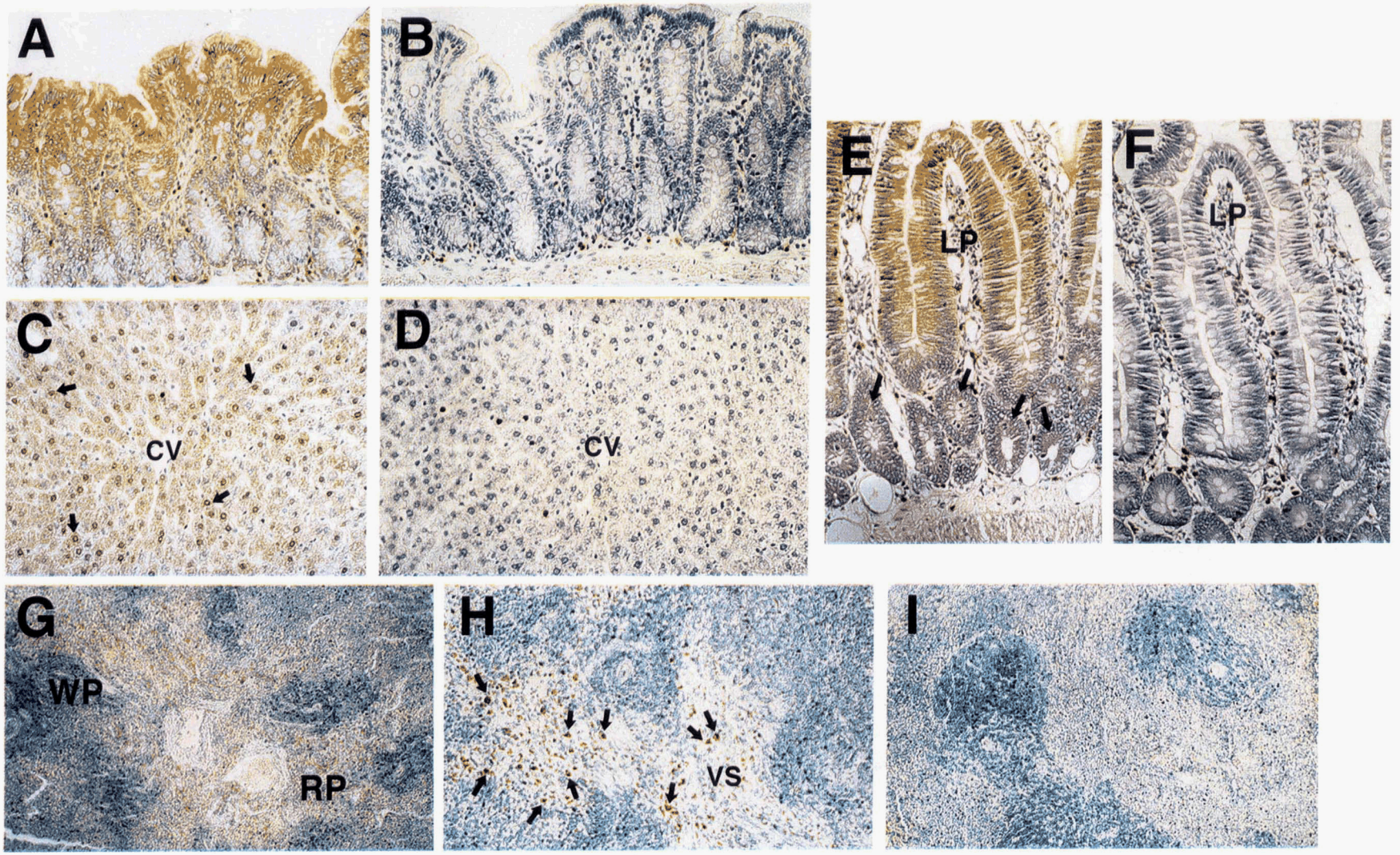


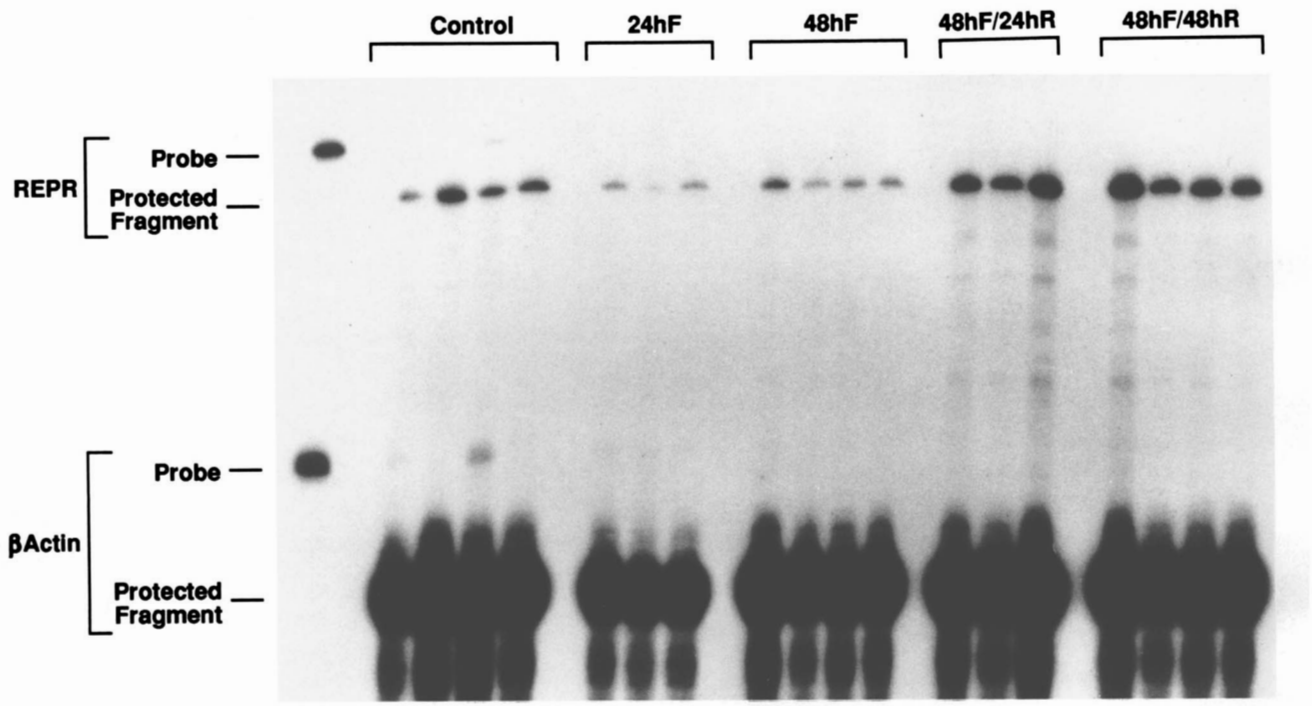
Fig. 8. Immunocytochemical localization of REPR in adult rat tissues. Tissue sections were prepared as detailed in Methods and reacted with a rabbit anti-REPR antipeptide antiserum, followed by a biotinylated anti-rabbit IgG and avidin-peroxidase color development. Panels A, C, E, G, H = antipeptide antisera; Panels B, D, F, I = peptide absorbed antiserum control. Panel A, B = colon, (100× magnification). Panel C, D = liver (100× magnification); CV, central vein. Arrows point to intense perinuclear staining of REPR. Panel E, F = proximal small intestine (100× magnification); LP, lamina propria. Arrows point to staining within crypts. Panels G, H, I = spleen (G, I 100×; H 200× magnification); WP, white pulp; RP, red pulp; VS, venous sinus. Arrows point to isolated cells within venous sinus endothelium with intense REPR immunoreactivity.

pulp. Higher magnification revealed intense staining of isolated cells lining the venous sinuses of the red pulp (Fig. 8G, H arrows).

REPR mRNA abundance in the setting of alterations in apoB mRNA editing

Previous studies have demonstrated that apoB mRNA editing is subject to developmental, hormonal, and dietary regulation in the rat (10–17). We have reexamined these observations in order to demonstrate whether apoB mRNA editing is regulated in parallel with the abundance of REPR mRNA. As shown in **Fig. 9,** upper panel, animals subjected to a 24 or 48 h fast showed a 1.7-fold decrease in hepatic REPR mRNA abundance when compared to controls fed ad libitum. Subsequent refeeding of a high carbohydrate diet for 24 or 48 h resulted in a 2.1- or 2.8-fold increase in REPR mRNA, revealing overall an approximately 5-fold range of hepatic REPR mRNA abundance in the adult rat. As shown in Fig. 9, lower panel, these changes were significantly correlated with hepatic apoB mRNA editing, suggesting that the regulation of apoB mRNA editing in this setting may be largely accounted for by the abundance of REPR. However, this suggestion remains to be confirmed by analysis of REPR protein.

