Alternative polyadenylation of apolipoprotein B RNA is a major cause of B-48 protein formation in rat hepatoma cell lines transfected with human apoB-100 minigenes

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Abstract The human apoB gene encodes an mRNA of 14121 nucleotides. In liver the apoB gene produces a full-length mature protein of 4,536 amino acids (B-100), whereas in the intestine this gene produces a truncated protein of 2,152 amino acids (B-48). B-48 results from RNA editing of nucleotide 6666 from C to U, thereby producing a stop codon at position 2153. Rat liver has been shown to contain apoB RNA editing capability resulting in production of both B-100 and B-48. To create an in vitro expression system for human B-100, a minigene with a wild type coding sequence for the entire B-100 protein (B-100/Gln) was stably transfected into rat hepatoma cells (McA-RH7777). Similarly, a minigene with mutation at nucleotide 6667 that allowed translation even after editing of nucleotide 6666 (B-100/Leu, nonstop mutant), a minigene with an additional nonsense mutation at nucleotide 7053 to produce B-50 (B-50/Leu), and a truncated wild type minigene with a stop signal at codon 3261 to produce B-74 and an mRNA of 10 kb (B-74/Gln) were also transfected. Very little full-length B-100 and B-74 was produced by any of the respective constructions, including the B-100/Leu with the nonstop mutation. Transfection with B-100/Gln, B-100/Leu and B-74/Gln constructions produced greater than 90% of apoB as B-48, whereas the B-50/Leu construction produced 76% B-50 and 24% B-48. The inability of the B-100/Leu construction to produce B-100 suggested an explanation for B-48 production other than RNA editing. Northern blot analysis showed that the RNA produced by all four transfectants was shortened to a size of about 7 kb. A 10-kb but no 7-kb RNA was observed in the B-74/Leu construction when transfected to Chinese hamster ovary cells suggesting cell type specificity in generation of a shortened RNA. The 3' end of apoB RNA from McA-RH7777 B-100/Leu transfectants was reverse transcribed, cloned, and sequenced. This revealed two species of RNA: one polyadenylated at or near nucleotide 6775 capable of coding for B-48, the other polyadenylated at nucleotide 7080 capable of coding for B-50. In 18% of the cDNA clones, nucleotide 6666 was edited from C to T. In 6 of 34 clones, addition of the poly(A) tail after nucleotide 6774 created a TAA stop codon, whereas no stop signals could be detected in the remaining clones. These studies suggest that lack of human B-100 expression in McA-RH7777 cells is due to alternative polyadenylation of apoB RNA at cryptic polyadenylation sites.—**Heinemann, T., S. Metzger, E. A. Fisher, J. L. Breslow, and L-S. Huang.** Alternative polyadenylation of apolipoprotein B RNA is a major cause of B-48 protein formation in rat hepatoma cell lines transfected with human apoB-100 minigenes. *J. Lipid Res.* 1994. **35:** 2200–2211.

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Apolipoprotein B (apoB) is the major protein component of low density lipoproteins (LDL) and is the ligand for the LDL receptor, which mediates cellular uptake of LDL (1). The human apoB gene contains 29 exons and is 43 kb in length (2). ApoB protein exists in two forms, B-100 and B-48, that differ by size and site of synthesis, the liver and intestine, respectively (1). ApoB mRNA, which is 14 kb in length, encodes a mature B-100 protein of 4,536 amino acids (3, 4). B-100 and B-48 are products of the same gene (1). A specific editing of C to U at nucleotide 6666 of B-100 RNA converts the glutamine (Gln) codon 2153 to a stop codon accounting for the production of B-48 (5, 6).

Elevated plasma LDL and apoB levels are major risk factors for atherosclerosis susceptibility and coronary heart disease, while moderately decreased levels of LDL cholesterol are generally associated with a decreased risk (7, 8). Specific apoB gene mutations have been shown to

Abbreviations: apoB, apolipoprotein B; LDL, low density lipoprotein; VLDL, very low density lipoprotein; CHO cells, Chinese hamster ovary cells; CMV, cytomegalovirus.

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either increase or decrease LDL cholesterol levels (7). Elucidation of apoB structure/function relationship may, therefore, enhance our understanding of the role of apoB in lipoprotein metabolism and atherosclerosis susceptibility. Expression of truncated apoB proteins in vitro has yielded insight into the role of the amino-terminal end of the protein in lipoprotein assembly and secretion (9–11). Several such studies demonstrate the use of rat hepatoma cells, McA-RH7777, for expression of human apoB proteins in vitro (10–12). The McA-RH7777 cell line retains two key properties of primary rat hepatocytes: secretion of apoB-containing VLDL particles, and a high rate of protein production (10, 13). However, unlike in humans, RNA editing activity of apoB RNA is present in both rat liver and intestine (14). Consequently, transfection of McA-RH7777 cells with an apoB-100 minigene construction yields mainly B-48 proteins (12). Base substitutions near the editing site fail to prevent editing of apo-B RNA in McA-RH7777 cells (15). The formation of B-48-like proteins, however, is abolished if the editing of nucleotide 6666 is allowed to occur in a truncated B-53 construction harboring a nonstop leucine (CTA) codon at position 2153 (12). When this leucine codon (CUA) is edited, a silent substitution that codes for leucine (UUA) results, thereby allowing translation to proceed beyond the editing site.

In this report, we describe our results obtained from expression of one wild type and two mutated B-100 minigene constructions and a B-74 truncated minigene construction in McA-RH7777 cells. All four minigene constructions, including the B-100 minigene containing a nonstop leucine mutation at codon 2153, failed to express a full-length B-100 protein. Instead, a B-48-like protein was produced from a shortened 7-kb apoB RNA generated by alternative polyadenylation at cryptic polyadenylation sites. A shortened apoB RNA, however, was not observed in Chinese hamster ovary (CHO) cells which do not exhibit RNA editing activity.

MATERIALS AND METHODS

Construction of apoB minigenes

ApoB-100 and B-74 minigenes were constructed as shown schematically in **Fig. 1.** To create a wild type B-100 minigene construction, a heterologous promoter derived from cytomegalovirus (CMV), and fragments from apoB cDNA and genomic clones were used. As shown in line 1 of Fig. 1A, clone pCMV3 contains the CMV promoter sequence and was derived from pCMVβ (Clontech Inc.). For subcloning purposes, a PacI site was engineered 3' to the SalI site in the vector. Clones pB274 (fragment A) and pB271 (fragment B) containing the cDNA from −10 to +2278 and +2279 to +5289, respectively, were obtained by cDNA synthesis from poly(A)$^+$ RNA derived from human hepatoma cells, HepG2 (lines 2 and 3 of Fig. 1A). Clone pB264 was subcloned from a 12-kb XhoI fragment of a genomic clone, CosB31 (16), and contains the genomic sequence from +5290 to approximately 1 kb of the sequence 3' to poly(A) signals of the apoB gene (line 4 of Fig. 1A). A linker containing a PacI site was inserted downstream to the 3' XhoI site, so that digestion with SalI and PacI enzymes would release a 10-kb fragment (fragment C). Fragment C contains most of exon 26, exons 27–29, introns 26–28, the termination codon, the poly(A) signals, and 1 kb of the downstream sequences. As shown in Fig. 1A, the final construction, pB384, was obtained by ligation of fragments A and B (line 5 of Fig. 1A); insertion of fragments A and B into pCMV3 vector (line 6 of Fig. 1A); and ligation of fragment C with the vector containing the CMV promoter, fragments A and B derived from line 6 (line 7 of Fig. 1A). Clone pB384, was analyzed by restriction enzyme digestions and partial DNA sequence analysis. Based on the expected size of apoB proteins expressed from this minigene and the amino acid residue at codon 2153, the construction was designated as B-100/Gln (line 1 of Fig. 1B).

