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Sequence Requirements for Apolipoprotein B RNA Editing in Transfected Rat Hepatoma Cells*

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Apolipoprotein (apo) B mRNA undergoes a novel tissue-specific editing reaction, which replaces a genomically templated cytidine with uridine. This substitution converts codon 2153 from glutamine (CAA) in apoB100 mRNA to a stop codon (UAA) in apoB48 mRNA (Powell, L. M., Wallis, S. C., Pease, R. J., Edwards, Y. H., Knott, T. J., and Scott, J. (1987) *Cell* 50, 831–840). To examine sequences in the human apoB mRNA required for the editing reaction, a series of deletion mutants around the cytidine conversion site was prepared and transfected into a rat hepatoma cell line (McArdle 7777). This cell makes both apoB100 and apoB48. Editing was detected by a primer extension assay on cDNA that had been amplified by the polymerase chain reaction. RNAs of between 2385 and 26 nucleotides spanning the conversion site underwent similar levels of conversion. Editing was confirmed by cloning and sequencing of cDNA corresponding to the transfected RNAs. Conversion did not occur in transfected human hepatoblastoma (HepG2) or epithelial carcinoma (HeLa) cell lines, which do not make apoB48. These results verify that apoB48 is generated by a genuine tissue-specific RNA editing reaction and show that 26 nucleotides of apoB mRNA are sufficient for editing.

Human apolipoprotein (apo)[1] B circulates in the blood in two different sized forms (Kane, 1983). ApoB100 containing 4536 amino acid residues is synthesized in the human liver and is required for the transport of endogenously synthesized triglyceride and cholesterol in the circulation (Knott *et al.*, 1986). ApoB48 is synthesized in the intestine in man and comprises residues 1–2152 of apoB100 (Chen *et al.*, 1987; Hospattankar *et al.*, 1987; Powell *et al.*, 1987). It is necessary for the absorption and distribution of dietary lipid. ApoB48 lacks the carboxyl-terminal domain that mediates the clearance of apoB100-containing lipoproteins by the low density lipoprotein receptor pathway (Knott *et al.*, 1986; Marcel *et al.*, 1987). Both proteins are the products of the same gene (Young *et al.*, 1986; Powell *et al.*, 1987). In apoB48 mRNA a genomically templated cytidine is substituted by a uridine or uridine-like nucleotide, which generates an in-frame stop codon (UAA) in place of glutamine (CAA) 2153 (Chen *et al.*, 1987; Hospattanker *et al.*, 1987; Powell *et al.*, 1987; Higuchi *et al.*, 1988). We and others have therefore concluded that the apoB RNA in human intestine undergoes a novel co- or post-transcriptional editing reaction. In rodents both forms of apoB are made by the same mechanism in the liver (Davidson *et al.*, 1988; Tennyson *et al.*, 1989), and the cytidine to uridine transition is highly regulated by thyroxine (Davidson *et al.*, 1988). In the present study we have therefore used the rat hepatoma cell line (McArdle 7777) to verify the RNA editing process and to show with deletion mutants that a sequence of 26 nucleotides is sufficient for the RNA modification reaction.

MATERIALS AND METHODS

ApoB Expression Vectors—A 2385-bp *Sal*I to *Xba*I fragment (nucleotides 5289–7674) and a 482-bp *Rsa*I fragment (nucleotides 6411–6893) of apoB cDNA were inserted into the expression vector pECE (Ellis *et al.*, 1986) to give plasmids pECE-SX and pECE-RS (Fig. 1). Other apoB cDNA clones, except pECE-26, were produced by cloning apoB cDNA polymerase chain reaction (PCR) products into *Cla*I, *Hin*dIII-restricted pECE-SX, which had been modified to remove a *Hin*dIII site in the polylinker by digestion with *Bgl*II and *Sal*I followed by blunt ending and religation. pECE-26 was produced by annealing oligonucleotides SW18 and -19 and then ligating into *Cla*I, *Hin*dIII-digested pECE-SX. RNA transcripts after transfection contained apoB mRNA from positions 5289 to 5847, the inserted cDNA sequence, and positions 7335 to 7674 (Fig. 1).

