Apolipoprotein B mRNA and lipoprotein secretion are increased in McArdle RH-7777 cells by expression of betaine-homocysteine S-methyltransferase

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The cDNA encoding rat betaine-homocysteine S-methyltransferase (BHMT) was isolated through production of monoclonal antibodies against protein fractions enriched with apolipoprotein B (apo B)-mRNA-editing complexes. BHMT mRNA was expressed predominantly in liver, and also in kidney, but not in small intestine. In stable McArdle RH-7777 (McA) cell lines expressing differing levels of BHMT, the editing efficiency of apo B mRNA was unchanged. Evaluation of apo B-mRNA expression revealed that steady-state levels were increased significantly and in parallel with BHMT protein expression. The highest levels of BHMT mRNA and BHMT enzyme activity expressed in stably transfected McA cells were comparable with

those found in rat hepatocytes. In contrast to the changes in apo B-mRNA abundance, levels of other apolipoprotein-encoding mRNAs and several liver-specific and ubiquitously expressed mRNAs were unchanged by BHMT expression. In the cell line expressing the highest level of BHMT, apo B-containing lipoprotein secretion was increased, indicating utilization of increased endogenous message. Results suggest that apo B-mRNA abundance in McA cells is related to the expression of BHMT, an enzyme important in homocysteine metabolism.

Key words: intestine, liver, metabolism, methionine, VLDL.

INTRODUCTION

Apolipoprotein B (apo B) is a critical protein component in the formation of triglyceride-rich lipoproteins, including very-low-density lipoproteins (VLDLs) and chylomicrons [1,2]. Lipoprotein assembly and secretion take place in the liver and intestine and involve complex pathways for the synthesis and degradation of both lipid and apolipoproteins. In humans, hepatically synthesized VLDL-apo B is a precursor to LDL-apo B, which has significant atherogenic potential. Therefore, identification of factors that regulate hepatic apo B gene expression and metabolism is important for understanding control of VLDL and LDL synthesis. After the apo B gene is transcribed, apo B secretion is regulated through post-transcriptional mechanisms that may include changes in apo B-mRNA stability [3], apo B-mRNA editing [4] and post-translational apo B degradation [5]. Utilizing a monoclonal-antibody strategy that identified an auxiliary factor involved in apo B-mRNA editing [4], we now identify a protein that is the rat homologue of human betaine-homocysteine S-methyltransferase (BHMT, EC 2.1.1.5). BHMT is a methyltransferase that catalyses the conversion of homocysteine (Hcy) to methionine using betaine as methyl donor [6]. The only other enzyme known to generate methionine from Hcy is methionine synthase (EC 2.1.1.13). BHMT is a 45-kDa cytosolic protein expressed primarily in liver and to a lesser extent in kidney [7,8]. We report that BHMT exhibits a novel protein function [9]. When BHMT cDNA is stably expressed in McArdle RH-7777 (McA) cells, no changes in apo B-mRNA-editing efficiency are observed, but apo B secretion is stimulated. Increased levels of apo B mRNA are also observed, suggesting that the increased mRNA is at least partially responsible for the

increase in apo B secretion. Northern-blot analysis reveals that expression of BHMT in stable McA cell lines raises BHMT mRNA to levels comparable with those observed in rat hepatocytes. This suggests that the transfections employed represent restoration of physiological levels of BHMT mRNA expression in McA cells rather than over-expression. The bifunctionality of BHMT in Hcy and apo B metabolism is discussed.

EXPERIMENTAL

Monoclonal antibody strategy for identifying apo B-mRNA-associated proteins

Apo B-RNA-editing 60 S pre-edited fractions were obtained by 10–50% glycerol gradient sedimentation of cytosolic S100 extracts from Sprague–Dawley rat (300–400 g) livers [10,11]. In addition to most of the liver's apo B-RNA-editing and editosome-assembling activity, this fraction contained the majority of apo B-RNA-binding activities. Three 6–8-week-old BALB/c female mice were immunized at intervals of 2–4 weeks with 100 µg of the 60 S pre-edited fraction, boosted 3 weeks later and then re-immunized several days prior to fusions [12]. Hybridoma-culture supernatants were pre-adsorbed with rat albumin and screened by radioimmunoassay using 0.1–1 µg of the 60 S pre-edited fraction as immobilized antigen. Antibody binding was detected using 2–10 µCi of ¹²⁵I-labelled sheep anti-mouse immunoglobulin per well (Amersham Life Sciences). Twenty hybridomas with supernatant reactivities in the upper 10% were cloned by limiting dilution and rescreened in a similar fashion against a 40% ammonium sulphate precipitate of the pre-