To prevent introduction of a stop signal at codon 2153 of the RNA transcribed from the apoB minigene constructions by RNA editing, an editing mutant was generated (B-100/Leu, line 2 of Fig. 1B). A single base substitution of T for A at nucleotide 6667 of the minigene, resulting in a Gln (CAA) to Leu (CTA) codon change, was created using a site-directed mutagenesis system (Amersham Inc.) based on the method of Sayers, Schmidt, and Eckstein (17). Editing of the RNA transcribed from this B-100 minigene in McA-RH7777 cells would result in a silent substitution, i.e., CUA (Leu) to UUA (Leu), rather than a stop codon (nonstop editing mutant). For mutagenesis, a 1.8-kb ClaI-HindIII fragment (line 4 of Fig. 1A) was subcloned, and then subjected to mutagenesis. The final B-100 nonstop editing mutant was achieved by substituting the wild type ClaI-HindIII fragment with the mutated fragment. The base substitution in the final construction, pB379, was verified by DNA sequence analysis. One of these editing mutant clones was found to contain an additional A to T base change in nucleotide 7053 resulting in an in frame stop codon (TGA) at amino acid 2282. The predicted size of the apoB protein expressed from this nonsense and editing double mutant (pB344) would be B-50 (B-50/Leu, line 4 of Fig. 1B).

A truncated B-74 construction was created by substituting the 10-kb fragment C in the B-100 minigene construction with a 4-kb SalI-AvrII fragment (fragment C') isolated from pB264 (line 4 of Fig. 1A). The last codon 3260 (GGT, Gly) in the fragment C' was reconstituted when engineered into a plasmid vector containing linker sequences including a TAA stop signal. The final construction, pB444, contains the CMV promoter, B-74 sequences, and a SV40 poly(A) signal (B-74/Gln, line 3 of Fig. 1B).

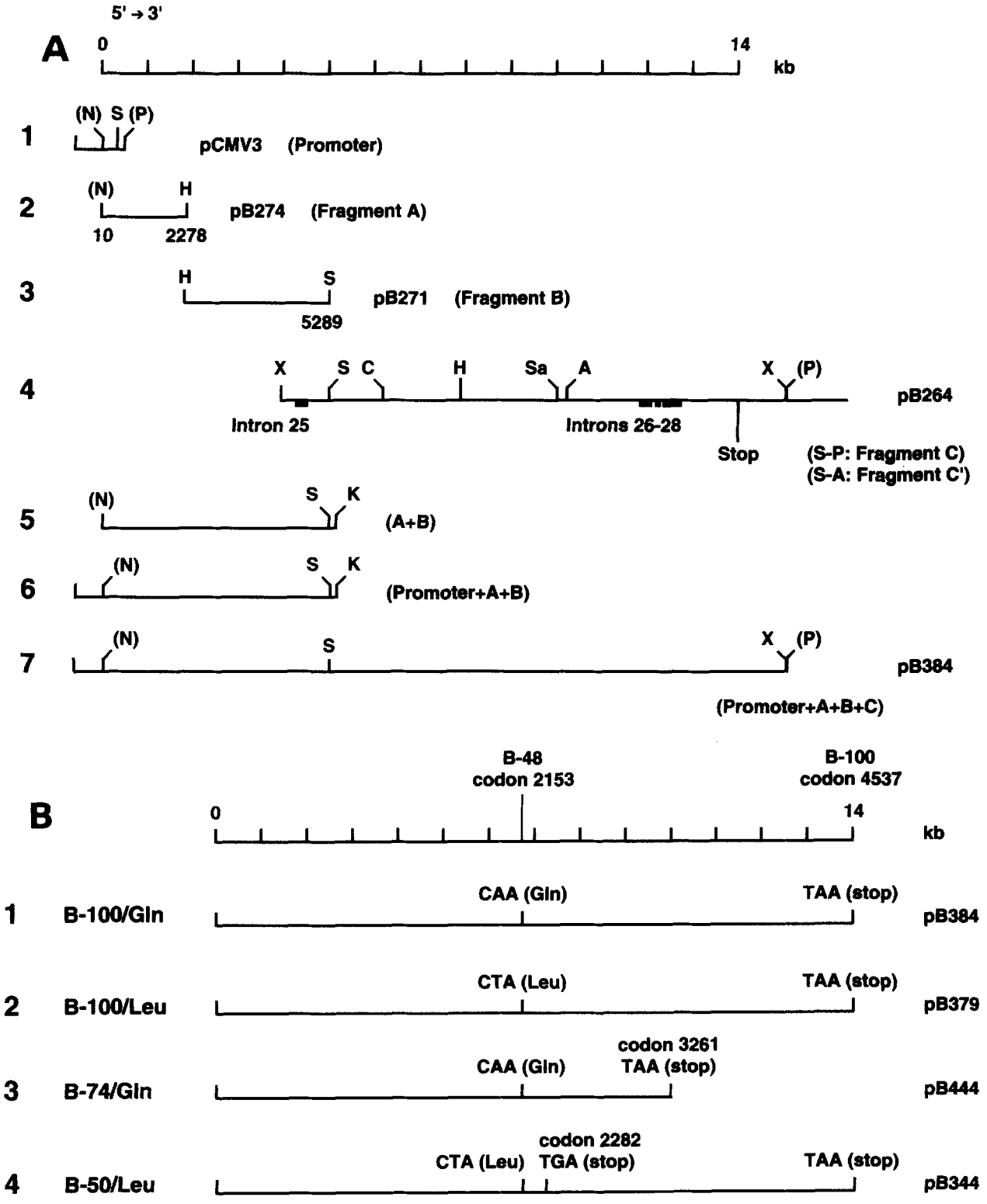

Fig. 1. Cloning strategy and schematic diagram of the apoB minigene constructions. Multiple cloning steps used for the construction of the apoB minigenes are depicted in Fig. 1A and are described in detail in Materials and Methods. Clone pCMV3 contains the CMV promoter sequences. Clones pB274 and pB271 are cDNA clones, while clone pB264 is a genomic clone. Restriction enzyme sites are shown in capital letters: A, AvrII; C, ClaI; H, HindIII; K, KpnI; P, PacI; S, SalI; X, XhoI. Parentheses indicate sites engineered for cloning purposes. Solid boxes indicate the positions of introns in pB264. The ClaI-HindIII fragment of pB264 was used for site-directed mutagenesis to create a leucine mutation at codon 2153. The SalI-AvrII fragment of pB264 was used in the construction of the B-74 minigene. All four minigenes are shown schematically in Fig. 1B. Based on the expected size of apoB proteins from the respective minigene construction and their amino acid residue at codon 2153, clones containing pB384, pB379, pB444, and pB344 are designated as B-100/Gln, B-100/Leu, B-74/Gln, and B-50/Leu, respectively.

Preparation of apoB stable transfectants

Both McA-RH7777 and CHO cells were maintained in Ham's F12 medium (Gibco BRL, Inc.) containing 10% fetal bovine serum (FBS). Cells were plated at 30% confluence with Dulbecco's modified Eagle's medium (DMEM) 1 day prior to transfection. ApoB minigene constructions were cotransfected with pSV2neo plasmid in a 20:1 molar ratio using calcium phosphate coprecipitation as described previously (18). About 48 h after transfection, G418 was added to medium for selection. Single foci of the resistant colonies were picked 10–14 days after selection. Genomic DNA was isolated from these transfectants and then subjected to Southern blot analysis (19). The 3-kb HindIII-SalI fragment of pB271 (line 3 of Fig. 1A) was used as a probe after radiolabeling with α^{32}P-dCTP (800 mCi/mmol, Amersham Inc.) by random priming method (20). Positive stable transfectants containing apoB minigene constructions were selected and maintained in the presence of G418 (400 μg/ml).