PCR—The following oligonucleotides were synthesized for PCR on an Applied Biosystems 380A DNA synthesizer: SW10 GGATCGATGAAAATGATATACAAATTGC 6585–6604; SW16 GATCGATGCAGACATATATGATAC 6648–6666; SW18 CGATACAATTTGATCAGTATATTAA 6662–6685; SW19 AGCTTTAATATACTGATCAAATTGTAT 6663–6685; SW17 GAAGCTTCTTTAATATACTGATC 6671–6688; SW13 GGAAGCTTGTAAATCATAACTATC 6686–6703; SW14 TGTCTTCCGTTCTGTAATGGC 5810–5830; SW15 GGTTTCATCTACAAACTGGTGG 7424–7445; DD3 AATCATGTAAATCATAATTATCTTTAATATACTGA 6774–6708. SW10, -16, and -18 are coding strands and the 5′ have *Cla*I-compatible ends, and SW13, -17, and -19 are complementary to apoB mRNA with *Hin*dIII-compatible 5′ ends. SW14 is coding strand, and SW15 is complementary to apoB mRNA. The coordinates refer to human mRNA.

cDNA template was amplified using *Thermophyllus aquaticus* DNA polymerase (2 units, New England Biolabs) in 100 μl of 100 mM Tris, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, and 0.2 mM each dATP, dCTP, dGTP, and dTTP. The DNA was initially denatured at 95 °C for 5 min followed by 25 cycles of denaturation at 92 °C for 0.6 min, annealing at 55 °C for 0.5 min, and extension at 72 °C for 2 min with a final incubation at 72 °C for 10 min using a Techne Heat/Cool Dri-Block PHC-1.

Transfections—Cell lines were transfected with plasmid DNA using the method of Chen and Okayama (1987). After 44 h cells were lysed in guanidinium isothiocyanate and total RNA prepared by ultracentrifugation through a 5.7 M CsCl cushion (Chirgwin *et al.*, 1979). RNA was treated with DNase I (23 units, Boehringer Mannheim) and *Pst*I (10 units, Bethesda Research Laboratories) in the presence of human placental RNase inhibitor to remove contaminating plasmid DNA. cDNA was synthesized using a commercially available kit (Amersham RPN 1256) and amplified by PCR using primers PCR 5 and PCR 8 as described previously (Powell *et al.*, 1987) for pECE-SX and pECE-

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[1] The abbreviations used are: apo, apolipoprotein; PCR, polymerase chain reaction; Pipes, 1,4-piperazinediethanesulfonic acid; bp, base pair(s).

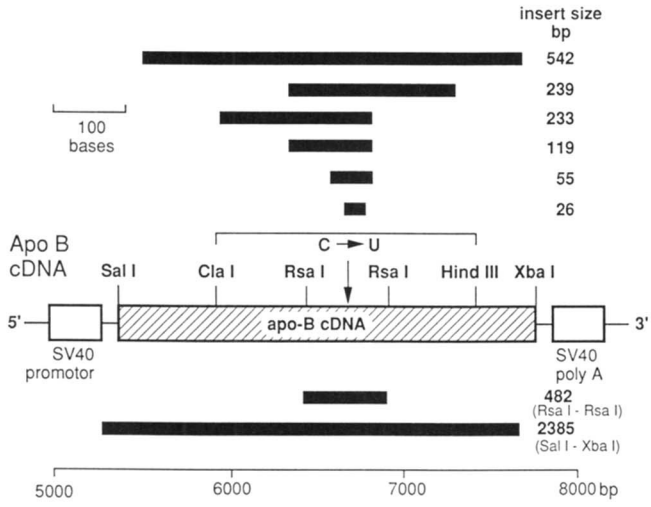

FIG. 1. **ApoB expression plasmids.** The vector, pECE, has a 300-bp sequence containing the SV40 promoter and a 235-bp sequence containing SV40 polyadenylation signals. The *thin lines* represent polylinker sequence between the control regions and apoB cDNA inserts. The 2385-bp *Sal*I,*Xba*I fragment of apoB cDNA was inserted into the *Sal*I,*Xba*I-restricted pECE to give pECE-SX, and the 482-bp *Rsa*I fragment was inserted into the *Sma*I site of pECE to give pECE-RS. Other clones were inserted into *Cla*I,*Hin*dIII-restricted pECE-SX.