Abbreviations used: apo B, apolipoprotein B; VLDL, very-low-density lipoprotein; McA, McArdle RH-7777; BHMT, betaine-homocysteine S-methyltransferase; HA, haemagglutinin; Hcy, homocysteine; 5'-RACE, rapid amplification of 5'-cDNA ends; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; RT, reverse transcriptase.

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editosome 60 S fraction as immobilized antigen. Five clones were identified as producing antibody with strong reactivity to a protein with an apparent molecular mass of 45 kDa, present in rat liver lysates. The most highly reactive antibody (anti-p45) was an IgG_{2b} isotype and was used in subsequent studies.

Cloning the cDNA encoding the 45 kDa protein

The anti-p45 monoclonal antibody was used to screen a λ gt-11 rat liver cDNA-expression library (Clontech). Five clones were identified after tertiary-level screening and confirmation by Western blotting of phage lysates for expression of p45- β -galactosidase fusion protein. *EcoRI* restriction fragments from each clone were subcloned into pSP73 (Promega) and sequenced bidirectionally (Sequenase V2.0, Amersham Life Sciences) using designated SP6, T7 and the cDNA-specific primers DA, DB and DE (DA, 5'-GGGGATGCATTGGTTGCAG-3', 5' at nt 410; DB, 5'-CAATGGCGGCACCTGCTTTT-3', 5' at nt 707; and DE, 5'-ATACGGGCCCTGAATGGT-3', 5' at nt 1660). The orientation of fragments within the recombinant λ clones was determined by cycle sequencing (*Taq*ence kit, Amersham Life Sciences) using vector-specific primers that flank the *EcoRI* site (λ gt11-5', 5'-GACTCCTGGAGCCCG-3'; and λ gt11-3', 5'-GGTAGCGACCGGCGC-3') and cDNA internal primers (DC, 5'-CCCCTGACTGTGGCAAAC-3', 5' at nt 828; and DD, 5'-CTCCTTCCGGGTGCACAC-3', 5' at nt 1358). The 5' end of the cDNA was obtained using rapid amplification of 5'-cDNA ends (5'-RACE) methodology [13]. The designated primer RACE-RT (5'-AGGTGTCTGACTCACACCTCCTGC-3', 5' at nt 448) was used in the synthesis of first-strand cDNA using rat liver total RNA and avian myeloblastosis virus reverse transcriptase (RT, Promega) according to the manufacturer's recommendations. Resultant cDNAs were 3' tailed using terminal deoxynucleotidyl transferase (Promega) and dGTP. PCR amplification of first-strand cDNA was achieved using an anchor primer (5'-CTCTCTAGAAATTC₁₅-3') and a nested primer (5'-GCCAGATATCTTCTCTGCCACG-3', 5' at nt 359) utilizing *Pfu* DNA polymerase (Stratagene). The sequences of eight 5'-RACE subclones were identical and contained an in-frame initiator AUG codon when ligated, through the *EcoRV* restriction site in the nested primer, to the partial cDNA sequence.

Plasmid constructions

The coding portion of the cDNA was subcloned into a modified pcDNAIII vector (InVitrogen) downstream of a sequence that encoded an initiator methionine and a nine amino acid influenza virus haemagglutinin (HA) epitope tag (YPYDVPDYA) [14].

