We previously showed that rat liver betaine-homocysteine methyltransferase (BHMT) mRNA content and activity increased 4-fold when rats were fed a methionine-deficient diet containing adequate choline, compared with rats fed the same diet with control levels of methionine (Park, E. I., Renduchintala, M. S., and Garrow, T. A. (1997) J. Nutr. Biochem. 8, 541–545). A further 2-fold increase was observed in rats fed the methionine-deficient diet with supplemental betaine. The nutrition studies reported here were designed to determine whether other methyl donors would induce rat liver BHMT gene expression when added to a methionine-deficient diet and to define the relationship between the degree of methionine restriction and level of methyl donor intake on BHMT expression. Therefore, rats were fed amino acid-defined diets varying in methionine and methyl donor composition. The effect of diet on BHMT expression was evaluated using Northern, Western, and enzyme activity analyses. Similar to when betaine was added to a methionine-deficient diet, choline or sulfonium analogs of betaine induced BHMT expression. The diet-induced induction of hepatic BHMT activity was mediated by increases in the steady-state level of its mRNA and immunodetectable protein. Using methyl donor-free diets, we found that methionine restriction was required but alone not sufficient for the high induction of BHMT expression. Concomitant with methionine restriction, dietary methyl groups were required for high levels of BHMT induction, and a dose-dependent relationship was observed between methyl donor intake and BHMT induction. Furthermore, the severity of methionine restriction influenced the magnitude of BHMT induction.

To study the molecular mechanisms that regulate the expression of BHMT, we have cloned the human BHMT gene. This gene spans about 20 kilobases of DNA and contains 8 exons and 7 introns. Using RNA isolated from human liver and hepatoma cells, a major transcriptional start site has been mapped using the 5′ rapid amplification of cDNA ends technique, and this start site is 26 nucleotides downstream from a putative TATA box.

Betaine-homocysteines S-methyltransferase (BHMT) (EC 2.1.1.5) catalyzes a methyl transfer from betaine to homocysteine (Hcy), forming dimethylglycine and Met, respectively. Betaine is an intermediate of choline oxidation, and the enzymes of this pathway are primarily found in the liver and kidney of mammals (1), although we have recently shown that BHMT is also expressed in the lenses of rhesus monkeys and humans (2). This enzyme has recently been shown to be zinc-dependent (3), and based on protein purification reports, it has been estimated that BHMT represents 0.5–2% of the total soluble protein in mammalian liver (4). Although these studies presumably used livers from animals that were consuming diets containing adequate Met, it is possible that BHMT can represent an even greater proportion of liver protein, because as reported here, when choline-containing diets are deficient in Met, there occur dramatic increases in hepatic BHMT protein content. However, regardless of dietary conditions, it is clear that BHMT is a major zinc metalloenzyme in liver.

Interest in the nutrient and genetic factors that influence Hcy metabolism has increased because elevated concentrations of this amino acid in blood have been correlated to the incidence of arteriosclerotic vascular disease and thrombosis. BHMT is one of two known mammalian enzymes that methylate Hcy, the other being the folate/vitamin B12-dependent Met synthase (EC 2.1.1.13). Of these two methyltransferases, it is known that genetic defects that reduce the flux through the Met synthase catalyzed reaction, or a deficient intake of either of its coenzyme vitamin precursors, result in elevated concentrations of blood Hcy (5, 6). Whether BHMT is required for normal Hcy metabolism is not known, because choline, the metabolic precursor of betaine, is not an essential nutrient unless the level of Met in the diet is severely deficient. Under normal nutritional status, the choline moiety can be synthesized by the S-adenosylmethionine-dependent conversion of phosphatidylethanolamine to phosphatidylcholine. A naturally occurring combined deficiency of Met and choline has not been described in humans and is not likely to occur because choline, primarily as phosphatidylcholine, is abundant in human diets. Furthermore, to date, there have been no inherited defects in the BHMT-catalyzed reaction described, and therefore, it has not been possible to ascertain whether such defects perturb Hcy metabolism in humans.

Although the significance of BHMT in Hcy homeostasis is not clear, it is known that the relative contribution of BHMT to Hcy remethylation can be influenced by diet. For example, it has...
been shown that the rate of Hcy remethylation is increased when choline or betaine is added to the diet of humans (7–9), and treating non-vitamin-responsive forms of homocystinuria with supplemental choline or betaine elicits a plasma Hcy-lowering response (5, 6, 10). As previously indicated by Mudd (11), these studies suggest that betaine concentrations in human liver are below that required to saturate BHMT, and present knowledge of hepatic betaine concentrations and the Michaelis constants of betaine for the human enzyme support this idea (3). In vitro studies designed to simulate Hcy metabolism in rat liver suggest that BHMT and Met synthase contribute equally to the conversion of Hcy to Met in that organ (12). Furthermore, we have shown that when a diet is otherwise nutritionally adequate, Met restriction dramatically elevates rat liver BHMT gene expression (13). This dietary induction of BHMT gene expression presumably enhances the methylation of the available Hcy in vivo, thus conserving the nutritionally essential carbon backbone of Hcy under conditions of Met deficiency. All of these studies suggest that BHMT has a quantitatively significant role in the hepatic conversion of Hcy to Met, although its significance in whole-body Hcy remethylation remains speculative.

