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Genomic Structure, Expression, and Chromosomal Localization of the Human Glycine *N*-Methyltransferase Gene

Yi-Ming A. Chen,*,[1] Li-Ying Chen,* Fen-Hwa Wong,* Cheng-Ming Lee,* Tai-Jay Chang,*,† and Teresa L. Yang-Feng‡

**Division of Preventive Medicine, Institute of Public Health, School of Medicine, National Yang-Ming University, Taipei, Taiwan 112, R.O.C.; †Department of Medical Research, Taipei Veterans General Hospital, Taiwan 112, R.O.C.; and ‡Department of Genetics, Yale University School of Medicine, New Haven, Connecticut 06511*

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The glycine *N*-methyltransferase (GNMT) gene encodes a protein that not only acts as an enzyme to regulate the ratio of *S*-adenosylmethionine to *S*-adenosylhomocysteine, but also participates in the detoxification pathway in liver cells. Previously, we reported that the expression level of GNMT was diminished in human hepatocellular carcinoma. In this study, the human GNMT gene was cloned and characterized. It contains six exons and spans about 10 kb. Instead of a TATA box, it has a transcriptional initiator located 801 bp upstream from the translation start codon. The gene was localized to chromosome 6p12 using fluorescence *in situ* hybridization. Northern blot analysis of 16 tissues from different human organs showed that GNMT was expressed only in liver, pancreas, and prostate. © 2000 Academic Press

INTRODUCTION

Glycine *N*-methyltransferase [GNMT (EC 2.1.1.20)] catalyzes the synthesis of sarcosine from glycine using *S*-adenosylmethionine as the methyl donor. The enzyme was first described by Blumenstein and Williams (1960) in guinea pig liver. In rabbit and rat livers, GNMT comprises 1–3% of the cytosolic proteins, and the enzyme is suggested to play an important role in the metabolism of methionine (Kerr, 1972; Cook and Wagner, 1981). In halophilic Methanoarchaea, GNMT plays a major role in osmoregulation (Lai *et al.*, 1999a). It has been postulated that rat GNMT is involved in gluconeogenesis and secretion function in both liver and kidney cells (Yeo and Wagner, 1994). In addition to its function as an enzyme, GNMT is a folate-binding protein (Cook and Wagner, 1984) and a cytosolic receptor for benzo[a]pyrene (Raha *et al.*, 1994; Bhat *et al.*, 1997). Among different species, so far only the rat GNMT gene has been isolated and characterized (Ogawa *et al.*, 1987).

Previously, the expression level of GNMT was found to be diminished in both human hepatocellular carcinoma (HCC) tissues and cell lines. Subsequently, the cDNA of human GNMT was isolated from a Taiwanese liver cDNA library (Chen *et al.*, 1998). To elucidate the mechanism of gene control and its association with HCC, the GNMT gene was isolated, and its structure and chromosomal localization were analyzed in this study.

MATERIALS AND METHODS

Isolation and characterization of the human GNMT gene (GNMT). A human placental genomic DNA library (Stratagene, La Jolla, CA), constructed in a λ phage FIX II, was used to isolate GNMT genomic clones. The human GNMT cDNA (Chen *et al.*, 1998) was used as a probe, and the hybridization and washing procedures were performed according to standard protocols (Sambrook *et al.*, 1989). Subsequently, the phage DNA was digested with different restriction enzymes and subcloned into the *Not*I or *Eco*RI site of pBluescript II KS (Stratagene). The subclones were sequenced using a DNA Sequencer (Applied Biosystems Model 373A, Version 1.0.2, Foster City, CA) with a Dye Terminator Cycle Sequencing Core Kit. The primers used in the sequencing reactions included M13 primers and primers designed from either human GNMT cDNA or genomic DNA sequences. Sequencing data were edited and analyzed using the MacDNASIS (Version 3.0, Hitachi Software Engineer Co., San Bruno, CA) and the NCBI Blast programs (http://ncbi.nlm.nih.gov/blast/).