RS transfections or SW14 and SW15 for the remaining transfections. These primers are specific for the human apoB sequence under the PCR conditions used.

Primer Extension Assay—After extraction with phenol/chloroform (1:1, v/v) and passage over a Sephadex G-50 spin column DNA samples from PCR (50 ng) were denatured at 95 °C for 3 min and annealed to 200 pg of ^{32}P-labeled oligonucleotide DD3 in 50 mM Pipes, pH 6.4, 0.2 M NaCl at 37 °C for 1 h. DNA samples were reverse-transcribed with 2 units/μl super reverse transcriptase (East Anglian Biotechnology) in 50 mM Tris-HCl, pH 8.2, 6 mM $MgCl_2$, 10 mM dithiothreitol, 0.5 mM each dATP, dCTP, and dTTP, and 0.25 mM dideoxy-GTP at 42 °C for 90 min. Products were analyzed on a 7.5% polyacrylamide urea suquencing gel and subjected to autoradiography at −70 °C with Cronex Lightning Plus intensifying screens (Du Pont). Autoradiographs were scanned using a Shimadzu dual wavelength thin layer chromatography scanner CS-930.

DNA Sequencing—Amplified DNA from the pECE-SX transfection was reamplified using primers SW10 and SW13 and from pECE-26 transfection with SW14 and SW15, digested with *Taq*I and *Hin*dIII, and ligated into *Cla*I,*Hin*dIII-digested m13tg131. Single-stranded M13 template DNA preparations isolated by standard methods were blotted onto nitrocellulose and hybridized with ^{32}P-oligonucleotides (1 × 10^9 cpm/μg), BGLN (C-specific) or BSTOP (T-specific) in 6 × SSC (0.15 M NaCl, 0.015 M sodium citrate, pH 7.2), 0.1% sodium dodecyl sulfate, 10 × Denhardt's reagent at 42 °C for 18 h as described by Powell *et al.* (1987). Filters were washed in 6 × SSC, 0.1% sodium dodecyl sulfate at room temperature for 30 min, then at 46 °C for 5 min, and autoradiographed at −70 °C with Cronex Lightning Plus intensifying screens. M13 template DNAs were sequenced using a sequenase kit as described by the manufacturer (United States Biochemical Corp.).

Mouse liver cDNA libraries (Clontech Laboratories Inc.) in phage λgt11 were plated on *Escherichia coli* 1088 at 42 °C and screened with a ^{32}P-labeled 2385-bp *Sal*I,*Xba*I fragment of human apoB cDNA (Fig. 1). Phage were plaque-purified and inserts subcloned into M13 for sequencing.

RESULTS

ApoB mRNA sequences required for editing were defined by transfection of human apoB cDNAs (Fig. 1) of varying lengths spanning the modification site into a rat hepatoma cell line, McArdle 7777 (McA-RH7777 ATCC CRL.1601), which synthesizes both apoB100 and apoB48 mRNAs.[2] RNA editing was detected by a primer extension assay of PCR-amplified cDNA prepared from RNA isolated from transfected McArdle 7777 cells. In this assay unmodified cDNAs terminate at position 6666. Modified cDNA molecules extend to position 6655 in humans and 6661 in rat (Fig. 2*A*), except for the clone with the smallest human apoB cDNA insert (pECE-26). The 5′ end of this clone is at 6662, and pECE-26 terminates at the adjacent upstream cytidine equivalent to position 6661 in this assay.

Rat and human apoB mRNAs were detected by the use of rat-specific or human-specific PCR primers. cDNA was synthesized from RNA isolated from untransfected McArdle 7777 cells and amplified by using rat specific primers, ND1 and ND2 (Davidson *et al.*, 1988). Both unedited (C at position 6666) and edited (T at position 6666) cDNAs could be detected. Densitometric estimation indicated 16% conversion to the edited form (Fig. 2, *Panel B, lane 1*). When human-specific primers are used (*lane 2*), no apoB mRNA was detected in untransfected cells.