Samples of fetal small intestine, liver, and kidney were examined for apoB mRNA editing and REPR mRNA abundance in order to evaluate the temporal pattern of these two parameters. As shown in **Fig. 10,** top panel, small intestinal apoB mRNA editing increased prenatally, beginning on gestational day 17–18, while the increase in REPR mRNA abundance began on gestational day 21. Analysis of hepatic apoB mRNA editing revealed a biphasic increase, first at gestational day 22 and a second



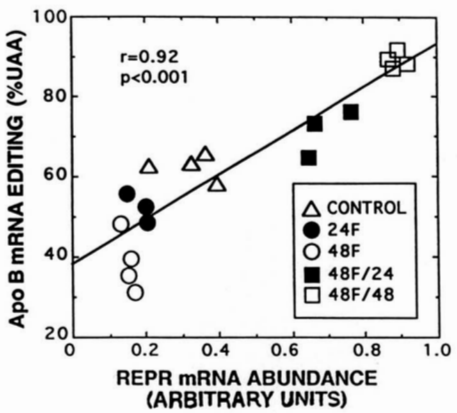


Fig. 9. Regulation of hepatic REPR mRNA abundance in response to fasting/refeeding. Upper panel: 50 μg total RNA from animals from each of the indicated groups was analyzed by RNase protection assay as described above and in the legend to Fig. 1. After digestion of the unhybridized probes, the reaction products were analyzed by 6% denaturing polyacrylamide electrophoresis. Lower panel: the extent of apoB mRNA editing, as determined by primer extension analysis of the same samples, was plotted against the values for REPR mRNA abundance and the correlation determined.