Primer extension. The transcription start of human GNMT was determined by primer extension according to the standard protocol (Sambrook *et al.*, 1989). Total RNAs were prepared from human tumorous and nontumorous (normal) liver cancer tissues as described previously (Chen *et al.*, 1998). Two primers, cap-1 and cap-2, which span the antisense nucleotide sequences from 640 to 606 (cap-1) and from 620 to 586 (cap-2) of the human GNMT gene, were used. The primers were radiolabeled with [γ-^{32}P]ATP at their 5′ termini using T4 polynucleotide kinase, and 2×10^5 cpm of each primer was hybridized separately to 30 μg of RNA from HCC tissue. The annealing temperatures for cap-1 and cap-2 were 30 and 32°C, respectively. The primer/RNA hybrids were then precipitated with ethanol, redissolved in reverse transcriptase buffer, and incubated with 50 units of murine MuLV reverse transcriptase (Stratagene) at 37°C for 2 h. To inhibit the synthesis of double-stranded DNA,

Sequence data from this article have been deposited with the EMBL/GenBank Data Libraries under Accession No. AF101475.

[1] To whom correspondence should be addressed. Fax: 886-2 28270576. E-mail: arthur@ym.edu.tw.

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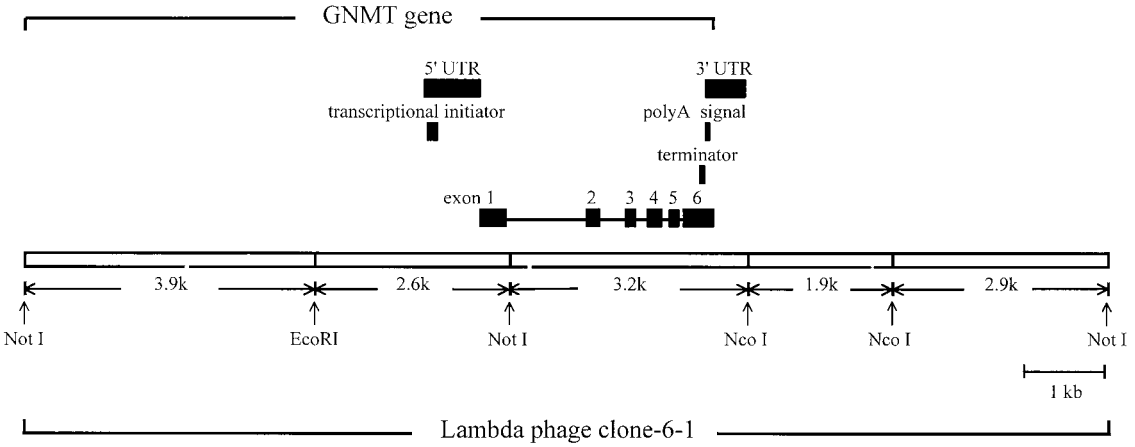

FIG. 1. Gene structure of the human GNMT gene.

actinomycin D (50 μg/ml) was added to the reaction. The mixture had been digested with DNase-free pancreatic RNase (Boehringer Mannheim, Mannheim, Germany) before it was subjected to electrophoresis. An end-labeled pGEM-3zf(+) and pUC/M13 forward sequencing primer (Promega Co., Madison, WI) was used as the size marker and run in parallel with the primer extension reaction on the gel.