The purpose of the nutrition studies reported here is to further clarify the interaction between dietary Met restriction and methyl donor intake on rat liver BHMT gene expression. We report here that dramatic changes in BHMT expression can be elicited when rats consume diets deficient in Met yet rich in methyl donor, i.e. choline, betaine, or sulfolium analogs of betaine. As an initial step to elucidate the molecular mechanisms that mediate any of the nutritional, hormonal, and tissue-specific expression of BHMT, and to ultimately characterize human genetic variants at the BHMT locus, we have characterized the organization of the human BHMT gene. Here, we report the intron-exon splice junctions of the gene, report the major transcriptional start site, and provide 3.2 kilobases of DNA sequence 5' to this transcriptional start site.

EXPERIMENTAL PROCEDURES

Materials—Human liver samples and human hepatoma cells ( Hep G2) were obtained from the Anatomic Gift Foundation (Laurel, MO) and the American Type Culture Collection (Manassas, VA), respectively. [α-32P]dCTP (3000 Ci/mmol) and nitrocellulose filters were obtained from Amersham Pharmacia Biotech. DNA restriction and modifying enzymes were purchased from Promega (Madison, WI), Boehringer Mannheim, and New England Biolabs (Beverly, MA). Oligonucleotides were synthesized at the University of Illinois' Biotechnology Center (Urbana, IL). Phage 1 artificial chromosome (PAC) clones were obtained from the Medical Resource Council of Canada Genome Resource Facility at the Hospital for Sick Children (Toronto, Ontario, Canada). Betaine hydrochloride was purchased from Sigma. Choline bitartrate, L-Met, and other chemicals used for the L-amino acid-defined diets were purchased from Dyets (Bethlehem, PA). Dimethylacetothetin (DMAT) (DMAT) was a gift from NutriQuest (Chesterfield, MO), and dimethylpropiothetin (DMPT) was purchased from TCI America (Portland, OR). PCR products were cloned into pCR1 (Invitrogen, San Diego, CA) or pGEM-T (Promega) plasmids. All other reagents were of the highest purity available from commercial vendors.

Diets and Animal Protocol—Three animal studies were performed to investigate the interaction between Met restriction and methyl donor intake on hepatic BHMT gene expression. These studies were approved by the University of Illinois' Laboratory Animal Care Advisory Committee.

The components of the amino acid-defined diets used in these studies have been previously described in detail (13) and are based on the American Society for Nutritional Sciences' nutrient recommendations for growing rats (14). The dietary treatment in Met (1.0, 1.5, 2.0, and 3.0 g/kg of diet) and methyl donor content. The methyl donors employed were choline, betaine, DMAT, and DMPT, and these compounds were present at levels ranging from 0 to 37.5 mmol per kg of diet, as indicated in Table I. DMAT and DMPT are sulfonium analogs of betaine; the latter is a plant metabolite found at high concentrations in marine algae (15) and is present in some terrestrial plants as well (16). These compounds were used only in study 1 and were added to the diet at levels that were isomethyl to the level of betaine used in the same study. All diets contained 3 g of cystine and 10 g of sucinylsulfathiazole per kg of diet. Sucinylsulfathiazole is an antibiotic that was added to inhibit the microbial metabolism of methyl donors in the gastrointestinal tract, but because of its use, all diets were supplemented with mendonied sodium bisulfite (50 mg/kg of diet).

The feeding trials were conducted using 3-week-old Sprague-Dawley rats (Harlan, Indianapolis, IN), which were housed as described previously (13). All rats were initially fed a control amino acid-defined diet for 3 days. The control diet contained adequate Met (3 g/kg) and choline (2.5 g/kg) and was devoid of other methyl donors. For the initial feeding period, rats were divided into 10 groups, each consisting of 3 replicate cages, and during the adaptation period, rats were randomly divided into experimental groups such that mean body weights among groups were not significantly different. When rats are fed diets severely restricted in any essential amino acid, they voluntarily decrease their food intake (grams/d) by 40–50% (17). Therefore, where indicated in Table I, food intake among some treatment groups was restricted to the average food intake of groups fed diets severely deficient in Met (1 g/kg of diet), which did not significantly differ from each other. Each group was given free access to water throughout the feeding trial, which varied from 10 to 16 days as indicated in Table I. Rats were killed and their livers stored at -80 °C until analyzed for BHMT activity, mRNA, and protein content.

Assay Procedures—Northern analysis was performed as described previously (13) except that the oligonucleotide probes for BHMT were made using a rat cDNA as template, rather than a porcine cDNA. mRNA content was quantified by phosphorimaging, and BHMT gene expression was normalized against rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (EC 1.2.1.12) expression.

Hepatic BHMT protein was visualized by Western analysis. Fifteen micrograms of crude liver extract were subjected to SDS polyacrylamide gel electrophoresis using a 5% stacking gel and a 12% separation gel using a Tris-glycine discontinuous buffer system. Duplicate gels were run, and protein from one gel was blotted onto nitrocellulose using a Tris-glycine-methanol transfer buffer and a semidyblotting apparatus. The second gel was Coomassie-stained for protein and densitometrically analyzed to confirm equal protein loading. Blotted protein was probed for BHMT using rabbit polyclonal antibodies prepared against highly purified recombinant human BHMT (3). The antigen and primary antibody interaction was detected using a peroxidase-coupled antirabbit IgG (Vector Laboratories, Burlingame, CA) and tetramethylbenzidine as the peroxidase substrate.