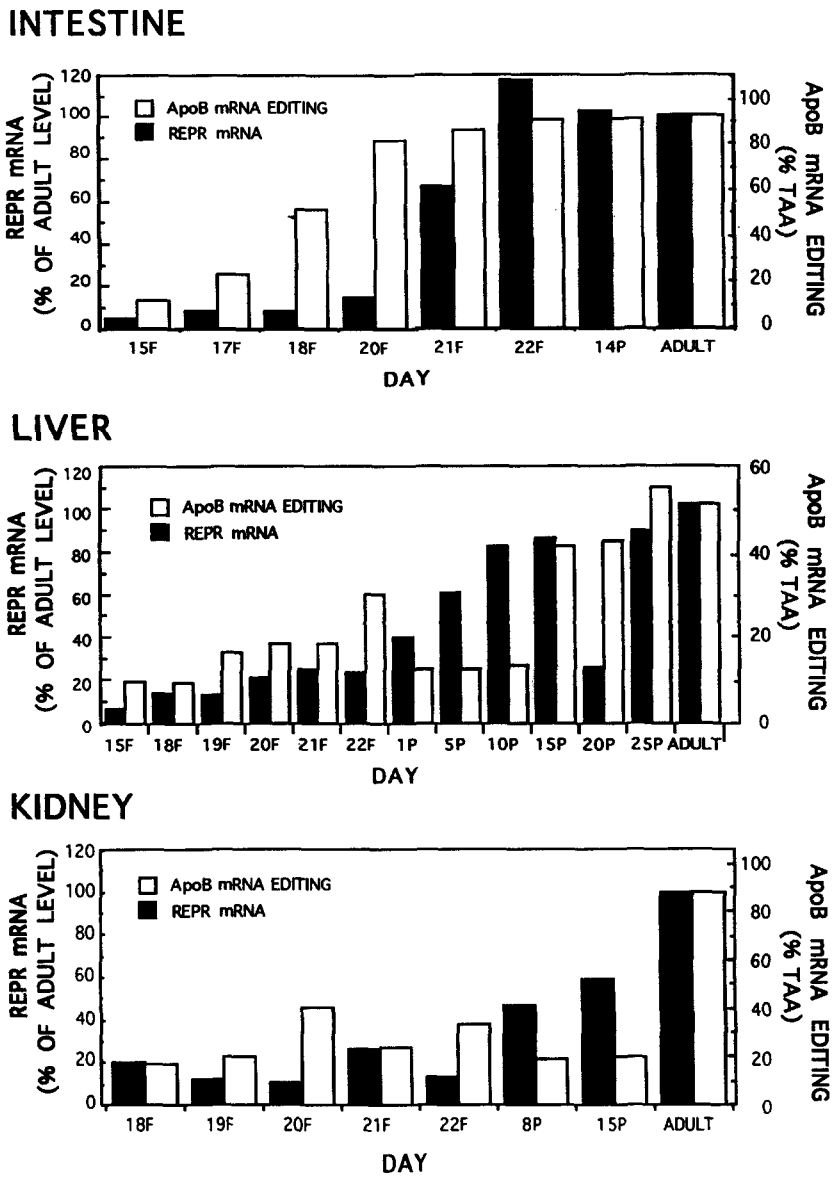


Fig. 10. Developmental regulation of apoB mRNA editing and REPR mRNA abundance in fetal and neonatal small intestine, liver and kidney. ApoB mRNA editing was determined after RT-PCR amplification of the region flanking the edited base (Methods) and the PCR products assayed by primer extension. ApoB mRNA editing is expressed as % TAA. RNA from the same tissue pool was subjected to RNase protection to determine REPR mRNA abundance. The results are presented as % of the respective adult level, a value assigned for each tissue at 100%.

peak beginning postnatally at day 15 (Fig. 10, middle panel). REPR mRNA abundance appeared to increase in step with the first peak of apoB mRNA editing, but demonstrated temporal dissociation postnatally, with a substantial increase in mRNA abundance from day 1 to 15 at the same time as apoB mRNA editing was unchanged. This temporal dissociation was less noticeable in the developing kidney (Fig. 10, lower panel).

Taken together, the data provide evidence that the regulation of apoB mRNA editing may be mediated via alterations in REPR gene expression and/or the modulated expression of other components. The mechanisms that may be operating to regulate the development of apoB mRNA editing are unknown at present but presumably include hormonal and nutritional alterations known to occur perinatally. In this regard, it should be emphasized that neonatal animals were studied without prior fasting.

DISCUSSION

ApoB mRNA editing, a site-specific cytidine deamination, involves the coordinated interaction of distinct protein components, one of which, REPR, most likely represents the catalytic component of the apoB mRNA editing enzyme. REPR was cloned by means of an expression strategy using chicken intestinal S-100 extracts as a source of complementation activity (6, 7). As chicken intestine contains only unedited apoB mRNA and as chicken intestinal S-100 extracts alone have no intrinsic editing activity, the data strongly imply the presence of additional factor(s) for the apoB mRNA editing reaction (6, 7). The present findings extend this earlier report concerning the tissue distribution of REPR mRNA and also demonstrate the presence of these auxiliary, or complementation factor(s) in numerous tissues in the rat.

Several features of these findings merit further discussion. REPR mRNA was found in every tissue examined, including tissues with very low levels of apoB mRNA. Earlier work demonstrated the presence of low levels of edited apoB mRNA in multiple human fetal tissues and more recent studies reported similar findings in the adult mouse (10, 19). As both of these reports relied upon RT-PCR, the relative abundance of apoB mRNA was presumed to be extremely low. The present report, using a sensitive RNase protection assay, demonstrates that apoB mRNA is indeed detectable in all rat tissues, albeit at low levels. These findings imply that, while a function for REPR in tissues such as spleen and kidney is unknown at present, a role in the editing of a low abundance apoB mRNA cannot be excluded, even though its significance with respect to lipoprotein secretion is likely to be minimal. Its presence in tissues such as colon and spleen, combined with the restricted cell-specific pattern of expression of both mRNA and protein, suggest the possibility that other functions may emerge for this gene, and these possibilities are currently being pursued. There may also be species considerations in attributing a biological function to REPR in these tissues, since the human homolog (HEPR) was found to be predominantly confined to the adult small intestine, with much lower levels of mRNA detectable in stomach and colon (20). In regard to the cell-specific distribution of REPR, both in situ hybridization and immunocytochemical studies demonstrate the presence of the gene product in all cells of the villus-crypt axis of the small intestine and in all cells of the hepatic lobule. Thus, the two most important sites of action of this gene product in the rat show no apparent local gradients of expression, although the possibility of a proximal to distal gradient in the small intestine was not specifically investigated. Immunocytochemical localization of REPR in the liver demonstrated intense perinuclear staining which is consistent with its presumed site of action, as inferred from the demonstration that apoB mRNA editing is an intranuclear event (21). This possibility is not diminished by the demonstration of a diffuse cytoplasmic staining pattern for REPR within the small intestinal enterocyte, as fixation and processing artefacts may preclude such resolution. Additionally, there is ample precedent for the import of proteins from the cytoplasmic compartment into the nucleus (22). Further studies are underway to resolve the issue of the subcellular localization of REPR using immunoelectron microscopy. Additionally, REPR mRNA was detectable in the red pulp of the spleen, findings confirmed by examination of the distribution of the protein by immunocytochemical identification. The identity of the cells expressing the highest levels of the protein within the venous sinuses of the red pulp is currently under investigation.