Chromosomal localization. Mapping Panel 1, consisting of 17 mouse–human hybrids and 1 Chinese hamster–human hybrid, was obtained from the NIGMS Cell Repository at the Coriell Institute for Medical Research (Camden, NJ). The characterization and human chromosome content of these hybrids are described in the NIGMS catalog. DNA samples were digested with *Pst*I, transferred to hybrid filters, and hybridized with ^{32}P-labeled GNMT cDNA probe as described previously (Yang-Feng *et al.*, 1985). Fluorescence *in situ* hybridization (FISH) was employed for regional assignment of GNMT. The pBluescript-GNMT-2.6 and pBluescript-GNMT-8.0 genomic subclones were labeled with biotinylated dUTP and used as a probe at a concentration of 50 ng/ml in the hybridization reaction. Hybridization to metaphase chromosomes, posthybridization washing, and signal detection with avidin–fluorescent isothiocyanate were carried out as described previously (Lichter *et al.*, 1991). For chromosome identification, metaphase chromosomes were cohybridized with a chromosome 6 centromere probe (Shaper *et al.*, 1992).

Northern blot analysis. Human multiple-tissue Northern blots were obtained from Clontech Laboratories (Palo Alto, CA) and used for RNA analysis. The human GNMT cDNA clone 9-1-2 was labeled with [α-^{32}P]dCTP using the Redi-Prime Kit (Amersham, Aylesbury, UK). The membrane had been treated with ExpressHyb solution (Clontech) for 30 min before it was hybridized with the radiolabeled probe (1×10^6 cpm/ml) at 68°C for 1 h. The membranes were washed four times with washing buffer A (2X SSC and 0.05% SDS) at room temperature and then washed two times with washing buffer B (0.1X SSC and 0.1% SDS) at 58°C for 30 min. The washed membranes were autoradiogrphed with Kodak XAR-5 film at −80°C. β-actin cDNA was used as a control probe in the Northern blot analysis.

RESULTS

Isolation of the Human GNMT Gene

To isolate the human GNMT genomic clone, a human placenta genomic library was screened with ^{32}P-labeled human GNMT cDNA clone 9-1-2. Approximately 1×10^6 plaques were screened with a human GNMT cDNA probe. After the tertiary screening, two clones containing GNMT signals were identified. Subsequently, clone 6-1 was digested with different restriction enzymes including *Not*I, *Nco*I, and *Eco*RI and subcloned into pBluescript II KS. Three of the subclones, pBluescript 3.9, pBluescript 2.6 and pBluescript 3.2, were identified by Southern blot for further analysis.

Gene Structure of Human GNMT

The sequencing data from different GNMT subclones were compiled, and the results showed that the human GNMT gene spans about 10 kb and comprises six exons (Fig. 1). The GenBank accession number for human GNMT is AF101475. The sizes of these six exons are 218, 128, 117, 143, 122, and 346 bp, respectively. The junctions between introns and exons were identified through sequence analysis. The amino acid sequence deduced from human GNMT is identical to that encoded by the GNMT cDNA clone 9-1-2, which we isolated previously (Chen *et al.*, 1998).

Sequence analysis showed that there are four Sp1-binding sites and one putative CAAT box in the upstream promoter region of the GNMT gene (Fig. 2). To locate the transcription start site, primer extension was performed with a primer (cap-1) that contains an antisense nucleotide sequence about 70–106 bp downstream from the putative initiator element. As shown in Fig. 3, a 106-bp radiolabeled band was found in the normal liver tissue (lane 2) but not in the tRNA control (lane 3). In comparison with the normal liver, the intensity of the signal in the HCC tissue (lane 1) was much weaker. Therefore, the 5′ terminus of GNMT RNA is located at nucleotide 5593 of the GNMT gene (arrow in Fig. 1A), which is 799 bp upstream from the translation start codon. This result has been confirmed by using another primer (cap-2) in the same experimental setting (data not shown).