BHMT activity in crude liver extracts was measured as described previously (4). Protein in liver extracts was measured by a Coomassie dye binding assay (Bio-Rad) using bovine serum albumin as standard. A unit of BHMT activity is defined as 1 nmol of Met formed per hour, but activities are expressed here relative to the mean BHMT activity of the appropriate control group for each study. Treatments 1, 7, and 14 were the control groups for Studies 1, 2, and 3, respectively, and the mean BHMT activities of these groups were given the relative value of 1.

Statistics—The data obtained from each nutrition study were analyzed by one-way analysis of variance. When analysis gave a significant F value (p < 0.05), treatment differences were evaluated using Fisher's least significant difference procedure.

Isolation of Genomic Clones Encoding the BHMT Gene—A human lung fibroblast cell line W138 genomic library in the Lambda FIX II vector (Stratagene, La Jolla, CA) was screened (106 plaques) with [32P]dCTP-labeled probes generated using the Redprime labeling system (Amersham Pharmacia Biotech) and a human BHMT cDNA as the template. The human cDNA was originally isolated in plBlauscript II SK (Stratagene), and this construct was named pTO9 (4). Four positive clones (A6, A8, A10, and A13) were identified following the tertiary screen. Phage from each plaque were amplified by the plate lysate method, and their respective DNAs were purified and characterized by restriction mapping and Southern hybridization. Radiolabeled probes for Southern analysis were made using a Psil 1531-bp fragment of the 5’ region and a 925-bp Psil-Xhol 3’ region fragment of the human cDNA as templates. The entire genomic DNAs, bordered by New sites, were cloned into plBlauscript II KS Phagemid to generate pE6P, pE7P, pE10P, and pE13P from the corresponding HindIII片段s. The four plasmids were XhoI-digested, and their respective DNAs were purified and cloned into pBlueScript II KS Phagemid. These clones were designated pE10P-1.5, pE10P-4.5, and pE10P-9.5, respectively. The 9.5-kb ScaI fragment of pE10P was further digested into smaller fragments and cloned in pBlueScript II KS Phagemid. The resulting clones, p10.9-5SxBiz-A, p10.9-5SxBiz-A, p10.9-5SxBiz-A, p10.9-5SxBiz-A, and p10.9-5SxBiz-A, were sequenced.
and p10-9.5 pSlal-B, as well as the other clones derived from pEP10, and 3.2 kilobases of DNA in the center region of pEP8 (promoter and distal upstream region), were analyzed by DNA sequencing. The sequencing for pEP8 began with primers designed from the most 5' region of pEP8.

PAC clones (G2, D8, A9, D11, M13, G19, and B23) were isolated using probes synthesized from the entire human cDNA as a template. The PAC DNA from these clones was purified and characterized by restriction mapping and Southern hybridization. Radiolabeled probes for Southern analysis were made using an EcoRI 835-bp fragment of the 5' region and a 1615-bp EcoK-Hind III 3' region fragment of the human cDNA. A 4.5-kb SacI fragment of PAC clone D11 was purified and cloned into plBluescript II KS phagemid. This clone was designated pEP-D11-4.5. A region of genomic DNA 3' to pEP-D11-4.5 was isolated by PCR using the following primers: 5'-GGCATAGTGCCACTGCTGTAATC-3', 5'-GCAGCTAAGGTATGAAGGTGT-3', and the 3' end long template PCR system (Boehringer Mannheim). The primers correspond to the 3' region of pEP-D11-4.5 and a region of DNA very near the 5' terminus of the human cDNA, respectively. The approximated 2.3-kb PCR product was cloned into a plasmid vector producing plasmid pEP-D11-2.3. Clones pEP-D11-4.5 and pEP-D11-2.3 were analyzed by DNA sequencing.

**DNA Sequencing and Intron Size Determination**—DNA was sequenced using an Applied Biosystems 373A automated DNA sequencer at the University of Illinois' Biotechnology Center (Urbana, IL). Exon-intron junctions in the pEP clones were determined by direct sequencing across the junctions using oligonucleotide primers designed from the cDNA sequence. Intron sizes were determined by sequencing through the regions, PCR amplification using oligonucleotide primers based on exon sequence, or by the combination of DNA sequence data in conjunction with estimates of DNA fragment sizes by their mobilities in agarose gels. The exon-intron junction sequences and 3.2 kilobases of DNA 5' to the translational start site (from pEP8) were determined by sequencing both DNA strands. The 5' region of the gene was analyzed using the Transcript Element Search Software with the Transfac version 3.3 data base (http://agave.humgen.upenn.edu/ess/index.html).

**5'-RACE Analysis of BHMT cDNA Ends**—Total RNA was isolated from human liver and Hep G2 cells using the Ultra-SpecII RNA isolation system (Biotex, Houston, TX). cDNA corresponding to the 5'-end of human liver mRNA was synthesized and amplified with the 5' RACE system (Life Technologies, Inc.) using the manufacturer's instructions. The primers used were 5'-GGCATAGTGCCACTGCTGTAATC-3' (gene-specific primer 1), 5'-GCAGCTAAGGTATGAAGGTGT-3' (gene-specific primer 2), 5'-TCCTCTCCATCGGCAAGACAAAC-3' (nested gene-specific primer) and the universal primers provided with the kit. The products were cloned into plasmid vectors and analyzed by DNA sequencing.