The present findings also demonstrate that the entire apoB mRNA editing complex is functional in some extrahepatic, extraintestinal tissues including kidney and, albeit at lower levels of activity, spleen. These findings extend other recent results demonstrating complementation activity for apoB RNA editing to be present in both chicken intestinal and human liver S-100 extracts, neither of which is competent to edit apoB RNA (7, 8). Taken together, the data offer convincing proof that components of the apoB mRNA editing enzyme are widely distributed, again raising the question of a function in such locations. The recent demonstration that the WT-1 gene undergoes a developmentally regulated U to C RNA editing conversion in the neonatal kidney raises the tempting speculation that other targets may exist for components of the apoB RNA editing machinery, although it should be emphasized that there is no information to suggest that these two examples of mammalian RNA editing are in fact related (23). There is little information concerning the nature of the complementation factor(s) alluded to above, which are currently identified on the basis of a functional assay. Independent reports have demonstrated the presence of proteins, in cell extracts prepared from tissues containing edited apoB mRNA, that can be UV cross-linked to the apoB RNA sequence (24–26). Several groups have demonstrated proteins of approximately 40–43 kDa and 66 kDa by SDS-PAGE, after UV cross-linking, in extracts from rat liver and small intestine (24–26) and these proteins have been proposed to be integral components of a higher order complex, referred to as an editosome (27). Although it is feasible, given the apparent specificity of this protein-RNA interaction, that these proteins may contribute to the complementation activity referred to above, proof of this hypothesis will await the further isolation and purification of these components.

The present studies provide information concerning the mechanisms that mediate the developmental and nutritional regulation of apoB mRNA editing in the rat. Previous studies established that rats subjected to fasting and subsequent refeeding of a high carbohydrate diet

demonstrated an increase in apoB mRNA editing in association with an increase in hepatic triglyceride synthesis (16). The present studies extend these findings by establishing that this modulation occurs in the setting of alterations in REPR gene expression, specifically a 5-fold range of REPR mRNA abundance. By contrast, recent studies have demonstrated that thyroid hormone modulation of hepatic apoB mRNA editing occurs without alteration in the abundance of REPR mRNA, suggesting the possibility that metabolic and hormonal modulation of rat hepatic apoB mRNA editing may involve different mechanisms (17). Presumably the thyroid hormone-dependent increase in hepatic apoB mRNA editing is mediated through regulation of the complementation factor(s), as evidenced by the increased activity of hepatic S-100 extracts isolated from thyroid hormone-treated rat livers (17), although the precise molecular mechanisms will await identification of these components.

The present studies also demonstrate that REPR mRNA abundance is developmentally regulated in the rat small intestine, liver, and kidney. These alterations in REPR mRNA abundance are temporally dissociated from the increases in apoB mRNA editing in these tissues and provide further support for the hypothesis that the regulation of apoB mRNA editing may be mediated through independent mechanisms, involving both REPR and the complementation factor(s). The pattern of developmental regulation of small intestinal and hepatic apoB mRNA editing is similar to that previously reported in the rat and mouse, but the nature of the developmental cues remains unknown (12, 19). In this context, previous studies (reviewed in ref. 28) have demonstrated that circulating levels of thyroxine increase in the neonatal rat during the first and second week of life and could thus potentially contribute to the observed pattern of increasing hepatic apoB mRNA editing at 10 and 15 days postnatally, in the setting of decreasing REPR mRNA abundance. On the other hand, the concomitant effects of alterations in circulating glucocorticoids require consideration even though recent work suggests that exogenous glucocorticoid administration to neonatal rats fails to modulate hepatic apoB mRNA editing (18, 28). The close temporal association of a perinatal increase in hepatic and intestinal triglyceride synthesis and secretion in the rat, described earlier (29), suggests the possibility that these events may be coordinated with alterations in apoB mRNA editing; elucidation of the underlying mechanisms coordinating these events may provide important insight into the development of hepatic and intestinal lipoprotein biogenesis. Developmental regulation of apoB mRNA editing activity was previously reported in both human and porcine small intestine but the pattern of this developmental increase indicated that the adult phenotype was reached prenatally, towards the end of the second trimester (10, 11). In this regard, studies are currently underway to investigate the developmental regulation of HEPR, the human homolog of REPR, in the fetal human small intestine.[2]