Metabolic labeling of cellular and medium proteins

ApoB stable transfectants were plated in plastic petri dishes (100 mm) and grown to confluence. Cells were washed twice with methionine-free (Met-free) DMEM and then labeled for 4 h in 5 ml Met-free DMEM containing 0.5 mCi ^{35}S-Trans label (ICN, Inc.). After labeling, medium was collected and protease inhibitors were immediately added to all samples. Final concentrations of inhibitors were as follows: aprotinin (100 KIU/ml), benzamidine (2 mM), caproic acid (1.3 mg/ml), EDTA (5 mM), glutathione (0.02%), leupeptin (10 μg/ml), D-phenylalanyl-L-prolyl-L-arginine (PPACK, 1.0 μM), pepstatin (10 μg/ml), polybrene (25 μg/ml), and soybean trypsin inhibitor (20 μg/ml). Medium samples were centrifuged to remove cell debris. Cells were washed twice with ice-cold phosphate-buffered saline (PBS), pH 7.4, and then scraped off the plates in 1 ml PBS. Cells were pelleted and resuspended in lysis buffer (150 mM NaCl, 50 mM Tris-HCl, pH 7.4, 5 mM EDTA, 62.5 mM sucrose, 0.5% sodium deoxycholate, and 0.5% Triton X-100) containing protease inhibitors as described above. Cells were lysed on a rocker at 4°C for 1 h. Debris was removed from cell lysates by centrifugation. Both medium samples and cell lysates were analyzed by immunoprecipitation.

Immunoprecipitation and SDS-polyacrylamide gel electrophoresis

Both medium samples and cell lysates from apoB transfectants were subjected to immunoprecipitation as described (21). The amount of protein labeled was quantified by trichloroacetic acid (TCA) precipitation as described (22). Equal amounts of TCA-precipitable cell lysates (2×10^7 cpm) and medium samples (5×10^6 cpm) were used. All samples were incubated with equal volume of dilution buffer (150 mM NaCl, 50 mM Tris-HCl, pH 7.4, 5 mM EDTA, 0.5% Triton X-100, 0.1% sodium dodecyl sulfate (SDS)), and a polyclonal rabbit anti-human apoB antibody at 4°C overnight. Rat serum was also added to the reaction mixture to remove antibody crossreactivity to endogenous rat apoB proteins synthesized in McA-RH7777 cells. Protein A-Sepharose (Pharmacia LKB, Inc.) was then added to samples and incubated at 4°C for 3 h. At this time, samples were washed three times with dilution buffer and resuspended in 1 × electrophoresis sample buffer (23). After washing, samples were boiled for 5 min prior to loading onto a 4% SDS-polyacrylamide gel (SDS-PAGE) (23). After electrophoresis, gels were subjected to fluorography, dried, and exposed to X-ray films at −70°C with intensifying screens. The relative amounts of apoB proteins expressed in the transfectants were determined by scanning the autoradiogram with a densitometer (LKB, Inc.).

RNA isolation and Northern blot analysis

Total cellular RNA was extracted from various cells lines using the guanidinium thiocyanate method according to Chomczynski and Sacchi (24). Cells plated in plastic petri dishes (100 mm) were grown to confluence and scraped off plates in 1 ml 4 M guanidinium thiocyanate. Cell lysates were extracted with a solution containing water-saturated phenol (Gibco BRL, Inc.), chloroform, and 200 mM sodium acetate, pH 4.2, prior to isopropanol precipitation. RNA samples were stored in a 70% ethanol/200 mM sodium acetate solution at −70°C.

Total cellular RNA samples from both McA-RH7777 transfectants (15 μg) and CHO transfectants (30 μg) were subjected to Northern blot analysis (25). RNA samples were electrophoresed in a 6% formaldehyde/0.8% agarose gel and then transferred to a nylon membrane. Membranes were baked at 80°C for 2 h and prehybridized at 65°C for 3 h in a buffer containing 5 × SSPE (1 × SSPE = 150 mM NaCl, 10 mM sodium phosphate, pH 7.4, 10 mM EDTA), 0.2% nonfat dry milk, 0.5% SDS, 100 μg/ml salmon sperm DNA, and 50% formamide. The membranes were then hybridized with either a 5' or a 3' apoB cDNA probe in the same buffer at 42°C overnight. The 5' probe, pB271, contained nucleotides 2279 to 5289 encoding the apoB protein sequence from B-16 to B-38. The 3' probe, pB522, contained nucleotides 7673 to 9849, encoding the apoB protein sequence from B-56 to B-73. Membranes were then washed in 0.1 × SSC/0.1% SDS (1 × SSC = 150 mM NaCl, 15 mM sodium citrate) at 65°C for 15 min and exposed to X-ray films at −70°C with intensifying screens.

Fractionation of total cellular RNA

Poly(A)$^+$ RNA was selected from total cellular RNA of the McA-RH7777 B-100/Leu transfectant by chromatography on oligo(dT) cellulose. A 1-ml column containing 25 mg oligo(dT) cellulose (Boehringer Mannheim) was

rinsed with ddH_2O and a washing solution containing 0.1 M NaOH and 5 mM EDTA. The column was then conditioned with 1 ml of high salt loading buffer containing 0.01 M Tris-HCl, pH 7.5, 0.5 M NaCl, 1 mM EDTA, and 0.1% SDS. Total cellular RNA (150 μg) was resuspended in 200 μl of this buffer and loaded onto the column after incubation at 65°C for 5 min. The effluent was heated to 65°C again and reapplied to the column. The column was then washed twice with 450 μl of high salt loading buffer with the effluent saved as the poly(A)$^-$ RNA fraction. After a wash with 800 μl of low salt loading buffer (0.01 Tris-HCl, pH 7.5, 0.1 M NaCl, 1 mM EDTA, 0.1% SDS), the poly(A)$^+$ RNA was eluted with 450 μl of elution buffer (0.01 M Tris-HCl, pH 7.5, 1 mM EDTA, 0.05% SDS). The poly(A)$^-$ RNA fraction was applied to a regenerated oligo(dT) column once more to ensure complete removal of poly(A)$^+$ RNA. Both poly(A)$^-$ RNA and poly(A)$^+$ RNA fractions were then precipitated in 70% ethanol/200 mM sodium acetate and stored at −70°C.

cDNA cloning by reverse transcription and polymerase chain reaction

Total cellular RNA from apoB stable transfectants was used for cDNA synthesis by reverse transcription followed by PCR amplification as described (26). For first strand synthesis, 5 μg of total cellular RNA was added to a reaction mix containing oligo $d(T)_{18}$ primer (100 pmol), dNTPs (0.25 mM), M-MLV reverse transcriptase (400 units, Gibco BRL), and RNasin (100,000 units, Promega) in a buffer containing 50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, and 10 mM dithiothreitol. Final reaction volume was 20 μl. The reaction mix was incubated at 42°C for 45 min. To increase the yield of first strand synthesis, the reaction mix was heat denatured at 90°C for 3 min followed by a second addition of M-MLV reverse transcriptase (400 units) and RNasin (100,000 units). The reaction was carried out at 42°C for an additional 45 min and then terminated by heating at 90°C for 3 min. Two human apoB-specific primers were synthesized for second strand synthesis. The first primer (#1489) contained sequences corresponding to cDNA nucleotides 6319 to 6339 (5'-AGATGTTCACTCCATTAACC-3'). The second nested primer (#259) contained sequences corresponding to cDNA nucleotides 6503 to 6525 (5'-TCTGAATTCATTCAATTGGGAGA-3'). For cloning purposes, a 3' oligo d(T) primer (#1827) containing engineered EcoRI and HindIII enzyme sites was also synthesized (5'-TATATAAGCTTGAATTC$(T)_{18}$-3'). For second strand synthesis, one tenth of the first strand reaction mixture was added to a mix containing a human apoB-specific primer #1489 (50 pmol) and 0.2 mM dNTPs in a buffer containing 10 mM Tris-HCl, pH 8.3, 1.5 mM $MgCl_2$, 0.1% gelatin, and 2 units of Taq DNA polymerase (Perkin-Elmer Cetus). Conditions were as follows: 94°C for 3 min, followed by 35 cycles of 94°C (1 min), 57°C (1 min), and 72°C (2 min), and a final extension period at 72°C for 10 min. Free nucleotides and primers were removed from the PCR reaction products by centrifugation in Centricon-100 filters (Amicon, Inc.). To increase reaction specificity, the purified PCR product was subjected to a second PCR reaction using two nested primers, #259 and #1827. The annealing temperature used for the second set of PCR was 55°C. For cloning, the PCR reaction mixtures were precipitated and resuspended in enzyme digestion buffers. Digested reaction mixtures were then extracted with chloroform, centrifuged through Centricon-100 filters, and ethanol precipitated. The digested products were ligated to pUC vectors and used to transform *E. coli* DH5α competent cells. Recombinant colonies were identified by colony hybridization as described (25). Colony filters were hybridized to apoB specific oligonucleotide probes, which were end labeled with [γ^{32}P]ATP using T4 polynucleotide kinase as described (25). To identify clones containing apoB sequences 3' to sequences coding for B-48, an oligonucleotide (#257) corresponding to cDNA nucleotides 6921 to 6943 was used as a probe. In order to identify all apoB recombinants bearing sequences coding for B-48 as well as B-50, the probe #257 was removed from colony filters and filters were rehybridized to a different probe (#1989) corresponding to cDNA nucleotides 6569 to 6588. Plasmid DNA from recombinants was isolated and then subjected to DNA sequence analysis.