Cell culture and lipoprotein analyses

The rat hepatoma cell line McA was maintained, transfected and stable clonal cell lines generated as described previously [15]. For lipoprotein-secretion analyses, stable transfected McA cells were seeded on to 60-mm dishes (2.5×10^5 cells/dish) and incubated until 50–60% confluent. Wild-type McArdle cells transfected with the pcDNAIII vector alone (designated as McA-CMV cells) served as control in these and all subsequent experiments. Cells were washed three times with Hanks balanced salt solution containing 0.2% (w/v) BSA and re-incubated in Dulbecco's modified Eagle's medium containing 0.2% (w/v) BSA for 20 h and then for a further 4 h. Media were collected and the cell monolayers washed twice with Hanks balanced salt solution and harvested as described previously [16]. Secreted apo B protein levels were determined by competitive radioimmunoassay using rat VLDL apo B as standard and a monoclonal antibody equally

reactive to apo B48 and apo B100 [12]. Protein concentrations of cell homogenates were measured by a modified Lowry procedure [17] and the concentration of medium apo B was calculated relative to total cell protein.

Protein analyses

Whole-cell protein was prepared from clonal cell lines using reporter lysis buffer (Promega) according to the manufacturer's recommendations. Equal amounts of protein (25 μ g), as determined by Bradford assay (BioRad), from each cell line were resolved by SDS/PAGE (10% gel) and transferred on to nitrocellulose. HA-epitope tagged proteins were detected using an anti-HA mouse monoclonal antibody (BabCo, Richmond, CA, U.S.A.) and a horseradish peroxidase-conjugated rabbit anti-mouse secondary antibody. Antibody reactivity was visualized using enhanced chemiluminescence (ECL, Amersham). Quantification of protein expression was performed using fluorescein-labelled rabbit anti-mouse secondary antibody (Cappel, West Chester, PA, U.S.A.) and detection by Fluoroimager analysis (Molecular Dynamics, Sunnyvale, CA, U.S.A.).

RNA isolation and analysis

Total cellular RNA was isolated from stable McA cell lines, primary rat hepatocytes and rat tissues using Tri-Reagent (Molecular Research Center, Cincinnati, OH, U.S.A.) according to the manufacturer's recommendations. Rat tissue-specific total RNAs were obtained from Ambion (Austin, TX, U.S.A.). RNAs were resolved through formaldehyde/agarose gels, transferred on to nylon filters and immobilized by UV-crosslinking. Filters were hybridized with random-primed labelled cDNA probes in ExpressHyb solution (Clontech) according to the manufacturer's recommendations and as described previously [18]. The probes used were as follows: an *EcoRI* restriction fragment from BHMT cDNA (nt 866–1317), and PCR amplicons from rat apo B (nt 6512–6719) [19], rat β 2-microglobulin (nt 136–383) [19] and rat ubiquitin (nt 2076–2772). Rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH; 316-bp cDNA), rat β -actin (126-bp cDNA) and rat cyclophilin (103-bp cDNA) were obtained from Ambion. Expressed-sequence-tag clones were obtained for transferrin (GenBank accession no. AA858975) and microsomal triglyceride-transfer protein (accession no. W13053). Partial rat cDNAs for apo AI, apo E and albumin were a gift of Dr. Lawrence Chan (Department of Cell Biology, Baylor College of Medicine, Houston, TX, U.S.A.) and Dr. Tom Sargeant (National Institutes of Health, Bethesda, MD, U.S.A.). Either filters were exposed to X-ray film or mRNA levels were quantified by PhosphorImager analysis.

Apo B-mRNA-editing assays

The proportion of editing upon apo B RNAs synthesized in McA cells was determined by the RT-PCR methodology as described previously [19]. Quantification was performed by PhosphorImager analysis.

BHMT enzyme assay

McA cells and primary rat hepatocytes were washed with PBS and then scraped into PBS containing 1 mM EDTA, 1 mM 2-mercaptoethanol and 10% (v/v) glycerol. Cell lysates were prepared by sonication and clarified by centrifugation at 10000 *g* for 15 min at 4 °C. Total lysate protein was determined by Bradford assay using BSA as standard. Dimethylacetothetin was synthesized and BHMT activity measured as described previously [20]. In brief, sample assays contained 5 mM D,L-Hcy, 2 mM

dimethylacetothetin (0.457 μ Ci), 50 mM Tris (pH 7.5), 5 mM 2-mercaptoethanol and cell lysate. Blanks contained all of the reaction components except cell lysate, and their values were subtracted from the sample values. Samples were assayed in duplicate and results varied less than 3%, and were thus averaged. BHMT activity (1 unit) is defined as the amount needed to produce 1 nmol of methionine per h. BHMT activities are expressed as specific activities (units/mg of total protein).