Chromosomal Localization of the GNMT Gene

The chromosomal localization of human GNMT gene was determined by somatic cell hybrid analysis and fluorescence *in situ* hybridization. Southern hybridization of the *Pst*I-digested DNA from hybrids detected a human specific fragment of 3.9 kb, two Chinese hamster bands of 1.5 and 1.25 kb, and a mouse fragment of

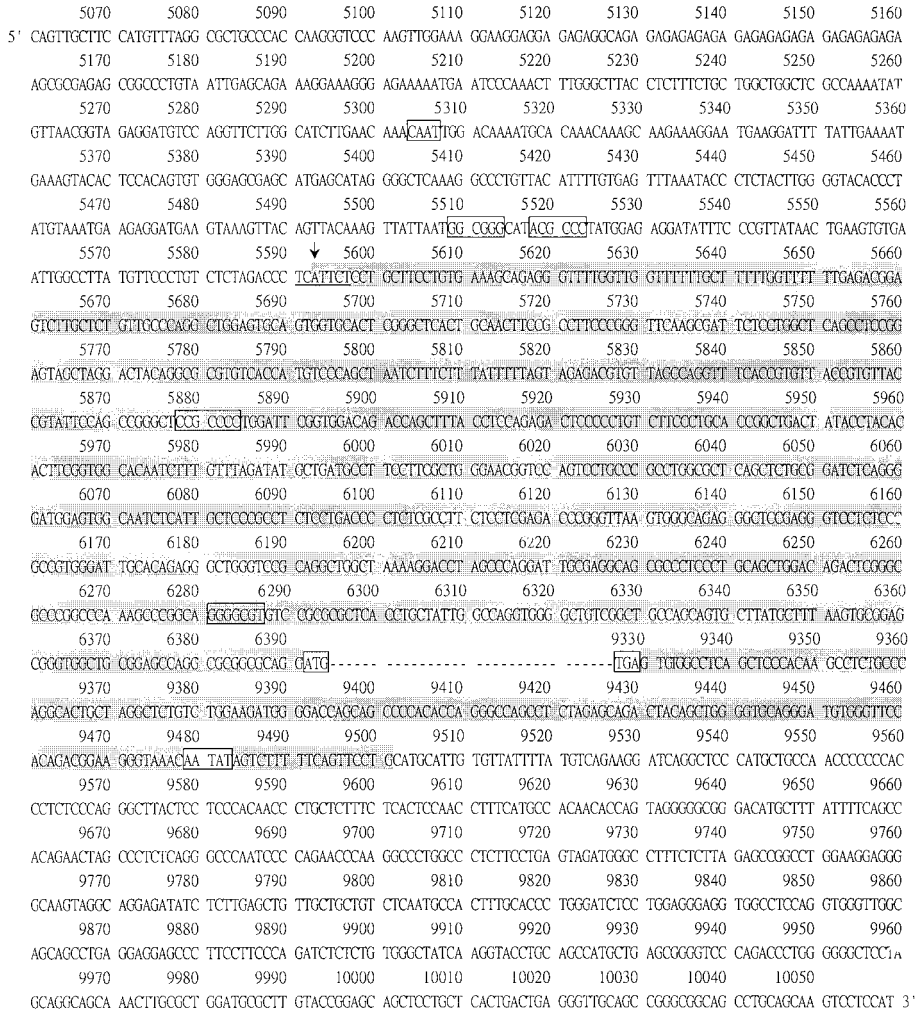

FIG. 2. Partial nucleotide sequence of the human GNMT gene. The putative CAAT box, Sp-1-binding site, translation start and stop codons, and poly(A) tail signal are in square boxes. The transcriptional initiator is underlined, and the transcription start site is marked by an arrow. The nucleotide sequence of the gene, 10,059 bp in length, has been deposited with GenBank under Accession No. AF101475.

2.8 kb. The 3.9-kb fragment was present only in the DNA from hybrids containing human chromosome 6. The regional localization of GNMT was further accomplished by using FISH as previously described (Shaper *et al.*, 1992). The human metaphase chromosomes were cohybridized with a biotinylated human GNMT genomic DNA probe and a chromosome 6 centromere probe. Twenty DAPI-banded cells were scored. The results showed that the GNMT signal was adjacent to the centromere signal and was present on both chromosomes 6 in all the cells analyzed. Therefore, the human GNMT gene was located at 6p12, near the centromere of chromosome 6 (Fig. 4).