DNA sequencing analysis

Multiple clones derived from cDNA synthesis were sequenced. Double-stranded plasmid DNA isolated from these clones was denatured by heat as described (27). The denatured DNA samples were sequenced with apoB sequence-specific primers using modified T7 DNA polymerase (Sequenase, United States Biochemical Co.) according to the dideoxy termination method of Sanger, Nicklen, and Coulson (28).

RESULTS

McA-RH7777 apoB transfectants expressed B-48-like proteins

Expression of human apoB proteins from McA-RH7777 transfectants was examined by metabolic labeling, followed by immunoprecipitation and SDS-PAGE. As shown in **Fig. 2,** no cellular B-100 protein was observed in either the B-100/Gln (wild type) or the B-100/Leu (nonstop editing mutant) transfectants (lanes 2 and 3 of left panel). Trace amounts of B-100 protein were observed in the medium fractions (lanes 2 and 3 of right panel). No B-74 protein was observed in either cell or medium fractions from the B-74/Leu transfectant (lane 4 of left and right panel). However, trace amounts of B-74

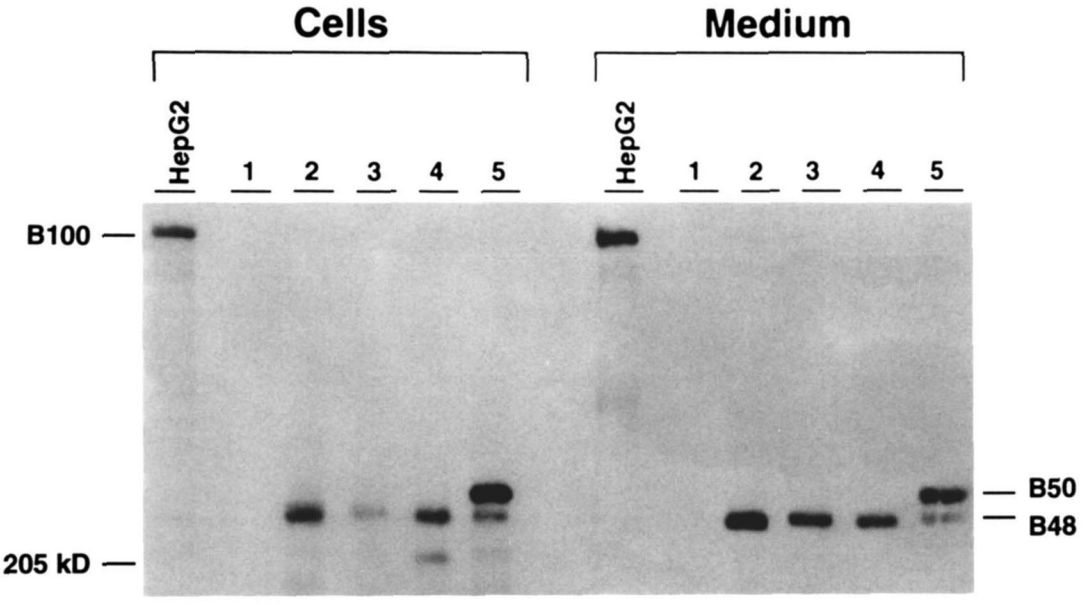

Fig. 2. ApoB expression from McA-RH7777 transfectants. Cell lysates and medium samples from McA-RH7777 apoB transfectants were subjected to immunoprecipitation followed by SDS-PAGE (see Materials and Methods). The fluorograph from the gel is shown. The molecular mass standard in kD is indicated on the left. Cell and medium samples from HepG2 cells were used as positive controls for the B-100 proteins. Cellular samples are shown on the left panel, and medium samples on the right panel. Lane 1: control McA-RH7777 cells. Lane 2: B-100/Gln transfectant. Lane 3: B-100/Leu transfectant. Lane 4: B-74/Gln transfectant. Lane 5: B-50/Leu transfectant. Proteins expressed by apoB transfectants are indicated on the right side of the figure.

protein from both cellular and medium fractions were visible after prolonged exposure (data not shown). In the B-50/Leu transfectant, a protein of expected B-50 size was detected in both cellular and medium fractions (lane 5 of left and right panel). Additional bands underneath the B-48-like protein were detected in the cell lysates of all apoB transfectants (lanes 2 to 5 of left panel). These bands were also observed in untransfected McA-RH7777 cells (lane 1) after prolonged exposure to autoradiogram (data not shown). These bands were often observed in untransfected McA-RH7777 cells in our experiments and most likely reflect nonspecific binding to the antibody. It is not surprising to see the expression of B-48 proteins in both B-100/Gln and B-74/Gln transfectants (lanes 2 and 4 of left and right panel) as human apoB RNA can be edited to B-48 RNA by the RNA editing activity present in McA-RH7777 cells. However, a high level of expression of B-48-like proteins in both B-100/Leu and B-50/Leu transfectants was also observed (lanes 3 and 5 of left and right panel). In the B-100/Leu transfectant, almost all apoB protein produced is B-48-like, while in the B-50/Leu transfectant, 24% of the total apoB protein is B-48-like and the remaining 76% is B-50. As editing would only result in a leucine to leucine silent mutation and not a stop codon, these results were unexpected. To rule out the possibility that the formation of B-48 protein was due to a cloning artifact, all of the minigene constructions were sequenced 1 kb spanning the editing site. No sequence errors were found. As the presence of B-48-like proteins is not likely due to introduction of a stop codon by RNA editing, we speculated that other mechanisms might play a role in the formation of these proteins.

A 7-kb apoB RNA was transcribed in the McA-RH7777 transfectants but not in the CHO transfectants

To determine whether the formation of B-48-like proteins in B-100/Leu and B-50/Leu transfectants was a transcriptional or post-transcriptional event, total RNA samples from apoB transfectants were subjected to Northern blot analysis (**Fig. 3A and B**). Expected sizes of the apoB mRNA are 14 kb for the B-100/Gln, B-100/Leu, and B-50/Leu transfectants and 10 kb for the B-74/Gln transfectant (Fig. 1B). Surprisingly, in McA-RH7777 cells only one species of apoB RNA from all four transfectants was detected using a 5' cDNA probe specific to human apoB (lanes 2 to 5 of Fig. 3A). The size of RNA was approximately 7 kb. Although this result was unexpected, it indicated that the formation of B-48-like proteins in apoB McA-RH7777 transfectants (Fig. 2) was likely due to a shortened RNA. To determine whether this phenomenon also occurs in other types of cells, RNA from four separate CHO B-74/Gln transfectants were analyzed by Northern blot analysis. As shown in Fig. 3, an expected 10-kb B-74 RNA was detected in the CHO transfectants (lanes 7 to 10 of Fig. 3A). These results showed that the occurrence of shortened apoB RNA was specific to a cell line exhibiting RNA editing activity.