**RESULTS**

The Influence of Diet on Rat Growth and BHMT Expression

The relative degree of Met deficiency was monitored by growth. Weight gain and feed efficiency (weight gain/feed intake) are shown in Table I. Rats given free access to the diets severely deficient in Met (1 g/kg) consumed significantly less food than those given free access to the diets containing adequate Met (3 g/kg). As expected, in Studies 1 and 2, the feed efficiencies of rats that were restricted-fed the control diets (treatments 2 and 8) were between those of rats given free access to the control diets (treatments 1 and 7) and those fed the Met-deficient diets (treatments 3 and 12). Overall, rats fed diets containing 1.0 or 1.5 g of Met/kg of diet (treatments 4–6, 11–13, and 14–17) had lower feed efficiencies than rats fed the diets containing 2.0 or 3.0 g of Met/kg of diet (treatments 2 and 8–10). These data indicate that the Met content of the former diets cannot support maximum growth and so in this regard are deficient in this amino acid. However, all rats gained weight, indicating that 1.0 g of Met/kg of diet is slightly above the minimum requirement of the weanling rat. The feed efficiency of rats fed the diet containing 2.0 g of Met/kg of diet (treatment 10) was approaching the feed efficiency of rats fed the control diet containing 3.0 g of Met/kg of diet (treatment 9). This indicates that 2.0 g of Met/kg of diet is approaching the minimum requirement for the weanling rat. These observations are consistent with Funk et al. (18), who showed that 2.5–3.0 g of Met/kg of diet was the minimum level of dietary Met that could support the maximum growth rate of weaning rats consuming an amino acid-defined diet and that rats consuming 2.0 g of Met/kg of diet were approaching that maximal growth rate. Furthermore, they observed linear growth responses between 1.0 and 2.0 g of Met/kg of diet.

The addition of various methyl donors to diets severely deficient in Met (1 g/kg) produced mixed results. In Study 1, the addition of 25 mmol of betaine/kg of diet slightly reduced feed efficiency, whereas the addition of an isomethyl level of DMAT clearly inhibited growth (treatments 4 and 5 versus treatment 3). The addition of DMPT resulted in a slight increase in feed efficiency (treatment 6 versus treatment 3). In Studies 2 and 3, the addition of choline to diets severely deficient in Met (1 g/kg) tended to decrease feed efficiency. Most of the data support the idea proposed by Storch et al. (9), who suggested that excess methyl donor consumption may increase Met requirements due to an enhanced oxidation rate of this amino acid.

Study 1 was performed to determine whether sulfonium analogs of betaine can induce hepatic BHMT expression when fed concomitant with a diet deficient in Met, as had been

| Table I | Dietary treatment | Weight gain | Feed efficiency |
|---------|------------------|-------------|-----------------|
|         | 5 g/kg Met       | 96±*        | 467±*           |
| Study 1 | 3.0 g/kg Met     | 96±*        | 467±*           |
|         | 3.0 g/kg Met     | 40±*        | 356±*           |
|         | 1.0 g/kg Met     | 17±*        | 158±*           |
|         | 1.0 g/kg Met + 25 mmol/kg betaine | 14±       | 134±*           |
|         | 1.0 g/kg Met + 37.5 mmol/kg DMAT | 7±        | 76±*            |
|         | 1.0 g/kg Met + 37.5 mmol/kg DMPT | 19±        | 189±*           |

*Study 1: Data are means of five rats fed the experimental diets for 16 days; average initial body weight was 55 g. All rats were given free access to the experimental diets, except treatment 2 rats, which were restricted-fed their diet to the level of food intake of treatment 3 rats. Study 2: Data are means of five rats fed the experimental diets for 10 days; average initial body weight was 51 g. Treatment 7 and 11–13 rats were given free access to the experimental diets, whereas treatment 8–10 rats were restricted-fed their diets to the average level of food intake of treatment 11–13 rats, which were similar. Study 3: Data are means of six rats fed the experimental diets for 14 days; average initial body weight was 49 g. Treatment 14–16 rats were given free access to the experimental diets, whereas treatment rats were restricted-fed their diet to the average level of food intake of treatment 14–16 rats, which were similar.

f, Within each study, means with unlike superscript letters are significantly different (p < 0.05).
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FIG. 1. A, diet-induced changes of hepatic BHMT activity and mRNA content (Study 1). Rats were fed the following experimental diets for 16 days: 1, Met-adequate (0.3%) diet; 2, Met-adequate diet; 3, severely Met-deficient (0.1%) diet; 4, severely Met-deficient diet containing 25 mmol of betaine/kg of diet; 5, severely Met-deficient diet containing 37.5 mmol of DMAT/kg of diet; and 6, severely Met-deficient diet containing 37.5 mmol of DMPT/kg of diet. All diets contained 2.5 mmol of choline/kg of diet. Liver RNA was isolated and probed for BHMT and GAPDH mRNA levels by Northern analysis and quantified by phosphorimaging. BHMT activity and the BHMT-GAPDH ratio of the control diet (treatment 1) were assigned a value of 1, and other treatment groups are expressed relative to this group. Values are means ± S.E. Means with unlike superscript letters are significantly different (p < 0.05). B, a representative Northern blot of the diet-induced changes in hepatic BHMT mRNA content (Study 1). One liver RNA sample was chosen at random from each dietary treatment group of Study 1 and probed for BHMT and GAPDH mRNA levels by Northern analysis as described under “Experimental Procedures.” BHMT and GAPDH mRNA were visualized by autoradiography, and the autoradiographic image was captured using the Foto/Analyst II Visionary system and Collage software (Fotodyne, New Berlin, WI).