RNA prepared from transfected McArdle 7777 cells was found to be contaminated with human apoB plasmid DNA used in the transfection. This was removed by using RNase-free DNase and *Pst*I. A *Pst*I site is present at position 6648 in the human apoB cDNA sequence. Successful removal of DNA was confirmed by PCR amplification of control-transfected RNA samples from which reverse transcriptase had

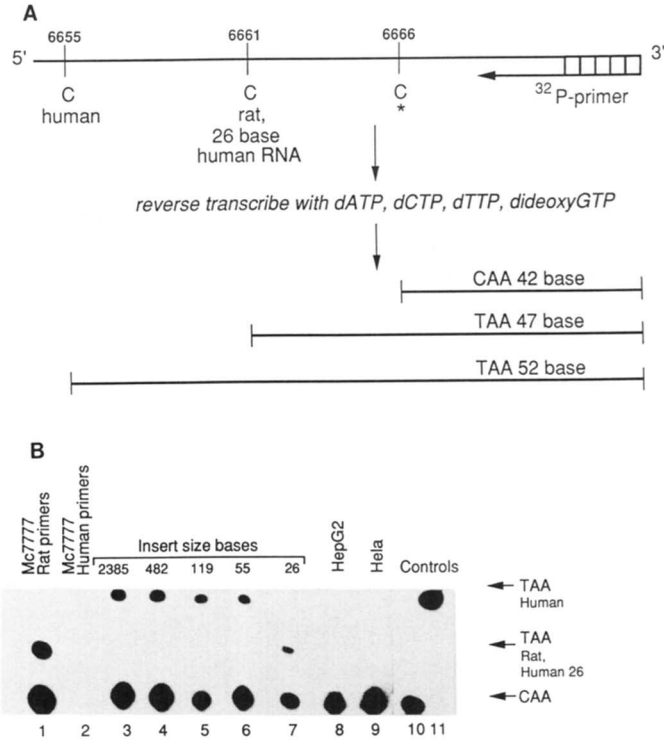

FIG. 2. **RNA editing detected by primer extension analysis.** *A*, schematic model of the primer extension assay. A ^{32}P-labeled primer is annealed to PCR-amplified apoB cDNA and extended with reverse transcriptase in the presence of dideoxy-GTP. Unmodified cDNAs terminate at position 6666 whereas modified cDNAs extend to position 6655 in humans and 6661 in rat. The human 26-bp insert also ends at 6661. *B*, primer extension analysis of PCR-amplified cDNA synthesized from RNA isolated from McArdle 7777 cells (*lanes 1–7*) transfected with: *lane 1*, no added DNA (rat endogenous conversion detected by using rat-specific apoB oligonucleotide primers ND1 and ND2 (Davidson *et al.*, 1988)); *lane 2*, no transfected DNA (human-specific primers 5 and 8 used for PCR); *lane 3*, pECE-SX; *lane 4*, pECE-RS; *lane 5*, pECE-119; *lane 6*, pECE-55; *lane 7*, pECE-26; *lane 8*, HepG2 cells transfected with pECE-119; *lane 9*, HeLa cells transfected with pECE-SX; *lane 10*, C control; *lane 11*, T control.

[2] M. S. Davies *et al.*, unpublished observations.

been omitted (data not shown). When apoB cDNAs were transfected into McArdle 7777 cells, RNA editing was demonstrated on inserts of 2385 and 482 nucleotides in length (Fig. 2*B*, *lanes 3* and *4*). Thus any information required for editing is contained within 482 nucleotides. Because smaller RNAs might have been unstable in the transfected cells, small cDNAs were inserted into the 2385-bp *SalI,XbaI* fragment with the central 1489-bp *ClaI* to *Hin*dIII fragment removed. RNA editing was found in all inserts down to a size of 26 nucleotides (Fig. 2*B*, *lanes 5–7*). The levels of editing, estimated by densitometry, were similar in all cases: 2385 bp (9.8%), 482 bp (12.4%), 119 bp (12.5%), 55 bp (13.4%), and 26 bp (8.1%). Results obtained with inserts of 542, 239, and 233 bp were similar (data not shown). These levels were slightly lower than the 16% endogenous conversion of rat apoB mRNA. DNA editing, that is conversion of C to T at position 6666 in plasmids upon transfection, was not detected on plasmid pECE-SX, which had been through transfection and PCR (data not shown).