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REFERENCES

1. Powell, L. M., S. C. Wallis, R. J. Pease, Y. H. Edwards, T. J. Knott, and J. Scott. 1987. A novel form of tissue-specific RNA processing produces apolipoprotein B-48 in intestine. *Cell.* **50:** 831–840.
2. Chen, S. H., G. Habib, C. Y. Yang, Z. W. Gu, B. R. Lee, S. Weng, S. R. Silberman, S. J. Cai, J. P. Deslypere, M. Rosseneu, A. M. Gotto, W. H. Li, and L. Chan. 1987. Apolipoprotein B-48 is the product of a messenger RNA with an organ-specific in-frame stop codon. *Science.* **238:** 363–366.
3. Greeve, J., L. Altkemper, J. H. Dieterich, H. Greten, and E. Windler. 1993. Apolipoprotein B mRNA editing in 12 different mammalian species: hepatic expression is reflected in low concentrations of apoB-containing plasma lipoproteins. *J. Lipid Res.* **34:** 1367–1383.
4. Bostrom, K., Z. Garcia, K. S. Poksay, D. F. Johnson, A. J. Lusis, and T. L. Innerarity. 1990. Apolipoprotein B mRNA editing: direct determination of the edited base and occurance in non-apolipoprotein B producing cell lines. *J. Biol. Chem.* **265:** 22446–22452.
5. Johnson, D. F., K. S. Poksay, and T. L. Innerarity. 1993. The mechanism for apoB mRNA editing is deamination. *Biochem. Biophys. Res. Commun.* **195:** 1204–1210.
6. Teng, B. B., C. F. Burant, and N. O. Davidson. 1993. Molecular cloning of an apolipoprotein B mRNA editing protein. *Science.* **260:** 1816–1819.
7. Giannoni, F., B. K. Bonen, T. Funahashi, C. Hadjiagapiou, C. F. Burant, and N. O. Davidson. 1994. Complementation of apolipoprotein B mRNA editing by human liver accompanied by secretion of apolipoprotein B48. *J. Biol. Chem.* **269:** 5932–5936.
8. Teng, B. B., and N. O. Davidson. 1992. Evolution of intestinal apolipoprotein B mRNA editing. Chicken apolipoprotein B mRNA is not edited, but chicken enterocytes contain in vitro editing enhancement factor(s). *J. Biol. Chem.* **267:** 21265–21272.
9. Navaratnam, N., J. R. Morrison, S. Bhattacharya, D. Patel, T. Funahashi, F. Giannoni, B. B. Teng, N. O. Davidson, and J. Scott. 1993. The p27 catalytic subunit of the apolipoprotein B mRNA editing enzyme is a cytidine deaminase. *J. Biol. Chem.* **268:** 20709–20712.
10. Teng, B. B., M. Verp, J. Salomon, and N. O. Davidson.

[2]As a final comment, it should be noted that the approved gene symbol assigned to the catalytic subunit of the mammalian apoB mRNA editing enzyme is APOBEC-1 (apoB mRNA editing enzyme, catalytic polypeptide #1). This symbol will be used in future reports in place of either of the currently used terms REPR (HEPR, etc.) or p27.