previously observed for betaine consumption (13). Enzyme activity levels and mRNA and protein contents were measured. The data for BHMT activity and mRNA content can be seen in Fig. 1A. Based on our previous reports (13, 19), we expected that rats consuming the diet containing 1 g of Met/kg of diet and supplemental betaine (treatment 4) would have significantly higher BHMT mRNA content and enzyme activity compared with rats fed the Met-adequate (3 g/kg of diet) control diet (treatment 1). As shown in Fig. 1A, about 8–9-fold changes were observed. Furthermore, feeding the sulfonium analogs of betaine, DMAT (treatment 5), and DMPT (treatment 6), concomitant with Met deficiency, also induced BHMT gene expression to a similar extent. In general, the relative level of activity mirrored mRNA content. Fig. 1B shows an autoradiograph of a representative Northern blot in which rat liver total RNA was probed for BHMT and GAPDH mRNA transcripts. The autoradiograph shows that the relative changes in BHMT gene expression were dramatic and easily quantified by phosphorimaging, as depicted in Fig. 1A. Fig. 2 shows a Western blot of total liver protein probed for BHMT. Three liver samples were chosen at random from rats fed either the control Met diet (treatment 1) or the Met-deficient diet containing supplemental betaine (treatment 4). The blot shows that the relative levels of BHMT protein were markedly affected by these dietary treatments, and this result qualitatively mirrored mRNA content and activity levels.

Study 1 also was designed to determine whether the large changes in BHMT expression were due to the significant reduction of food intake that accompanies the dietary restriction of Met. As shown in Fig. 1A, although the rats restricted-fed the control diet (treatment 2) had levels of BHMT expression about 50% higher than those of rats given free access to the same diet (treatment 1), this change in expression is small compared with those changes observed when rats were fed diets containing 1 g of Met/kg of diet and supplemental levels of methyl donor (treatments 4–6), in which 6–9-fold increases were observed.

In Study 1, the rats fed the diet solely deficient in Met (treatment 3) had BHMT activities that were approximately 2-fold higher than the rats fed the diet containing adequate Met (treatment 1); however, changes in mRNA content were not detected. In our previous study (13), we reported a 4-fold change in BHMT mRNA content and activity when rats were fed a diet solely deficient in Met. However, in that study, the level of dietary choline was twice that used in this follow-up study, i.e. 5 versus 2.5 mmol of choline/kg of diet. Whether coincident or not, these observations lead us to hypothesize that Met restriction per se does not induce BHMT expression, but that some level dietary methyl donor is also required, concomitant with Met restriction, before an induction of BHMT expression will be observed. Furthermore, as we previously suggested (19), there may be a methyl donor dose-dependent relationship to its induction level. Study 2 was designed to test these hypotheses.

Study 2 dietary treatments used choline as the sole methyl donor. The results of Study 2 (Fig. 3A) showed that when dietary Met was adequate (treatment 8), a moderate level of choline intake (5 mmol/kg) had no effect on BHMT expression compared with rats consuming the same diet devoid of choline (treatment 9). Furthermore, when rats were fed diets devoid of choline, Met intake ranging from adequate (3 g/kg) to severely deficient (1 g/kg) had no effect on BHMT expression (treatments 9–11). However, when choline was added to diets severely deficient in Met, a significant induction of BHMT expression was observed, and the effect appeared to be dose-dependent (treatments 11–13). In this study, the changes observed in BHMT activity were also mediated by changes in mRNA content and immunodetectable protein (not shown), as observed with betaine and the sulfonium analogs of betaine in Study 1.

As in Study 1, in Study 2, the feed-restricted rats consuming the control diet (treatment 8) had about 70% higher levels of BHMT activity compared with rats given free access to the same diet (treatment 7). Although feed restriction caused a
The maximum relative BHMT induction levels observed in Study 1 (treatment 4) was approaching twice that observed in Study 3 (treatment 16), although the dietary treatments were similar. Treatment 4 (Study 1) was limiting in Met (1 g/kg) and contained excess betaine (25 mmol/kg), and treatment 16 (Study 3) was limiting in Met (1 g/kg) and contained excess choline (20 mmol/kg). Therefore, although high induction of BHMT expression was consistently observed when rats were fed diets severely deficient in Met containing supplemental methyl donor, we observed an unexplained variation in the magnitude of induction between studies.

Organization of the Human BHMT Gene and 5' Region—A Lambda Fix II human genomic library was screened as described under "Experimental Procedures." Four clones (A6, A8, λ10, and A13) were obtained, and all were found to have some degree of overlapping sequence based on restriction mapping and Southern analysis. The linear arrangement of two of these clones can be seen in Fig. 4. The entire genomic inserts of the clones, bordered by NotI sites, were inserted into pBluescript II KS phagemids to generate pEP6, pEP8, pEP10, and pEP13 clones. The genomic inserts varied in size from 16 to 19 kilobases. The restriction maps of pEP6, pEP8, and pEP13 indicated that these clones were nearly identical, whereas pEP10 contained additional flanking sequence. Therefore, pEP8 and pEP10 were chosen for further characterization. The pEP10 clone was fragmented further as described under "Experimental Procedures." The pEP8 and pEP10 clones, and fragments thereof, were analyzed by DNA sequencing. Both pEP8 and pEP10 contained the 5' coding region of the gene, yet were missing varying portions of the 3' region. The pEP8 clone was found to contain exons 1 and 2, but it encoded only part of intron 2. The pEP8 clone was estimated to have an additional 8 kb of DNA 5' to the start codon found in exon 1, as originally identified in the human cDNA (4). The pEP10 clone was found to encode exons 1–6 but only part of intron 2 (Fig. 4). In summary, pEP8 and pEP10, both of which were derived from Lambda Fix II genomic inserts, together encoded exons 1–6 and about 8 kb of DNA 5' to exon 1. The sequence encoded by exons 1–6 correspond to about two-thirds of the open reading frame, or about half of the sequence that makes up the entire human cDNA.