RESULTS

Rat BHMT mRNA is expressed predominantly in liver

A partial cDNA was obtained by screening a rat liver cDNA-expression library using a monoclonal antibody raised against apo B-RNA-binding proteins. The 5' end was completed by 5'-RACE techniques (see the Experimental section). The cDNA contained a 70 nucleotide, 5'-untranslated leader and a 3'-untranslated region greater than 600 nucleotides (Figure 1) and

encodes a protein comprising 407 amino acids with a predicted (Expasy pI/MW tool, available at <http://www.expasy.ch>) and apparent relative molecular mass of 45 kDa (see below, Figure 5b). BLAST searches with this cDNA against the GenBank database identified 80% nucleotide similarity and 95% amino acid similarity of the deduced amino acid sequence with human BHMT. The rat tissue-specific mRNA expression of the cDNA (Figure 2) revealed predominant expression in liver with minor expression in kidney, which corresponds with the tissue-specific enzyme activity profile of BHMT [7]. Moreover, transfected McA cells exhibited increased BHMT enzymic activity (see below). We have identified this cDNA as rat BHMT (GenBank accession no. AF038870).

Expression of BHMT increases apo B-mRNA abundance

As monoclonal antibodies were developed against proteins with apo B-RNA-binding activity [10], changes in steady-state apo B-mRNA levels were determined when BHMT was expressed in

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1 caagcctttgtgagaccgctcctgtccagtcgcagctggcttcagcgccactcaggacaccggaag ATG GCA
   CCG ATT GCC GGC AAG AAG GCC AAG AGG GGA ATC TTA GAA CGC TTA AAT GCT GGC GAA GTC
23 P I A G K K A R G I L E R L N A G E V
   GTG ATC GGA GAT GGG GGA TTT GTC TTT GCA CTG GAA AAG AGG GGC TAC GTA AAG GCT GGA
23 V I G D G G F V F A L E K R G Y V K A G
   CCC TGG ACC CCA GAG GCT GCG GTG GAG CAC CCC GAG GCA GTT CCG CAG CTT CAT CGG GAG
43 P W T P E A A V E H P E A V R Q L H R E
   TTC CTC AGA GCT GGA TCG AAC GTC ATG CAG ACC TTC ACT TTC TAT GCA AGT GAG GAC AAG
63 F L R A G S N V M Q T F T F Y A S E D K
   CTG GAA AAC CGA GGG AAC TAC GTG GCA GAG AAG ATA TCT GGG CAG AAG GTC AAT GAA GCT
83 L E N R G N Y V A E K I S G Q K V N E A
   GCT TGT GAC ATT GCA CGG CAA GTT GCT GAC GAA GGG GAT GCA TTG GTT GCA GGA GGT GTG
103 A C D I A R Q V A D E G D A L V A G G V
   AGT CAG ACA CCT TCC TAC CTC AGC TGC AAG AGT GAG ACG GAA GTT AAA AAG ATA TTT CAC
123 S Q T P S Y L S C K S E T E V K K I F H
   CAA CAG CTT GAG GTC TTC ATG AAG AAG AAT GTG GAC TTC CTC ATT GCA GAG TAT TTT GAA
143 Q Q L E V F M K K N V D F L I A E Y F E
   CAT GTT GAA GAA GCC GTG TGG GCA GTC GAG GCC TTA AAA ACA TCC GGG AAG CCT ATA CGC
163 H V E E A V W A V E A L K T S G K P I A
   GCT ACC ATG TGC ATC GGA CCT GAA GGA GAT CTA CAT GGC GTG TCT CCT GGA GAG TGC GCA
183 A T M C I G P E G D L H G V S P G E C A
   GTG CGT TTG GTA AAA GCA GGT GCC GCC ATT GTC GGT GTG AAC TGC CAC TTC GAC CCC AGC
203 V R L V K A G A A I V G V N C H F D P S
   ACC AGC TTG CAG ACA ATA AAG CTC ATG AAG GAG GGT CTG GAA GCA GCT CGG CTG AAG GCT
223 T S L Q T I K L M K E G L E A A R L K A
   TAC TTG ATG AGC CAC GCC CTG GCC TAC CAC ACC CCT GAC TGT GGC AAA CAG GGA TTT ATT
243 Y L M S H A L A Y H T P D C G K Q G F I
   GAT CTC CCA GAA TTC CCC TTT GGA TTG GAA CCC AGA GTT GCC ACC AGA TGG GAT ATT CAA
263 D L P E F P F G L E P R V A T R W D I Q
   AAA TAC GCC AGA GAG GCC TAC AAC CTG GGG GTC AGG TAC ATT GGC GGC TGC TGC GGA TTT
283 K Y A R E A Y N L G V R Y J G G C C G F
   GAG CCC TAC CAC ATC AGG GCC ATT GCA GAG GAG CTC GCC CCA GAA AGG GGA TTT TTA CCA
303 E P Y H I R A I A E E L A P E R G F L P
   CCA GCT TCA GAA AAA CAT GGC AGC TGG GGA AGT GGT TTG GAC ATG CAC ACC AAA CCC TGG
323 P A S E K H G S W G S G L D M H T K P W
   ATC AGG GCA AGG GCC AGG AAA GAA TAC TGG CAG AAT CTT CGA ATA GCT TCG GGC AGA CCG
343 I R A R A R K E Y W Q N L R I A S G R P
   TAC AAT CCT TCG ATG TCC AAG CCG GAT GCT TGG GGA GTG ACG AAA GGG GCA GCA GAG CTG
363 Y N P S M S K P D A W G V T K G A A E L
   ATG CAG CAG AAG GAA GCC ACC ACT GAG CAG CAG CTG AGA GCG CTC TTC GAA AAA CAA AAA
383 M Q Q K E A T T E Q Q L R A L F E K Q K
   TTC AAA TCC GCA CAG TAG ccacaggccagcggttcggggcggaattcctccaggtccgggcccacagtgtgcacc
403 F K S A Q *
   ggaaggagaaggcatctcctaaccacgcttgggttgatgccggttacacctgtgattgggtgctagttagacaaaatgg
   agtcacagatagcatttcacagttacaaaactacgctttagaattttacctagaaggagaaggagaagttccacagtaa
   atcctgaacacatttcctacgtgcctgtgcattacaggcgacaggagtcactgcagcgaagagaaagtcacccgacgt
   caatctcatttcagatagggggataggacacacacctccacgagtgacatagaaccttcagggaacggtatcataagtgac
   acagcaaccatctatctaaagatgcttcccaagtggttcacagatcttttgagcaggacccttaggcagaaacacac
   acaccagccctgtaaaacttaacagataactgatccattctgttaattctgttaattctgttctgtctgactgtcttccat
   ttcattaataaaaaacatgccggttgaaacacctcaaaaaaaaaaaaaaaaaaagaattcc 1891