1990. Apolipoprotein B messenger RNA editing is developmentally regulated and widely expressed in human tissues. *J. Biol. Chem.* **265:** 20616–20620.
11. Teng, B. B., D. D. Black, and N. O. Davidson. 1990. Apolipoprotein B messenger RNA editing is developmentally regulated in pig small intestine: nucleotide comparison of apolipoprotein B editing regions in five species. *Biochem. Biophys. Res. Commun.* **173:** 74–80.
12. Wu, J. H., C. F. Semenkovich, S. H. Chen, W. H. Li, and L. Chan. 1990. Apolipoprotein B mRNA editing: validation of a sensitive assay and developmental biology of RNA editing in the rat. *J. Biol. Chem.* **265:** 12312–12316.
13. Giannoni, F., F. J. Field, and N. O. Davidson. 1994. An improved reverse transcription-polymerase chain reaction method to study apolipoprotein gene expression in Caco-2 cells. *J. Lipid Res.* **35:** 340–350.
14. Davidson, N., L. M. Powell, S. C. Wallis, and J. Scott. 1988. Thyroid hormone modulates the introduction of a stop codon in rat liver apolipoprotein B messenger RNA. *J. Biol. Chem.* **263:** 13482–13485.
15. Seishima, M., C. L. Bisgaier, S. L. Davies, and R. M. Glickman. 1991. Regulation of hepatic apolipoprotein synthesis in the 17 alpha-ethinyl estradiol-treated rat. *J. Lipid Res.* **32:** 941–951.
16. Baum, C. L., B. B. Teng, and N. O. Davidson. 1990. Apolipoprotein B messenger RNA editing in the rat liver: modulation by fasting and refeeding a high carbohydrate diet. *J. Biol. Chem.* **265:** 19263–19270.
17. Inui, Y., F. Giannoni, T. Funahashi, and N. O. Davidson. 1994. REPR and complementation factor(s) interact to modulate rat apolipoprotein B mRNA editing in response to alterations in cellular cholesterol flux. *J. Lipid Res.* **35:** 1477–1489.
18. Inui, Y., A. M. L. Hausman, N. Nanthakumar, S. J. Henning, and N. O. Davidson. 1992. Apolipoprotein B messenger RNA editing in rat liver: developmental and hormonal modulation is divergent from apolipoprotein A-IV gene expression despite increased hepatic lipogenesis. *J. Lipid Res.* **33:** 1843–1856.
19. Higuchi, K., K. Kitagawa, K. Kogishi, and T. Takeda. 1992. Developmental and age-related changes in apolipoprotein B mRNA editing in mice. *J. Lipid Res.* **33:** 1753–1764.
20. Hadjiagapiou, C., F. Giannoni, T. Funahashi, S. F. Skarosi, and N. O. Davidson. 1994. Molecular cloning of a human small intestinal apolipoprotein B mRNA editing protein. *Nucleic Acids Res.* **22:** 1874–1879.
21. Lau, P. P., W. Xiong, H. J. Zhu, S. C. Chen, and L. Chan. 1991. Apolipoprotein B mRNA editing is an intranuclear event that occurs posttranscriptionally coincident with splicing and polyadenylation. *J. Biol. Chem.* **266:** 20550–20554.
22. Pante, N., and U. Aebi. 1993. The nuclear pore complex. *J. Cell Biol.* **122:** 977–984.
23. Sharma, P. M., M. Bowman, S. L. Madden, F. J. Rauscher III, and S. Sukumar. 1994. RNA editing in the Wilms' tumor susceptibility gene, WT1. *Genes & Dev.* **8:** 720–731.
24. Lau, P. P., S. H. Chen, J. C. Wang, and L. Chan. 1991. A 40 kilodalton rat liver nuclear protein binds specifically to apolipoprotein B mRNA around the RNA editing site. *Nucleic Acids Res.* **18:** 5817–5821.
25. Navaratnam, N., R. Shah, D. Patel, V. Fay, and J. Scott. 1993. Apolipoprotein B mRNA editing is associated with UV crosslinking of proteins to the editing site. *Proc. Natl. Acad. Sci. USA.* **90:** 222–226.
26. Harris, S. G., L. Sabio, E. Mayer, M. F. Steinberg, J. W. Backus, J. D. Sparks, C. E. Sparks, and H. C. Smith. 1993. Extract-specific heterogeneity in high-order complexes containing apolipoprotein B mRNA editing activity and RNA-binding proteins. *J. Biol. Chem.* **268:** 7382–7392.
27. Smith, H. C. 1993. Apolipoprotein B mRNA editing: the sequence to the event. *Semin. Cell Biol.* **4:** 267–278.
28. Henning, S. J. 1981. Postnatal development: coordination of feeding, digestion and metabolism. *Am. J. Physiol.* **241:** G199–G214.
29. Jamdar, S. C., M. Moon, S. Bow, and H. J. Fallon. 1978. Hepatic lipid metabolism age-related changes in triglyceride metabolism. *J. Lipid Res.* **19:** 763–770.