To obtain the remaining 3' region of the gene, PAC clones were isolated using probes made from the entire human cDNA. Seven clones (G2, D8, A9, D11, M13, G19, and B23) were isolated. Using SacI digestion patterns (not shown), PAC clones were divided into those that did (G2 and D11) and those that did not (D8, A9, M13, G19, and B23) contain the diagnostic 9.5-kb fragment characterized in pEP10 (Fig. 4). PAC clones D8 and D11 were chosen as representatives of each group and analyzed by Southern blot using probes synthesized from the EcoRI (835 bp) and EcoRI-XhoI (1615 bp) fragments of the human cDNA, which encoded a 5' and a 3' region of the cDNA, respectively. The 5' probe encoded exons 1–5 but only a portion of the 5' region of exon 6, whereas the 3' probe encoded the 3' region of exon 6 and the remainder of the cDNA. A 4.5-kb SacI fragment of D11 hybridized to both probes and was analyzed further by DNA sequencing. As hoped, sequence analysis of this clone overlapped pEP10-1.5 and encoded exon 7 and the 5' portion of intron 7. Exon 8, the last exon of the human gene, was PCR-amplified using a primer designed from the known 5' sequence of exon 7 and another primer designed from the end of the 3' untranslated region of the human cDNA. The resulting 2.3-kb PCR product was sequenced and found to encode the remaining 3' region of intron 7, and all of exon 8. Exon 8
encodes the last 59 amino acids of the open reading frame and the entire 3' untranslated region of the human cDNA. In summary, the PAC D11 clone was used to isolate a DNA fragment (pEP-D11-4.5) which overlapped the 3'-end of the l-derived pEP-10-1.5 clone and encoded exon 7 and part of the 5' region of intron 7. The PAC D11 clone was also used to PCR amplify the remaining 3'-end of intron 7 and exon 8, the latter being the last exon of the human BHMT gene.

A diagram of the human BHMT gene can be seen in Fig. 4. It consists of 8 exons and 7 introns, and all intron-exon splice junctions follow the GT-AG rule (Table II). Both strands of the 5'-flanking region of the gene and the intronic sequence flanking each exon have been sequenced, and these sequences have been deposited in the GenBank data base. In general, the exon sequence of the gene was in good agreement with our previously published cDNA sequence (4); however, two nucleotide differences have been detected. Using the numbering used for the original cDNA, nucleotides 23 and 742 are both A in the human cDNA but are both G in the genomic sequence reported here. The first difference is in the 5' untranslated region of the cDNA, and the second difference is found in exon 6. The A to G change in exon 6 corresponds to a Gln-to-Arg amino acid change. At this juncture, it is unknown whether these two differences represent polymorphisms or mutations.

5'-RACE analysis of RNA isolated from either human liver or human hepatoma cells (Hep G2) indicated only one major mRNA species in liver. A total of seven PCR amplification products were sequenced, and each ended 77 bases 5' to the first nucleotide of the start codon in the original cDNA. These 5'-RACE products had a purine at the terminal 5' position, and this extended the 5' untranslated region of our previously published cDNA sequence by 51 nucleotides. mRNAs generally begin with a purine, and therefore this A at position −77 relative to the start ATG is the major transcriptional start site used in liver.

The 5' genomic region of human BHMT is shown in Fig. 5. This sequence was analyzed for consensus transcription factor sequences, and many putative transcription factor binding sites were found. Centered 26 bases 5' to the major transcriptional start site is a putative TATA-binding protein site. The location of this TATA-binding protein site in relation to the 5' end of the mRNA is consistent with this being the primary promoter region in liver. Analysis of this entire region of DNA indicates the presence of four consensus TATA boxes, but only the one noted above was identified by the TSSG and TSSW human polymerase II promoter region and start-of-transcription analysis programs as a potential promoter. Just 5' to this TATA box are four putative Sp1 sites and one putative activator protein-2 site. Just 3' to this TATA box are two putative Sp1 sites. Regions 5' to this TATA box contain putative binding sites for HNF-1, HNF-3, and CAAT enhancer-binding protein, all of which have been identified as liver-specific or liver-enriched transcription factors. It is interesting to note that there are putative transcription factor binding sites for homeobox 4c, 4d, and 4e (22). These sites are centered 794 bases 5' to the transcriptional start site. Homeobox transcription factors are known to be important in early embryonic and fetal development, and the BHMT mRNA has been detected in fetal tissue (GenBank accession number W97296). Also shown in Fig. 5 are several consensus sites for steroid hormone receptors, including glucocorticoids, progesterone, estrogen, and androgen binding sites. Hydrocortisone has been reported to increase hepatic BHMT gene expression (21), whereas there have been no reports on the effects progesterone or androgens have on BHMT expression.
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**TABLE II**
Exon-intron, organization of the human BHMT gene