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Figure 1 The cDNA and derived amino acid sequence of rat BHMT

The numbers on the left represent amino acids from the ATG start codon, number 1. The asterisk indicates the in-frame stop codon, and lower-case letters designate the sequence of the 3'-untranslated region. GenBank accession number AF038870.

Figure 2 Tissue-specific expression of rat BHMT

Total RNA (7.5 μ g per lane) from various rat tissues were electrophoretically fractionated and transferred on to nylon membranes followed by hybridization with 32 P-labelled BHMT partial cDNA. Equal RNA loading was verified by reprobing with a GAPDH probe (results not shown).

Figure 3 BHMT protein expression and apo B-mRNA abundance

(a) HA-tagged BHMT protein expression in McA-CMV cells (null) and in McA-BHMT cell lines C, K, J and G was assayed by Western-blot analysis of cell extracts with a monoclonal anti-HA antibody. (b) Apo B-mRNA expression in stable transfected cell lines were analysed by Northern blotting. GAPDH mRNA varied less than 10% between lanes (results not shown).

the rat hepatoma cell line, McA. McA cells both synthesize and secrete apo B as well as editing apo B mRNA at moderate levels, making them valuable in studies of apo B metabolism. Stable clonal McA cell lines that expressed HA-epitope-tagged BHMT were isolated. Transfection of the vector alone served as a null control (CMV). Four representative cell lines (C, K, J and G) were selected from 20 isolates as expressing increasing levels of epitope-tagged BHMT, as determined by Western blotting of equal amounts of whole-cell extracts (Figure 3a). Quantification of exogenous BHMT expression, as determined by the HA tag, indicated that cell line G expressed 35 times more HA-BHMT than cell line C.