RNA editing was unequivocally confirmed by DNA sequence analysis of M13 clones derived from PCR-amplified cDNAs prepared from McArdle 7777 cells transfected with the largest (2385 bp) and the smallest (26 bp) cDNA inserts (results not shown). Dot blots of M13 template DNAs were probed with ^{32}P-labeled T-specific (BSTOP) or C-specific (BGLN) oligonucleotides (Powell *et al.*, 1987). Oligonucleotide BSTOP hybridized to 5 out of 44 (11.4%) of the templates containing the largest insert. All of these were sequenced and contained T at position 6666 in the apoB cDNA. There were no other nucleotide changes between positions 6605 and 6685. 11 out of 93 (11.8%) templates containing the 26-bp insert hybridized to the T-specific oligonucleotide. 5 of these were sequenced and found to contain T at position 6666. The insert DNA terminated at position 6662 confirming that these clones represented the 26-bp insert.

Human hepatoblastoma cells (HepG2) only synthesize apoB100 mRNA (Powell *et al.*, 1987). When the 119-bp (pECE-119) and 2385-bp (pECE-SX) insert cDNAs were transfected into HepG2 cells (Fig. 2*B*, *lane 8*), human epithelial carcinoma cells (HeLa) (Fig. 2*B*, *lane 9*), and Chinese hamster ovary cells (data not shown), no RNA editing was detected.

Humans, rabbits (Powell *et al.*, 1987), rats (Davidson *et al.*, 1988), and mice (this study) make both forms of apoB by the same cytosine to uridine transition in RNA. We have, therefore, examined the sequence of the apoB gene in the 150-bp region surrounding the stop codon. All four species show a high level of sequence conservation (Fig. 3) sharing 90% homology. This sequence is enriched in adenosine and uridine (74%) compared to most other regions of apoB mRNA. The mean adenosine and uridine content of apoB mRNA is 57%.

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      6608                                                    6657
Human AGATGATGCCAAAATCAACTTTAATGAAAAACTATCTCAACTGCAGACAT
Rabbit G---A----------------A----------G--------T-------
Rat   ----AG---------------G----------C--------TG------
Mouse ----AG---------------C----------C--------TG------

                                                        6697
          *
Human ATATGATACAATTTGATCAGTATATTAAAGATAGTTATGATTTACATGAT
Rabbit --G-------------------------------A--T----C--------
Rat   -CGC-----------------------G-----A-------GC---G--C
Mouse -CGC-----------------------------A-------CC------C

                                                        6757
Human TTGAAAATAGCTATTGCTAATATTATTGATGAAATCATTGAAAAATTAAA
Rabbit --T----------A----G------A---C-------G----------
Rat   --A----G-A--------C-G----------AG-----------GC----
Mouse --A----G-A--------G-G----------CG-----------G-----
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FIG. 3. **DNA sequence in the region of the converted nucleotide.** Nucleotide sequences of human, rabbit, rat, and mouse apoB cDNAs. Differences are only shown for nonhuman species. The *asterisk* denotes modified base.

DISCUSSION

These studies verify that apoB48 is generated by an RNA editing reaction. This has been demonstrated on human apoB RNA expressed in the rat hepatoma cell line McArdle 7777, which produces both apoB100 and apoB48 in common with the rat liver. The proportion of human apoB RNA modified in the hepatoma was slightly lower than that of the endogenous rat RNA. The editing reaction shows cell-type specificity in that apoB mRNA modification was not demonstrated in the human hepatoblastoma cell line HepG2, which produces only apoB100, or in HeLa and Chinese hamster ovary cell lines that do not produce apoB.