| N | Size | Junction | Exon-intron junctiona |
|---|------|----------|-----------------------|
|   | bp   | No.      | Size                  |
| 1 | 110  | 1        | −77–33                |
|   |      |          | GCC AAG AAG23         |
|   |      |          | Ala-Lys-Lys11         |
| 1 | 133  | 2        | 34–166                |
|   |      |          | CCA GAA GCAG166       |
|   |      |          | Pro-Glu-Ala-Val-166   |
| 1 | 119  | 3        | 167–285               |
|   |      |          | GAAG ATA TCT285       |
|   |      |          | Lys-Ile-Ser285        |
| 1 | 192  | 4        | 286–477               |
|   |      |          | GATT GCA GAG477       |
|   |      |          | Ile-Ala-Glu159        |
| 1 | 148  | 5        | 478–625               |
|   |      |          | GTG AAA GCA G625      |
|   |      |          | Val-Lys-Ala-Gly625    |
| 1 | 183  | 6        | 626–808               |
|   |      |          | TTC CCA TTT G808      |
| 1 | 229  | 7        | 809–1037              |
|   |      |          | TTAGA GCA AG1037      |
| 1 | 1352 | 8        | 1038–2389             |
|   |      |          | Arg-Ala-Arg146        |
|   |      |          | Junction              |
|   |      |          | GTGagcttc             |
| 1 | 121  | 1        | 1−3.8                 |
|   |      |          | gtaatttag             |
| 1 | 133  | 2        | 2−3.0                 |
|   |      |          | acetecag              |
| 1 | 119  | 3        | 3−0.92                |
|   |      |          | cctttag               |
| 1 | 192  | 4        | 4−0.677               |
|   |      |          | ctgatcag              |
| 1 | 148  | 5        | 5−4.5                 |
|   |      |          | cctacacag             |
| 1 | 183  | 6        | 6−1.526               |
|   |      |          | cttaacag              |
| 1 | 229  | 7        | 7−2.9                 |
|   |      |          | gttcacacag            |
| 1 | 1352 | 8        | 8−3.4                 |
|   |      |          | gttacacag             |

**DISCUSSION**

Choline may be a significant source of one carbon units in metabolism because it is abundant in human diets and its oxidation interfaces with Met and methylenetetrahydrofolate synthesis. Choline oxidation takes place primarily in the liver and kidney with enzymes found in the mitochondria and cytoplasm. The complete oxidation of choline results in four of its five carbons entering the one-carbon pool. In the cytosol, one carbon directly becomes the labile methyl carbon of Met via the BHMT-catalyzed reaction. In the mitochondrion, three other one-carbon units enter the folate pool at the oxidation state of formaldehyde by reactions catalyzed by dimethylglycine dehydrogenase, sarcosine dehydrogenase, and the glycine cleavage system, respectively. It has been established that folate metabolism in the mitochondrion functions in part to produce formate, which leaves this organelle to become activated into the folate one-carbon pool of the cytoplasm (23, 24). In the cytoplasm these one carbon units are used for purine and thymidylate biosynthesis and the folate- and vitamin B6-dependent methylation of Hcy. Because choline is abundant in the diet of humans, primarily as phosphatidylcholine, its oxidation may contribute substantially to the total one carbon needs of the liver and kidney.

The regulation of hepatic choline oxidation is not completely understood. Free choline resides at a metabolic branch point; it can be incorporated into phospholipids, converted to acetylcholine, or oxidized to glycine. Quantitatively, the use of choline for acetylcholine synthesis is negligible. The competition for choline to be incorporated into phospholipids or proceed through the oxidation pathway is between cytosolic choline kinase and transport into the mitochondrion; the latter is reportedly the rate-limiting step in the irreversible oxidation of choline to betaine (25). The K_m of rat liver choline kinase for choline is about 0.03 mM (26), and that for choline transport into rat liver mitochondrion has been estimated to be about 0.022 mM (27). Choline concentrations in rat liver range from 0.05 to 0.25 mM (28, 29), and presumably, higher levels are attained when dietary choline is supplemented in the diet. Taken together, these data suggest that free choline in liver is preferentially incorporated into phospholipids and that choline oxidation functions as a spillover pathway that is sensitive to dietary choline intake and subsequent tissue levels. In fact, metabolic tracer studies using rat liver slices have estimated that about 90% of the free choline proceeds through the oxidation pathway (30). We are interested in BHMT because it resides at an interface between choline oxidation and sulfur amino acid and one-carbon metabolism, and it is possible that these pathways are coordinately regulated in part by the BHMT-catalyzed reaction. We have chosen to focus our initial efforts on the influence nutrition has on BHMT gene expression because previous work indicated that activity levels of hepatic BHMT are affected by diet.