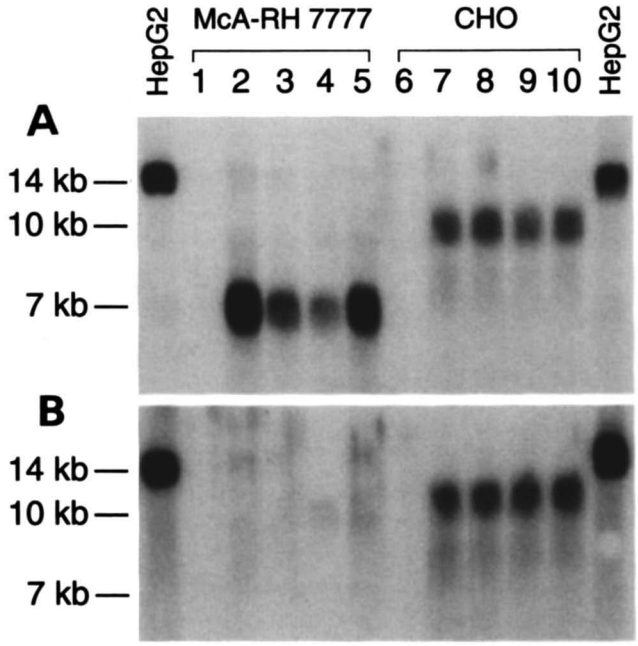

Fig. 3. Northern blot analysis of RNA samples from McA-RH7777 and CHO apoB transfectants. Total RNA isolated from McA-RH7777 and CHO apoB transfectants was subjected to Northern blot analysis and hybridized to either a human apoB probe (pB271) containing cDNA sequences from nucleotide 2279 to 5289 (A) or to a human apoB probe containing cDNA sequences from nucleotide 7673 to 9849 (B). A 14-kb apoB RNA was detected from control HepG2 cells by both probes. RNA samples from control McA-RH7777 and CHO cells are shown on lane 1 and lane 6, respectively. RNA samples from McA-RH7777 apoB stable transfectants are shown in lanes 2 to 5 of Fig. 3, A and B: lane 2, B-100/Gln transfectant; lane 3, B-100/Leu transfectant; lane 4, B-74/Gln transfectant; and lane 5, B-50/Leu transfectant. RNA samples from four different CHO B-74/Gln transfectants are shown in lanes 7 to 10 of Fig. 3, A and B.

Another Northern blot with samples identical to those in Fig. 3A was hybridized with a 2.2-kb cDNA probe containing sequences coding for B-56 to B-73 (Fig. 3B). This was performed to rule out the possibility that a full-length 14-kb apoB message in McA-RH7777 transfectants was cleaved at the sequence near the B-48 editing site by a nuclease resulting in two 7-kb RNA species indistinguishable by size. As shown in Fig. 3B, the 3' probe hybridized only to the 10-kb apoB RNA from four separate CHO B-74/Gln transfectants (lanes 7 to 10), but not to the 7-kb apoB RNA from McA-RH7777 transfectants (lanes 2 to 5). These results showed that the latter was in fact derived only from the 5' half of the apoB RNA. In addition, the probe hybridized to trace amounts of a 14-kb full-length message from the McA-RH7777 transfectants containing full-length minigene sequences (lanes 2, 3, and 5 of Fig. 3B) and a 10-kb message in the B-74/Gln transfectant (lane 4 of Fig. 3B), consistent with the small amount of secreted full-length protein detected by immunoprecipitation as shown in Fig. 2.

The 7-kb apoB RNA was polyadenylated

To determine whether the 7-kb apoB RNA was polyadenylated, total cellular RNA from McA-RH7777 B-100/Leu transfectants was separated into poly(A)$^+$ and poly(A)$^-$ fractions by oligo(dT) cellulose chromatography. Both RNA fractions along with total cellular RNA were subjected to Northern blot analysis using a 5' human apoB cDNA probe (pB271), as shown in **Fig. 4.** A total of 15 μg total cellular RNA (lane 2) and poly(A)$^-$ fraction (lane 5) was used. A total of 0.18 μg of poly(A)$^+$ RNA was used (lane 3 of Fig. 4), as the percentage of poly(A)$^+$ RNA recovered from total cellular RNA was about 1.23%. Additional samples with higher or lower concentrations of RNA from poly(A)$^+$ (0.37 μg, lane 4) and poly(A)$^-$ (10 μg, lane 6) fractions were also used. As shown in Fig. 4 the 7-kb apoB RNA was present in both total cellular RNA (lane 2) and poly(A)$^+$ RNA fraction (lanes 3 and 4), but not in the poly(A)$^-$ fraction (lanes 5 and 6). This indicated that most if not all of the 7-kb apoB RNA in McA-RH7777 transfectants was in fact polyadenylated. The migration difference of apoB RNA observed in total cellular RNA (lane 1) and poly(A)$^+$ RNA (lanes 2 and 3) was likely attributed to different amounts of RNA loaded on the gel and was not a reflection of size differences. Trace amounts of a 7-kb apoB RNA were also observed in the HepG2 cells (lanes 1 and 7) suggesting that generation of a shortened 7-kb apoB RNA also occurred in this cell line.

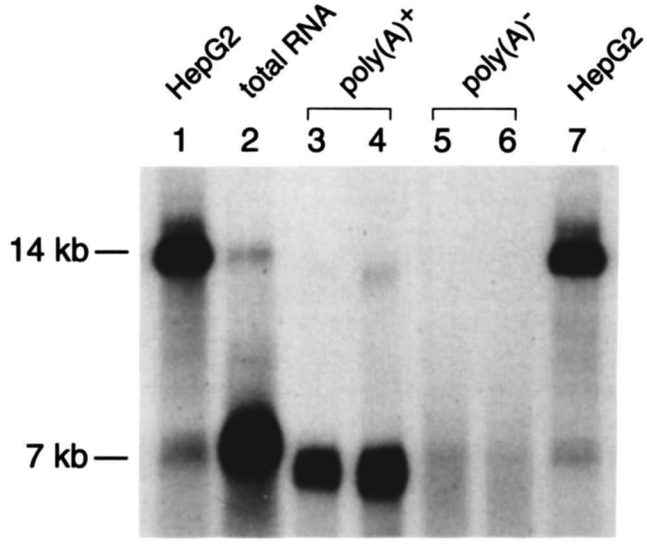

Fig. 4. Northern blot analysis of poly(A)$^+$ and poly(A)$^-$ RNA fractions from McA-RH7777 B-100/Leu transfectant. Total cellular RNA (15 μg, lane 2), poly(A)$^+$ RNA (0.18 μg, lane 3; 0.37 μg, lane 4) and poly(A)$^-$ RNA (15 μg, lane 5; 10 μg, lane 6) from the McA-RH7777 B-100/Leu transfectant was subjected to Northern blot analysis using a 5' human apoB probe (pB271). A 14-kb full-length B-100 RNA from HepG2 cells was used as size marker (lanes 1 and 7). The difference in migration between total cellular RNA and poly(A)$^+$ RNA was caused by differences in the amount of RNA loaded on the gel as described in Results.