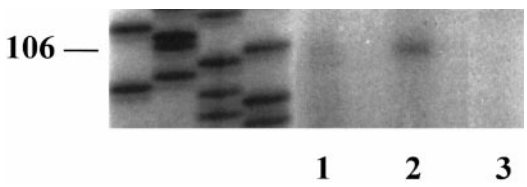

FIG. 3. Identification of the transcription start site of the human GNMT gene. The RNA samples used in the primer extension were HCC tissue (lane **1**), normal liver tissue (lane **2**), and tRNA (lane **3**). A partial nucleotide sequence of plasmid pGEM-3zf(+) was used as the size marker and run in parallel with the reaction. The number shown in the left margin represents the size of the band.

Tissue Expression of the GNMT Gene

The GNMT gene expression in multiple adult and fetal tissues was determined by Northern blot analysis using GNMT cDNA clone 9-1-2 as a probe. As shown in Fig. 5, the GNMT mRNA, of about 1.8 kb, was expressed only in the adult liver, adult pancreas, adult prostate, and fetal liver. The level of human GNMT gene expression was highest in the adult liver, and it was absent in other human tissues tested including heart, brain, placenta, lung, skeletal muscle, kidney, spleen, thymus, testis, ovary, small intestine, colon, peripheral blood leukocytes, fetal brain, fetal lung, and fetal kidney.

DISCUSSION

GNMT is present in different species including halophilic Methanoarchaeon (Lai *et al.*, 1999b), *Saccharo-*

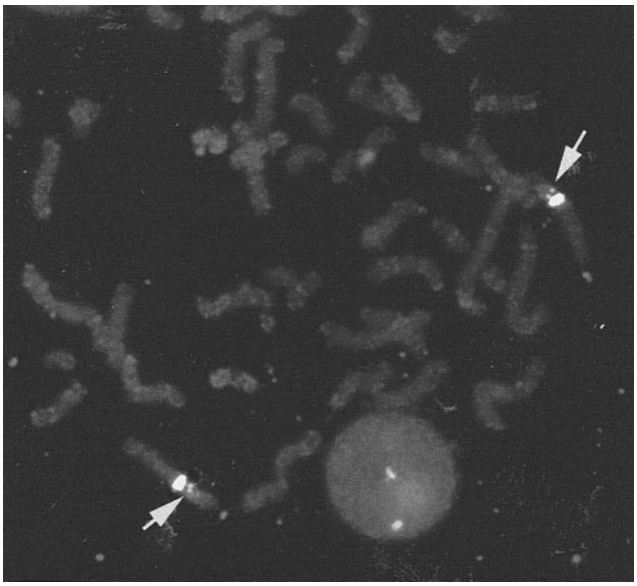

FIG. 4. A representative metaphase after *in situ* hybridization with a biotinylated GNMT probe. The metaphase was cohybridized with a chromosome 6 centromere probe. The arrows indicate the specific GNMT hybridization signals detected by avidin-fluorescent isothiocyanate.

myces cerevisiae (Bork *et al.*, 1992), and mammals (Blumenstein and Williams, 1960; Ogawa *et al.*, 1993). The present study is the first to isolate and characterize the human GNMT gene. As shown in Fig. 1, human GNMT contains 6 exons whose average length is 179 ± 34 bp, ranging from 117 to 346 bp. It is very similar to the rat GNMT gene (Ogawa *et al.*, 1987); both of the genes have the same numbers and almost identical sizes of exons and introns.

