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) to stop codon (UAA) in apoB 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) 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; Hospattanker 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, 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 SalI to XbaI fragment (nucleotides 5289-7674) and a 482-bp SalI to RflI 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 mRNA polymerase chain reaction (PCR) products into ClaI, HindIII-restricted pECE-SX, which had been modified to remove a HindIII site in the polylinker by digestion with SfiI and SalI followed by blunt ended ligation. pECE-26 was produced by annealing oligonucleotides SW18 and -19 and then ligating into ClaI, HindIII-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 GATCGATGCATAATATATGATAC 5810-5830; SW11 GGTGG 7424-7445; DD3 AGCTTGTAAATCATAACTATC 6686-6703; SW14 TGTCTTCC-GTTCTGTAATGGC 6774-6708. SW10, -16, and -18 are coding strands complementary to apoB mRNA with HindIII-compatible 5' ends. SW14 is coding strand, and positions 7335 to 7674 (Fig. 1).

HindIII-restricted pECE-SX, which had been modified to remove a HindIII site in the polylinker by digestion with SfiI and SalI followed by blunt ended ligation. pECE-26 was produced by annealing oligonucleotides SW18 and -19 and then ligation into ClaI, HindIII-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).

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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 2985-bp SalI,XbaI fragment of apob cDNA was inserted into the SalI,XbaI-restricted pECE to give pECE-SX, and the 482-bp inserts. The 2385-bp SalI,XbaI fragment of apoB cDNA was inserted into the pECE-SX sequence.

RS transfections or SW14 and SW15 for the remaining transfections.

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. 2A), 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.

Translated 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 PstI. A PstI 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

RESULTS

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Note: The text includes a diagram and a table that are not represented in the natural text.
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. 2B, 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 SoilXhol fragment with the central 1489-bp ClaI to HindIII fragment removed. RNA editing was found in all inserts down to a size of 26 nucleotides (Fig. 2B, 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 plasmid upon transfection, was not detected on plasmid pCE-SX, which had been through transfection and PCR (data not shown).

RNA editing was unambiguously 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 32P-labeled T-specific (BSTOP) or C-specific (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. 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 apoB mRNA (Powell et al., 1987). When the 119-bp (pECE-119) and 2385-bp (pECE-SX) insert cDNAs were transfected into HepG2 cells (Fig. 2B, lane 8), human epithelial carcinoma cells (HeLa) (Fig. 2B, 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%.

**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 mRNA 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 (6682–6687) 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 pCE-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, 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 (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 adenine 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, 2745–2755

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

Chirgwin, J. M., Przybyle, 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

* M. S. Davies et al., unpublished result.
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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