Polyadenylation occurred at various sites in the 7-kb RNA

The size of the apoB RNA from the examined McA-RH7777 apoB transfectants indicated that apoB RNA terminated at approximately 7 kb 3' to the start of transcription. As there are several cryptic polyadenylation signals clustered in nucleotides 6681–7055 of the apoB cDNA sequence (4), as shown schematically in **Fig. 5**, we speculate that these cryptic poly(A) signals were utilized to generate shortened polyadenylated apoB transcripts. To prove this possibility, total cellular RNA isolated from the McA-RH7777 B-100/Leu transfectant was used for cDNA synthesis by reverse transcription followed by PCR amplification. An oligo $d(T)_{18}$ and a human apoB-specific primer were used for the cDNA synthesis. The apoB-specific primer was located approximately 300 to 600 bp 5' to possible polyadenylation sites (nucleotides 6681–7055) for the 7-kb RNA. PCR-amplified products were analyzed on agarose gels, stained with ethidium bromide, and visualized under UV light. One major product of approximately 300–350 bp, suggesting a polyadenylation site in the vicinity of the cDNA that encodes B-48, and a minor product of approximately 600 bp, suggesting a polyadenylation site in the cDNA encoding B-50, were observed when total cellular RNA isolated from the B-100/Leu transfectant was used as a template (data not shown). The same preparation of the RNA sample from the B-100/Leu transfectant was also subjected to cDNA synthesis with the omission of first strand synthesis followed by PCR amplification, and no specific PCR products were observed. This indicated that there was no DNA contamination in the RNA preparation from the B-100/Leu transfectant and that the amplified cDNA products are in fact reverse transcripts from the apoB RNA.

Amplified PCR products containing apoB cDNA derived from the B-100/Leu transfectant were used directly for cloning. Recombinant clones were identified by colony hybridization using [γ^{32}P]ATP-labeled oligonucleotide probes corresponding to sequences in the vicinity of cDNA encoding either B-48 or B-50. About 250 colonies hybridized to the B-48 oligonucleotide probe and 11 of them also to the B-50 specific probe. Plasmid DNA was isolated from 23 of the B-48 clones and from all of the B-50 clones. All B-48 recombinants contained an insert of 300–350 bp, whereas the B-50 recombinants had an insert of about 600 bp. Sequence analysis of the B-48 clones showed that the sites for polyadenylation varied. In 6 clones poly(A) sequences were added after cDNA nucleotide 6777, which is 21 bp 3' to the cryptic poly(A) signal ATTAAA at nucleotide 6752 (**Fig. 6A**). In 6 other clones the poly(A) tails were added after nucleotide 6774, in 9 clones after nucleotide 6771, in 1 clone after nucleotide 6747, and in 1 clone after nucleotide 6651. In all clones, the sequence of the editing site at nucleotide 6666 was also examined. In 4 of the 23 B-48 clones (18%) nucleotide 6666 was edited to T and had TTA sequences for the leucine codon 2153. Eighteen clones were not edited and the remaining clone was polyadenylated before the editing site. Among the 4 edited clones, 3 were polyadenylated after nucleotide 6771 and 1 after nucleotide 6777. In the 6 clones polyadenylated after nucleotide 6774, the addition of a poly(A) tail created a TAA-stop codon, whereas no stop codons were found in the sequences of the other cDNA clones.

All of the B-50 clones were polyadenylated after nucleotide 7079. As shown in Fig. 6B, a second cryptic poly(A) signal (AATAAA) at nucleotide 7055 was apparently used. In two out of 11 clones (18%) the leucine codon 2153 was edited (TTA), whereas the remaining clones had a non-edited codon (CTA). No stop signals or other modifications in these fragments were detected by DNA sequence analysis.

These DNA sequencing data showed that 6 out of a total of 34 clones examined (18%) were edited at codon 2153, suggesting that the RNA editing activity was active and recognized the proper nucleotide for editing in the McA-RH7777 B-100/Leu transfectant. There was no difference in the percentage of edited cDNA clones observed between B-48 clones and B-50 clones. More than one cryptic poly(A) signal was apparently used resulting in alternative polyadenylation of apoB RNA in McA-RH7777 apoB transfectants. In the population of B-48 clones, polyadenylation after nucleotide 6771 appeared to

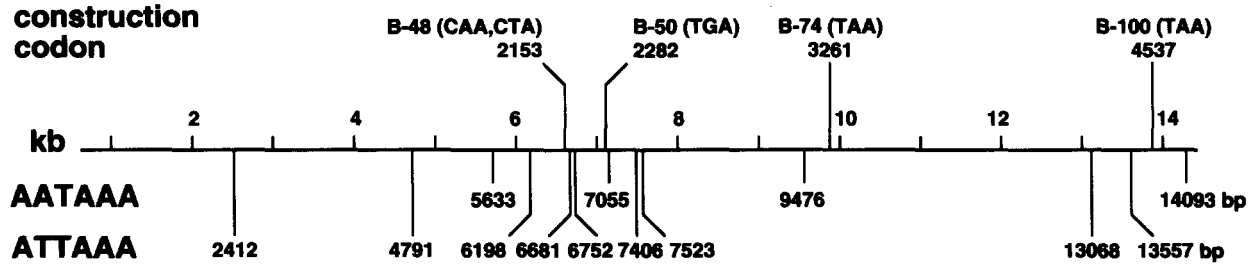

Fig. 5. Schematic diagram of stop codons and cryptic poly(A) signals in apoB minigene constructions. The sites and nucleotide sequences of stop codons for the four apoB minigene constructions are indicated in the map. Nucleotide positions for cryptic poly(A) signal sequences are shown beneath the gene. Positions for the AATAAA and the ATTAAA motifs are indicated in base pairs (bp). One of two natural poly(A) signals in the 3' untranslated region of the apoB cDNA is also shown (16).

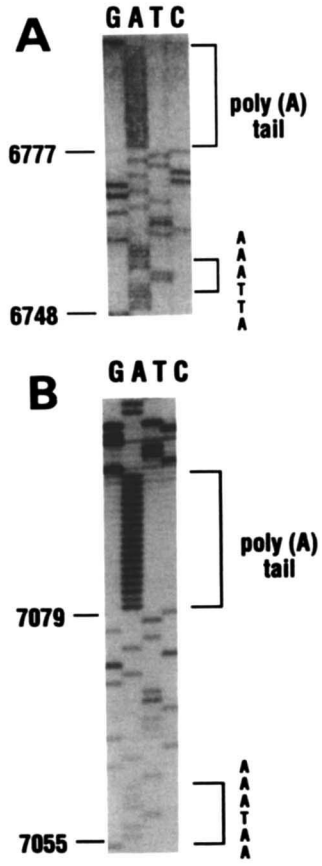

Fig. 6. Sequence analysis of cDNA clones. cDNA clones derived from the B-100/Leu transfectant were subjected to DNA sequence analysis. Fig. 6A shows the sequence from a cDNA clone identified by colony hybridization with oligonucleotide probe corresponding to cDNA encoding B-48. Fig. 6B shows the sequence of a cDNA clone identified by colony hybridization with oligonucleotide probe corresponding to cDNA encoding B-50. The cryptic poly(A) signals ATTAAA and AATAAA are indicated in Fig. 6, A and B, respectively. cDNA nucleotide numbers upstream to the poly(A) tail are also shown.

be slightly favored and the percentage of edited clones was found to be higher in this subpopulation when compared to cDNA clones polyadenylated at other sites. The size difference between the major B-48 and minor B-50 RNA species (about 300 bp) could not be distinguished by Northern blot analysis.

DISCUSSION

Expression of full-length apoB-100 proteins from minigenes transfected into rat hepatoma McA-RH7777 cells is hampered due to conversion of CAA to UAA at codon 2153 by a specific RNA editing mechanism (5, 6). However, substitution of codon 2153 with a leucine codon (CTA) allowed expression of full-length B-53 proteins after transfection with a truncated B-53 minigene (12). It was shown that RNA editing of the Leu codon (CUA) would give rise to a silent substitution (UUA) instead of a stop codon. In this report, we showed that McA-RH7777 cells transfected with a B-100 minigene construction containing a Leu substitution at codon 2153 failed to express full-length B-100 proteins. Instead, large amounts of B-48-like proteins were produced. Northern blot analysis detected a 7-kb RNA, rather than the expected 14-kb RNA. A 7-kb apoB RNA was also observed in a McA-RH7777 transfectant containing a truncated B-74 construction, whereas in four different CHO B-74/Gln transfectants the expected 10-kb apoB RNA was present. Sequence analysis of cDNA clones indicated that cryptic poly(A) signals downstream of the editing site were used for polyadenylation thereby resulting in a shortened apoB RNA. The formation of B-48-like proteins, therefore, resulted from an alternative polyadenylation of apoB RNA and occurred only in a cell line exhibiting RNA editing activity.