Total cellular RNA was isolated from the four BHMT cell lines and McA-CMV cells and apo B-mRNA abundance determined by Northern-blot analysis (Figure 3b). Steady-state apo B-mRNA expression paralleled increases in HA-BHMT from null cells (McA-CMV) to the highest-BHMT-expressing cell line with proportions of 1:2.6:2.9:7.2:11.7, respectively. The in-

Figure 4 The effect of BHMT expression is specific for apo B mRNA

Total RNA (25 μ g) from the null vector control (McA) and McA-BHMT cell line G was resolved through a formaldehyde/agarose gel. The gel was stained with ethidium bromide, digitally photographed and the RNAs transferred on to a nylon filter by capillary blotting. Ten separate identical filters were probed with the indicated cDNAs and then, after stripping, were reprobed with cyclophilin cDNA. Fold increases in specific mRNAs between McA and McA-BHMT cell line G were determined after each signal was normalized to cyclophilin mRNA. MTP, microsomal triglyceride-transfer protein.

crease in apo B-mRNA abundance, as normalized to GAPDH mRNA, was observed consistently in multiple RNA preparations and from McA-BHMT cell lines K and G maintained in culture for different periods of time. In three independent experiments, RNA was isolated from McA-CMV and McA-BHMT cell lines K and G grown in parallel cultures. Apo B-mRNA levels were analysed in triplicate and quantified. After stripping, the blots were rehybridized with an 18 S RNA probe. Apo B-mRNA abundance was significantly increased 3.1 ± 0.63 ($\pm$ S.E.M.) times for the K cell line and 3.9 ± 0.53 times for the G cell line, each compared with the McA-CMV control ($P < 0.025$, paired *t* test).

The effect of BHMT is apo B-mRNA-specific

To determine whether the effect of BHMT expression was specific for apo B mRNA, Northern-blot analysis was performed on total RNA from McA-CMV cells and McA-BHMT cell line G, which expressed the highest level of BHMT mRNA (Figure 4). Levels of several different mRNAs, including those that encode other apolipoproteins, as well as liver-specific and ubiquitously expressed transcripts, were analysed. Cell line G expressed a 14-fold increase in BHMT mRNA compared with the McA-CMV cells and demonstrated a 4-fold increase in apo B mRNA. The mRNA abundance of no other transcript, including three directly involved in lipoprotein metabolism (apo AI, apo E and microsomal triglyceride-transfer protein), was affected by exogenous BHMT expression. Quantification of rRNA demonstrated equivalent loading of undegraded RNA in each lane. As there is no single RNA species with an invariant expression level in all situations, the mRNAs of two housekeeping genes, cyclophilin

and β -actin, were used to normalize levels of mRNA expression and were equivalent between McA-CMV and McA-BHMT cell line G (results not shown). These data suggest that the effect of BHMT expression in McA cells has specificity for apo B mRNA.

Expression of BHMT increases apo B secretion by McA cells

As BHMT expression induced an increase in steady-state levels of apo B mRNA, we compared the relative rates of apo B secretion by McA-CMV and McA-BHMT cell line G. The 0–20-h apo B secretory rate was increased 1.5 times ($P < 0.05$) and the 20–24-h secretory rate was increased 1.6 times ($P < 0.05$) compared with that observed in McA-CMV cells in parallel cultures. This increase in apo B secretion was consistent with the increase in apo B-mRNA abundance.

Rat liver expresses high levels of BHMT mRNA

We compared BHMT expression in McA-CMV and McA-BHMT cell line G with that of rat liver and hepatocytes to determine BHMT expression relative to control cells. The signal for β -actin mRNA showed a 10-fold difference between liver and freshly isolated hepatocytes compared with that observed in the McA cell lines. Liver-specific versus cytoskeletal gene expression can exhibit an inverse relationship during establishment of cultured cell lines from primary rat hepatocytes and can also vary significantly depending upon the extracellular matrix upon which the hepatocytes are cultured [21]. Using rRNA levels to normalize BHMT expression, results indicate that the highest level of BHMT mRNA expression in cell line G was comparable with that observed in rat liver and hepatocytes. These data indicate that the increased apo B-mRNA abundance observed in BHMT-transfected McA cells was not related to over-expression of BHMT.