There have been numerous studies investigating the effects of nutrition on hepatic BHMT activity (13, 19, 31–34). The earliest studies used rats and showed that the specific activity of BHMT varies with the dietary intake of sulfur amino acids, choline, and betaine (31–33). The greatest changes were observed with Met deficiency and Met deficiency in combination with excess dietary choline where up to 3-fold increases of BHMT activity were observed. We subsequently observed similar responses of BHMT activity in chicken liver when chicks were fed varying levels of sulfur amino acids, choline, and betaine (19). Although supplemental levels of Met, either with or without excess methyl donor, slightly stimulated chick liver BHMT activity, as was observed in rats (31), we confirmed that BHMT activity is most dramatically up-regulated when the diet is deficient in Met. Furthermore, the magnitude of induction was greater in our chicken study than that previously reported for rats, reaching 6-fold induction levels. A more recent study from our laboratory showed that 8–10-fold increases in BHMT activity can be achieved by feeding rats amino acid-defined diets deficient in Met containing excess betaine (13). In this study, the diet-induced changes were shown to be mediated by changes in the steady-state levels of mRNA. Finally, we recently reported a preliminary investigation using pigs (34), and the results suggest that in this species the liver enzyme is refractory to changes in diet, but the kidney enzyme is inducible under conditions of Met deficiency with supplemental choline or betaine, although to a much lower extent than what we observed with the chicken and rat liver enzymes. Taken together, the data are consistent across species and indicate that BHMT activity is influenced by Met, choline, and betaine intakes. In all of the previous studies, it was concluded that Met restriction induces BHMT activity. However, none of the studies to date have controlled for the dramatic changes in food intake that accompany the consumption of diets deficient in...
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The data we report here show that changes in BHMT activity are primarily due to the changes in the nutrient content of the diet rather than a physiological response to reduced food intake. These changes in activity are due to changes in mRNA content and immunodetectable protein. Furthermore, our data indicate that although high levels of BHMT induction require Met restriction, Met restriction alone is not sufficient; there is a dose-dependent relationship between the level of BHMT induction and the amount of methyl donor in a diet limiting in Met. We also show that the magnitude of induction is related to the severity of Met deficiency. Although the methyl donor-dependent induction of BHMT expression requires Met restriction, this induction occurs at a level of Met intake that is above the maintenance requirement for the weanling rat. Taken together, these data support the idea proposed by Finkelstein et al. (31) that BHMT functions to conserve the backbone of Hcy under conditions of Met deficiency; however, BHMT primarily does so only when Met is also a source of methyl donor in the diet, shown here to include choline, betaine, or sullonium analogs of betaine.

The requirement for both low dietary Met and adequate methyl donor levels for the high induction of BHMT expression may be a regulatory control to prevent the futile cycling of methyl groups. Because a diet devoid or low in choline necessitates the synthesis of this compound using methyl groups. Because a diet devoid or low in choline necessitates the synthesis of this compound using methyl groups derived from Met only to in turn oxidize it would be a futile cycle. In contrast, the induction of BHMT when dietary Met is scarce Met supplies. Synthesizing choline from methyl groups available in choline, betaine, or sulfonium analogs of betaine.

Met, and all of the studies in which a major induction was observed used choline-containing diets.
probability of Hcy being remethylated to Met, rather than have Hcy proceed through the transsulfuration pathway.

Further studies will be required to determine what mechanisms mediate the diet-induced changes in steady-state levels of hepatic BHMT mRNA. Earlier reports indicate that actinomycin D could partially block BHMT induction in rat liver (20) or cultured cells (35), provided varying levels of dietary or medium Met, respectively. It can be inferred from these earlier studies that the diet-induced increases we observed in BHMT mRNA are due in part to changes in transcription. The influence of diet or physiological state on BHMT mRNA turnover has not been studied. In order to begin studies on the transcriptional regulation of BHMT expression, we have cloned the 5′-flanking region of the human BHMT gene into a luciferase reporter vector and have confirmed that changes in medium sulfur amino acid and betaine concentrations cause significant changes in BHMT promoter activity in Hep G2 transfectants.2 The molecular signals that initiate these putative changes in BHMT transcription remain to be identified.

In addition to the 5′-flanking region of the human BHMT gene, we have isolated several overlapping clones encoding the human BHMT gene (Fig. 4). The intron-exon junctions were mapped and sequenced, and the intron sizes were determined. The intronic sequences flanking each exon are reported and all intron-exon junctions follow the GT-AG rule. The major transcriptional start site in liver has been determined using 5′-RACE, and computer analysis of the 5′-flanking region has identified many putative transcription factor binding sites (Fig. 5). Centered 26 nucleotides upstream from the transcriptional start site is a TATA box. This TATA box was the only one identified as a putative promoter using TSSG and TSSW programs. The 5′-flanking region also contains a significant number of potential steroid transcription factor binding sites. Some of the glucocorticoid response elements are presumably functional because glucocorticoids have been shown to increase rat liver BHMT activity (20) and mRNA levels (21). Several transcription factor binding sites known to be important in liver-specific expression were also identified, including HNF-1, HNF-3, and CAAT enhancer-binding protein. Although a genetic deficiency of BHMT activity has not been described, it is possible that mutations or polymorphisms in the gene exist that reduce BHMT activity and increase plasma Hcy levels and thus increase vascular disease risk. We are unaware of any attempts to identify individuals deficient in BHMT activity, most likely because the organs that express this enzyme, the liver and kidney, are not routinely biopsied and have not been specifically sampled for this purpose. The sequence of the human BHMT gene reported here will permit investigators to screen for gene variants using genomic DNA isolated from blood or any other easily isolated cell type. The availability of the 5′-flanking region of the gene will allow further studies on the nutrient-, hormone-, and tissue-specific regulation of BHMT gene transcription.

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