Recently, Johnson and Innerarity (29) reported an identical species of polyadenylated apoB RNA in McA-RH7777 cells transfected with a nonstop B-100/Leu minigene as described in this study. However, the amount of B-48 of total apoB proteins produced in their B-100/Leu transfectant was about 70%, compared to almost 100% produced in our B-100/Leu transfectant. This suggests that the extent of alternative polyadenylation varies in different McA-RH7777 transfectants. It is also possible that the level of expression of the transfected apoB minigenes affects the efficiency of alternative RNA polyadenylation.

A 7-kb apoB RNA also has been observed in intestinal cells of humans and rabbits (5, 6). In the human intestine, poly(A) signals in positions 6752 and 7055 were shown to be utilized for the generation of alternatively polyadenylated intestinal apoB RNA (5, 6). Both of these poly(A) signals were apparently also responsible for alternative polyadenylation of apoB RNA in the B-100 minigene transfectant described in the current study. The combined results indicated that alternative polyadenylation of apoB RNA occurs both in vivo and in vitro.

As a short 7-kb apoB RNA was observed in a McA-RH7777 B-74/Gln transfectant but not in CHO B-74/Gln transfectants, this suggests that alternative polyadenylation of apoB RNA occurred only in cells with endogenous RNA editing activity. Previous studies also have shown that a 7-kb apoB RNA is detected only in intestinal cells but not in human HepG2 hepatoma cells, which exhibit a negligible amount of RNA editing activity (5, 6, 30, 31). Our Northern analyses (Fig. 4) suggest that there is a negligible amount of 7-kb apoB RNA present in HepG2 cells. In another study, an apoB fragment containing 354 bp of apoB cDNA sequences (nucleotides 6507–6860) encompassing the RNA editing site was inserted in frame into the apoE cDNA sequence (32). A full-length apoE,B chimeric message was detected in CHO transfectants,

whereas both a full-length and a shorter chimeric message were detected in human CaCo-2 cells of intestinal origin. This shorter chimeric message was edited and polyadenylated 3' to the poly(A) signals in apoB cDNA sequences.

Therefore, there is strong evidence that the presence of RNA editing activity is coincident with alternative polyadenylation of apoB RNA. It was suggested that replacement of C by U at nucleotide 6666 in the intestinal apoB RNA allows the recognition of cryptic polyadenylation signals present in the apoB RNA (32). These signals are clustered within 1 kb downstream of the editing site as shown in Fig. 5. However, editing and alternative RNA polyadenylation are most likely two separate mechanisms relying on the presence of intact editing machinery. As described previously, all human intestinal apoB RNA is edited to a stop codon in residue 2153, yet only 85% consists of a shortened 7-kb RNA (5). Hence, the conversion of nucleotide 6666 from C to U does not ensure consequent alternative polyadenylation of the RNA. Consistent with this, our study showed that only 18% of the apoB cDNA clones derived from the shortened apoB RNA of the B-100/Leu construction were edited.

In eukaryotic genes both poly(A) and termination signals are generally required for termination of the transcription by RNA polymerase II (33, 34). Termination sites are located downstream of poly(A) signals at varying distances ranging from a few base pairs to more than 4 kb (35, 36). These elements are considered to destabilize the transcription complex by slowing the kinetic rate of transcription, thereby initiating the termination process (37). It was shown that multiple functional termination elements acting in concert may be necessary to ensure an efficient termination of transcription (36, 38). It is not clear by which mechanism the shortened RNA is created in the McA-RH7777 apoB transfectants; however, it might be caused by premature termination of RNA transcription. In this respect it is interesting that in the B-50/Leu transfectant both B-48 and B-50 proteins were expressed and the amount of B-50 proteins was substantially higher than that of B-48-like proteins. As the nonsense mutation in this B-50 construction was a result of base substitution at nucleotide 7053, this mutation might decrease the efficiency of premature termination of RNA transcription by affecting a sequence possibly involved in the termination process, thereby allowing transcription to proceed beyond the cryptic polyadenylation signal at nucleotide 7055. Other 3' sequences might be relevant for premature transcription termination. The B-50/Leu construction used in this study contained the entire apoB coding sequence. However, expression of a B-48 protein was not detected when a truncated B-53/Leu minigene was transfected in McA-RH7777 cells (12). Furthermore, a recent report showed that B-48-like proteins were produced when McA-RH7777 cells were transfected with truncated minigene constructions ranging from B-60 to B-94 (39). In light of these additional findings we speculate that the absence of the 3' apoB sequence downstream of cryptic poly(A) signals in the B-53/Leu minigene might have prevented premature termination of apoB transcription.

A highly unusual finding in our study was that only 6 out of 34 cDNA clones derived from the B-100/Leu transfectants had an in-frame stop codon (TTA) created by the addition of a poly(A) sequence after nucleotide 6774, and no stop codons were found in any of the remaining 28 clones. To our knowledge, this finding is unprecedented. It remains unresolved whether the absence of a stop codon affected translation termination or whether the translated B-48 proteins contained a poly-lysine peptide (AAA), translated from the poly(A) tail, at the carboxy terminus of the protein.

In summary, we have identified alternative polyadenylation of RNA as a major mechanism, in addition to RNA editing, that accounts for the formation of B-48-like proteins in B-100 minigene transfected McA-RH7777 cells. Although the mechanisms responsible for the generation of shortened apoB RNA transcripts await further investigation, we propose that expression of a full-length B-100 protein might be achieved by the introduction of silent substitutions in cryptic poly(A) signal sequences. Mutations in poly(A) signals have been shown to affect transcription termination drastically in mammalian genes (33, 40). The elimination of active cryptic poly(A) signals in apoB in conjunction with a nonstop leucine substitution in codon 2153 might overcome both hindrances of alternative RNA polyadenylation and RNA editing so as to facilitate full-length B-100 expression. The current study also suggests that an in vitro expression system for B-100 in McA-RH7777 cells allows studies relevant to the alternative polyadenylation of apoB RNA described in human intestine, adding to its value as a tool for investigation of the intracellular metabolism of B-100.

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REFERENCES

1. Kane, J. P., and R. J. Havel. 1989. Disorders of the biogenesis and secretion of lipoproteins containing the B apolipoproteins. *In* The Metabolic Basis of Inherited Disease. C. R. Scriver, A. R. Beaudet, W. S. Sly, D. Valle, J. B. Stanbury,