To confirm that the cDNA encoded functional BHMT, enzyme activity was assayed in transfected McA cells. BHMT activities of cell lysates derived from McA wild-type cells, McA-CMV cells and McA-BHMT cell lines K and G were 0.16, 0.07, 1.56 and 2.17 units/mg of protein, respectively. BHMT activity of rat hepatocyte lysates was 4.38 units/mg of protein. These results indicate that the wild-type McA cells expressed very little BHMT, whereas cell line G expressed about half of that observed in rat hepatocytes. This suggests that the observed increases in apo B-mRNA abundance were not related to over-expression of BHMT relative to physiological levels present in rat liver.

Intestinal expression of a BHMT-related protein

As liver and intestine are the major sites of apo B-mRNA expression, we determined whether BHMT mRNA was also expressed in intestinal cells. Figure 5(a) indicates that BHMT mRNA was undetectable by Northern-blot analysis of intestinal RNA. However, when rat liver and intestinal cell extracts were analysed for BHMT expression by Western blot using the original anti-p45 monoclonal antibody, two specific bands were observed with molecular masses of 45 and 40 kDa in liver and intestine, respectively (Figure 5b). Additional Northern-blot analysis was performed using a probe complementary to the 5' end of BHMT (nt 70–866), but no signal was observed for intestine. As the two probes covered the entire coding region of the BHMT mRNA, the lack of a BHMT-specific signal in intestine suggests that the intestine's shared epitope with BHMT was not due to an alternatively spliced mRNA variant of BHMT. Results suggest that a BHMT-related protein may be present in intestine.

Figure 5 Rat liver expresses high levels of BHMT mRNA

(a) Total RNA (25 μ g) from each of the indicated sources was resolved through a formaldehyde/agarose gel. The gel was stained with ethidium bromide, digitally photographed and the RNAs transferred on to a nylon filter by capillary blotting. The filter was probed sequentially with BHMT and β -actin specific cDNAs. (b) Protein (60 μ g) from S100 extracts of rat liver and intestine were resolved by SDS/PAGE and transferred on to nitrocellulose. Membranes were reacted with anti-p45 monoclonal antibody and reactivity was detected with anti-mouse 125 I-IgG and visualized by autoradiography. Relative protein masses are indicated (kDa).

Expression of BHMT does not affect apo B-mRNA editing

Apo B-mRNA editing is a significant process in the cellular metabolism of apo B mRNA and protein. Cytidine at nucleotide 6666 (Cyt-6666) is deaminated to uridine, thereby altering a glutamine codon to a stop codon, resulting in the translation of a protein that is only 48% of the full-length apo B. This process is mediated by the editosome, a multiprotein complex [22]. Since a fraction containing this complex was used as the antigen preparation for the generation of the monoclonal antibodies, we determined whether our cell lines expressing BHMT exhibited altered apo B-mRNA-editing efficiencies. Using RT-PCR, a 544-bp transiently transfected human apo B amplicon that contained Cyt-6666 was amplified from each of the McA cell lines [19]. Editing efficiencies at Cyt-6666 were determined by poisoned primer-extension assay and averaged 12.0 and 14.0% in wild-type McA and McA-CMV cells, respectively. In the C, K, J and G cell lines expressing BHMT, editing efficiencies averaged 11.3, 12.3, 16.6 and 15.1%, respectively, indicating that expression of BHMT in McA cells did not affect apo B-mRNA editing.

DISCUSSION

A two-stage screening strategy is currently being used to examine the role of auxiliary factors in regulation of apo B-mRNA editing and metabolism. In the first stage, monoclonal antibodies to apo B-RNA-binding activities are prepared and used to screen cDNA-expression libraries for proteins that affect apo B mRNA or protein metabolism. In the second stage, corresponding