The RNA derived from pECE-26 is sufficient to confer efficient RNA editing. This RNA contains 26 nucleotides (6662–6687) that span the conversion site and which are fully conserved across several species, together with distant apoB flanking sequences. These specific flanking sequences are not required for conversion of the 482-nucleotide RNA derived from pECE-RS, which lacks them and yet contains all information required for efficient editing. Whether the flanking sequences in pECE-26 provide structures necessary for RNA editing is not established. However, the RNA derived from pECE-26 is predicted to form a stem-loop using its new 5′-flanking sequence with the edited nucleotide found in the 6-nucleotide loop,[3] and a highly conserved 27-nucleotide (6656–6682) stem-loop is found in the other larger RNAs with edited nucleotide occurring in an 8-nucleotide loop[3] (Zucker and Steigler, 1981). Therefore, to establish the precise sequence requirements and whether these secondary structures are needed for editing of apoB mRNA we have initiated a program of site-specific mutagenesis.

The 150-base sequence encompassing the RNA editing site contains 74% uridine and adenosine nucleotides. No other region of the 14.5-kilobase apoB mRNA has such a high uridine and adenosine content. The dinucleotide UpA is generally excluded from RNA destined to be expressed as mRNA in the cytoplasm (Beutler *et al.*, 1989). Scarcity of this dinucleotide may reflect a need for mRNA stability and the need to avoid the action of UpA-specific cytoplasmic ribonucleases found in certain cells. The UpA-rich sequence encompassing the modification site is possibly spared for functional reasons.

We conclude that the conserved 26-nucleotide UpA-rich region spanning the apoB mRNA is sufficient to confer RNA editing and may be required for the binding of the enzyme or ribonucleoprotein complex which mediates the modification reaction.

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REFERENCES

Beutler, E., Gelbart, T., Han, J., Koziol, J. A., and Beutler, B. (1989) *Proc. Natl. Acad. Sci. U. S. A.* **86,** 192–196

Chen, C., and Okayama, H. (1987) *Mol. Cell. Biol.* **7,** 2745–2752

Chen, S.-H., Habib, G., Yang, C.-Y., Gu, Z.-W., Lee, B. R., Weng, S.-A., Silberman, S. R., Cai, S.-J., Deslypere, J. P., Rosseneu, M., Gotto, A. M., Jr., Li, W.-H., and Chan, L. (1987) *Science* **238,** 363–366

Chirgwin, J. M., Przbyla, A. E., MacDonald, R. J., and Rutter, W. J. (1979) *Biochemistry* **18,** 5294–5299

Davidson, N. O., Powell, L. M., Wallis, S. C., and Scott, J. (1988) *J. Biol. Chem.* **263,** 13482–13485

[3] M. S. Davies *et al.*, unpublished result.

Ellis, L., Clauser, E., Morgan, D. O., Edery, M., Roth, R. A., and Rutter, W. J. (1986) *Cell* **45,** 721–732
Higuchi, K., Hospattankar, A. V., Law, S. W., Meglin, N., Cartwright, J., and Brewer, H. B., Jr. (1988) *Proc. Natl. Acad. Sci. U. S. A.* **85,** 1772–1776
Hospattankar, A. V., Higuchi, K., Law, S. W., Meglin, N., and Brewer, H. B., Jr. (1987) *Biochem. Biophys. Res. Commun.* **148,** 279–285
Kane, J. P. (1983) *Annu. Rev. Physiol.* **45,** 637–650
Knott, T. J., Wallis, S. C., Powell, L. M., Pease, R. J., Lusis, A. J., Blackhart, B., McCarthy, B. J., Mahley, R. W., Levy-Wilson, B., and Scott, J., (1986) *Nucleic Acids Res.* **14,** 7501–7504
Marcel, Y. L., Innerarity, T. L., Spilman, C., Mahley, R. W., Protter, A. A., and Milne R. W. (1987) *Arteriosclerosis* **7,** 166–175
Powell, L. M., Wallis, S. C., Pease, R. J., Edwards, Y. H., Knott, T. J., and Scott, J. (1987) *Cell* **50,** 831–840
Tennyson, G. E., Sabatos, C. A., Higuchi, K., Meglin, N., and Brewer, H. B., Jr. (1989) *Proc. Natl. Acad. Sci. U. S. A.* **86,** 500–504
Young, S. G., Bertics, S. J., Scott, T. M., Dubois, B. W., Curtiss, L. K., and Witztum, J. L. (1986) *J. Biol. Chem.* **261,** 2995–2998
Zucker, M., and Stiegler, P. (1981) *Nucleic Acids Res.* **9,** 133–148