J. B. Wyngaarden, and D. S. Frederickson, editors. McGraw-Hill Publications, New York. Vol. I. 1139–1164.
2. Blackhart, B. D., E. M. Ludwig, V. R. Pierotti, L. Caiati, M. A. Onasch, S. C. Wallis, L. Powell, R. Pease, T. J. Knott, M-L. Chu, R. W. Mahley, J. Scott, B. J. McCarthy, and B. Levy-Wilson. 1986. Structure of the human apolipoprotein B gene. *J. Biol. Chem.* **261:** 15364–15367.
3. Knott, T. J., R. J. Pease, L. M. Powell, S. C. Wallis, S. C. Rall, T. L. Innerarity, B. Blackhart, W. H. Taylor, Y. Marcel, R. Milne, D. Johnson, M. Fuller, A. J. Lusis, B. J. McCarthy, R. W. Mahley, B. Levy-Wilson, and J. Scott. 1986. Complete protein sequence and identification of structural domains of human apolipoprotein B. *Nature.* **323:** 734–738.
4. Yang, C-Y., S-H. Chen, S. H. Gianturco, W. A. Bradley, J. T. Sparrow, M. Tanimura, W-H. Li, D. A. Sparrow, H. DeLoof, M. Rosseneu, F-S. Lee, Z-W. Gu, A. M. Gotto, and L. Chan. 1986. Sequence, structure, receptor-binding domains and internal repeats of human apolipoprotein B-100. *Nature.* **232:** 738–742.
5. Chen, S-H., G. Habib, C-Y. Yang, Z-W. Gu, B. R. Lee, S-A. 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.
6. 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.
7. Young, S. G. 1990. Recent progress in understanding apolipoprotein B. *Circulation.* **82:** 1574–1594.
8. Sniderman, A., S. Shapiro, D. Marpole, B. Skinner, B. Teng, and P. O. Kwiterovich, Jr. 1980. Association of coronary atherosclerosis with hyperapobetalipoproteinemia (increased protein but normal cholesterol levels in human plasma low density (β) lipoproteins). *Proc. Natl. Acad. Sci. USA.* **77:** 604–608.
9. Spring, D. J., L. W. Chen-Liu, J. E. Chatterton, J. Elovson, and V. N. Schumaker. 1992. Lipoprotein assembly. *J. Biol. Chem.* **267:** 14839–14845.
10. Yao, Z., B. D. Blackhart, M. F. Linton, S. M. Taylor, S. G. Young, and B. J. McCarthy. 1991. Expression of carboxyl-terminally truncated forms of human apolipoprotein B in rat hepatoma cells. *J. Biol. Chem.* **266:** 3300–3308.
11. White, A. L., D. L. Graham, J. LeGros, R. J. Pease, and J. Scott. 1992. Oleate-mediated stimulation of apolipoprotein B secretion from rat hepatoma cells. *J. Biol. Chem.* **267:** 15657–15664.
12. Yao, Z., B. D. Blackhart, D. F. Johnson, S. M. Taylor, K. W. Haubold, and B. J. McCarthy. 1992. Elimination of apolipoprotein B-48 formation in rat hepatoma cell lines transfected with mutant human apolipoprotein B cDNA constructs. *J. Biol. Chem.* **267:** 1175–1182.
13. Tanabe, S., H. Sherman, L. Smith, L. A. Yang, R. Fleming, and R. Hay. 1989. Biogenesis of plasma lipoproteins in rat hepatoma McA-RH7777: importance of diffusion-mediated events during cell growth. *In Vitro.* **25:** 1129–1140.
14. Tennyson, G. E., C. A. Sabatos, K. Higuchi, N. Meglin, and H. B. Brewer, Jr. 1989. Expression of apolipoprotein B mRNAs encoding higher- and lower-molecular weight isoproteins in rat liver and intestine. *Proc. Natl. Acad. Sci. USA.* **86:** 500–504.
15. Chen, S-W., X. Li, W. S. L. Liao, J. H. Wu, and L. Chan. 1990. RNA editing of apolipoprotein B mRNA. *J. Biol. Chem.* **265:** 6811–6816.
16. Huang, L-S., and J. L. Breslow. 1987. A unique AT-rich hypervariable minisatellite 3' to the apoB gene defines a high information restriction fragment length polymorphism. *J. Biol. Chem.* **262:** 8952–8955.
17. Sayers, J. R., W. Schmidt, and F. Eckstein. 1988. 5'-3' Exonucleases in phosphorothioate-based oligonucleotide-directed mutagenesis. *Nucleic Acids Res.* **16:** 791–802.
18. Graham, F. L., and A. J. van der Erb. 1973. A new technique for the assay of infectivity of human adenovirus 5 DNA. *Virology.* **52:** 456–467.
19. Southern, E. M. 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J. Mol. Biol.* **98:** 503–517.
20. Feinberg, A. P., and B. Vogelstein. 1983. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. *Anal. Biochem.* **132:** 6–13.
21. Dixon, J. L., S. Furukawa, and H. N. Ginsberg. 1991. Oleate stimulates secretion of apolipoprotein B-containing lipoproteins from HepG2 cells by inhibiting early intracellular degradation of apolipoprotein B. *J. Biol. Chem.* **266:** 5080–5086.
22. Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl, editors. 1992. Current Protocols in Molecular Biology. Green Publishing Associates and Wiley-Interscience. New York. Vol. II. 10.18.
23. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature.* **227:** 680–685.
24. Chomczynski, P., and N. Sacchi. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate–phenol–chloroform extraction. *Anal. Biochem.* **162:** 156–159.
25. Maniatis, T., E. F. Fritsch, and J. Sambrook. 1982. Molecular Cloning: a Laboratory Manual. Cold Spring Harbor, New York.
26. Witsell, A. L., and L. B. Schook. 1990. Clonal analysis of gene expression by PCR. *BioTechniques.* **9:** 318–322.
27. Andersen, A. S., A. F. Petterson, and T. B. Kjeldsen. 1992. A fast and simple technique for sequencing plasmid DNA with sequenase using heat denaturation. *BioTechniques.* **13:** 678–679.
28. Sanger, F., S. Nicklen, and A. R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA.* **74:** 5463–5467.
29. Johnson, D. F., and T. L. Innerarity. 1993. Human apolipoprotein B cryptic polyadenylation sites are activated in McA-RH7777 cells. *Circulation* **(Suppl.). 88:** I-132, abstr. 699.
30. Higuchi, K., A. V. Hospattankar, S. W. Law, N. Meglin, J. Cortright, and H. B. Brewer, Jr. 1988. Human apolipoprotein B (apoB) mRNA: identification of two distinct apoB mRNAs, an mRNA with the apoB-100 sequence and an apoB mRNA containing a premature in-frame translational stop codon, in both liver and intestine. *Proc. Natl. Acad. Sci. USA.* **85:** 1772–1776.
31. Boström, K., Z. Garcia, K. S. Poksay, D. F. Johnson, A. J. Lusis, and T. L. Innerarity. 1990. Apolipoprotein B mRNA editing. *J. Biol. Chem.* **265:** 22446–22452.
32. Boström, K., S. J. Lauer, K. S. Poksay, Z. Garcia, J. M. Taylor, and T. L. Innerarity. 1989. Apolipoprotein B48 RNA editing in chimeric apolipoprotein EB mRNA. *J. Biol. Chem.* **264:** 15701–15708.
33. Logan, E., E. Falck-Pedersen, J. E. Darnell, Jr., and T. Shenk. 1987. A poly(A) addition site and a downstream termination region are required for efficient cessation of transcription by RNA polymerase II in the mouse β^{maj}-globin gene. *Proc. Natl. Acad. Sci. USA.* **84:** 8306–8310.

34. Connelly, S., and J. L. Manley. 1989. RNA polymerase II transcription termination is mediated specifically by protein binding to a CCAAT box sequence. *Mol. Cell. Biol.* **9:** 5254–5259.
35. Ashfield, R., P. Enriquez-Harris, and N. Proudfoot. 1991. Transcription termination between the closely linked human complement genes C2 and factor B: common termination factor for C2 and c-myc? *EMBO J.* **10:** 4197–4207.
36. Hagenbüchle, O., P. K. Wellauer, D. L. Cribbs, and U. Schibler. 1984. Termination of transcription in the mouse α-amylase gene amy-2^a occurs at multiple sites downstream of the polyadenylation site. *Cell.* **38:** 737–744.
37. Richardson, J. P. 1993. Transcription termination. *Crit. Rev. Biochem. Mol. Biol.* **28:** 1–30.
38. Tantravahi, J., M. Alvira, and E. Falck-Pedersen. 1993. Characterization of the mouse β^{maj} globin transcription termination region: a spacing sequence is required between the poly(A) signal sequence and multiple downstream termination elements. *Mol. Cell. Biol.* **13:** 578–587.
39. McLeod, R. S., Y. Zhao, S. L. Selby, J. Westerlund, and Z. Yao. 1994. Carboxyl-terminal truncation impairs lipid recruitment by apolipoprotein B-100 but does not affect secretion of the truncated apolipoprotein B-containing lipoproteins. *J. Biol. Chem.* **269:**.2852–2862.
40. Whitelaw, E., and N. Proudfoot. 1986. α-Thalassaemia caused by a poly(A) site mutation reveals that transcriptional termination is linked to 3' end processing in the human α2 globin gene. *EMBO J.* **5:** 2915–2922.