cDNAs are stably transfected into McA cells followed by evaluation of apo B mRNA, apo B-mRNA-editing efficiency and apo B secretion. A protein with a molecular mass of 45 kDa was identified that increased apo B-mRNA abundance and apo B secretion in McA cells but did not influence apo B-mRNA-editing efficiency. Nucleotide and amino acid BLAST searches of GenBank revealed that the corresponding cDNA had high similarity with human BHMT [20]. Correlation of the tissue-specific expression of the new cDNA with the tissue-specific enzyme-activity profile [7] and the increased BHMT enzyme activity of transfected McA cells indicates that this cDNA encodes rat BHMT. BHMT is the enzyme responsible for conversion of betaine and Hcy to dimethylglycine and methionine, respectively [6]. Thus we have identified a novel relationship between apo B-mRNA and BHMT expression. We have also identified an intestinal protein that shares an epitope with hepatic BHMT with a molecular mass of 40 kDa. Intestinal and hepatic cells are the major apo B-producing cells in mammals, but BHMT activity is restricted to liver and kidney [7] and is not detected in small intestine [23]. It is possible that the intestinal 40-kDa protein may exhibit an activity related to apo B-mRNA and protein without the associated BHMT methyltransferase activity. Formal proof of this possibility awaits the cloning of the corresponding cDNA.

The large increase in apo B-mRNA abundance in BHMT-transfected McA cells is novel and unexpected but is significant, given the positive correlation of apo B-mRNA abundance with increasing expression of BHMT. Moreover, the physiological significance of this observation is strengthened by finding comparable levels of BHMT mRNA in the highest-expressing cell line and in rat hepatocytes. The magnitude of the increased apo B mRNA is greater than the modest changes in apo B-mRNA levels and secretion rates observed with hormonal and metabolic perturbation of apo B-producing cells (reviewed in [24]). For example, ethanol increases intracellular apo B and apo B secretion by HepG2 cells, but with only a doubling of apo B mRNA [25]; 25-hydroxycholesterol increases apo B mRNA by 55% in HepG2 cells [26] and transforming growth factor β -1 only doubles apo B mRNA in Caco-2 cells [27]. There are three instances *in vivo* where large increases in apo B mRNA are observed, such as reported here. These include a 6-fold increase in livers derived from humans with abetalipoproteinaemia [28], a 7-fold increase in rat intestine during development [29] and a 6-fold increase in livers of oestradiol-treated roosters [30]. The mechanism by which BHMT leads to increases in apo B-mRNA abundance and apo B secretion remains to be elucidated.

Rat hepatic BHMT activity is regulated by dietary factors and is increased by high-protein [31] and methionine-deficient diets [32]. Combinations of dietary treatments can increase hepatic BHMT mRNA levels 9-fold [33,34]. Since ethanol increases BHMT activity [35] as well as apo B mRNA and apo B secretion [25], there is a physiological precedent for our observations. Interestingly, dietary betaine, which increases BHMT activity [36], protects against ethanol-induced fatty liver [37], suggesting a potential role for BHMT in lipid mobilization and secretion.

The correspondence of BHMT expression and apo B-mRNA abundance suggests a potential role for BHMT activity in increasing apo B-mRNA stability or *apo B* gene transcription. One possibility is that BHMT binds and stabilizes apo B mRNA. Apo B mRNA has been demonstrated to bind a number of proteins, including those involved in site-specific mRNA editing [10,38–40] and others that may be involved in mRNA stability [3]. BHMT expressed in and purified from *Escherichia coli*, however, does not bind directly to the 449 nt spanning the apo B-mRNA-mooring sequence (results not shown). We cannot rule

out the possibility that BHMT binds to other sites on apo B mRNA or interacts with apo B-mRNA-binding proteins. Ongoing studies are focused on determining the mechanism and include analysis of the effect of BHMT expression on apo B-mRNA stability and *apo B* gene transcription.

Our results demonstrate a relationship of BHMT with apo B that leads to increased apo B-mRNA levels and apo B secretion rates in hepatically derived cells. BHMT, methionine synthase and cystathionine synthase (EC 4.2.1.22) regulate the branch-point metabolism and intracellular levels of Hcy, a known risk factor for atherosclerosis [41]. Considering that betaine decreases plasma Hcy levels, presumably by enhancing hepatic BHMT activity [33,36], understanding the regulation of the *bhmt* gene and its relationship with apo B metabolism becomes important. Our findings suggest that there is a novel relationship between a factor involved in Hcy metabolism and apo B, both of which are associated with macrovascular disease in humans.

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