In the upstream promoter region of the GNMT genome, there were four Sp1-binding sites and one putative CAAT box (Fig. 2). Although there is a putative TATA box (nucleotides 5545–5550) in the promoter region, it is questionable because its location is too far from the transcription start site, and its sequence (TATAAC) does not match the consensus sequence of a TATA box, TATAA/TA. The alternative transcriptional initiator was identified through consensus sequence analysis and primer extension experiments. As shown in Fig. 2A, the nucleotide sequence of the initiator, TCATTCT (nucleotides 5593–5599 of the human GNMT gene), matches the degenerate initiator consensus sequence (PyPyA+1NT/APyPy) of eukaryotic genes very well (Javahery *et al.*, 1994). In addition, the size of the band detected in the primer extension indicated that the transcription start site is in the initiator (Fig. 2). GNMT gene expression was found to be down-regulated in HCC (Chen *et al.*, 1998), which is also reflected in the primer extension experiment. As shown in Fig. 3, the intensity of the 106-bp band in normal liver tissue is much stronger than that in HCC tissue.

The predicted GNMT mRNA sequence deduced from GNMT is identical with the GNMT cDNA clone isolated from a Taiwanese liver cDNA library (Chen *et al.*, 1998). However, it is different from the Japanese GNMT cDNA (Ogawa *et al.*, 1993) in the following three aspects. First, there are 140 nucleotides in the 5′-untranslated region of the Japanese cDNA that were not found in the human GNMT sequence. Sequence analysis using the Blast program (http://www.ncbi.nlm.nih.gov/blast/) indicated that the 140-bp sequence from the Japanese GNMT cDNA had 99% homology with that of the 25S rRNA gene of *S. cerevisiae*. Second, in the coding region, there were four nucleotide differences resulting in one amino acid change (amino acid residue 24, aspartic acid for the human GNMT gene, and glutamic acid for the Japanese GNMT cDNA). However, in the rabbit, pig, and rat, the predicted amino acid residue 24 of their GNMT proteins was consistently aspartic acid (Ogawa *et al.*, 1993). Therefore, it may be a mistake in the Japanese GNMT cDNA. Third, in the 3′-terminal region of the Japanese GNMT cDNA, there are extra 2- and 4-bp insertions in nucleotides 9334–9335 and 9472–9473, respectively, of the human GNMT sequence. The inserted sequences may be artifacts since they were not found in the Taiwanese GNMT cDNA.

In terms of tissue expression, among 20 adult or fetal organs, the GNMT gene is expressed only in adult liver, adult pancreas, adult prostate, and fetal liver (Fig. 5). The level of human GNMT gene expression in adult liver is higher than that in fetal liver. Previously, Kerr (1972) reported that GNMT enzyme activity is greatest in mammalian liver, followed by the pancreas and kidney. In fetal rabbit liver, the GNMT enzyme activity appears at a low level at about 20 days postfertilization, it rises to higher levels after birth and reaches a maximum in the adult liver (Heady and Kerr, 1975). Through immunohistochemical staining, it was shown that GNMT is present in the periportal region of the liver, the exocrine tissue of the pancreas, and the proximal convoluted tubules of the kidney (Yeo and Wagner, 1994). Therefore, GNMT gene expression is tissue-specific and possibly related to embryonic development. In this study, there was no signal detected

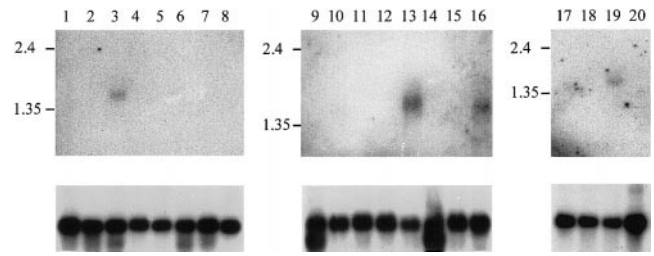

FIG. 5. Northern blot analyses of GNMT mRNA in tissues from different human organs. Lane **1,** spleen; lane **2,** thymus; lane **3,** prostate; lane **4,** testis; lane **5,** ovary; lane **6,** small intestine; lane **7,** colon; lane **8,** peripheral blood leukocytes; lane **9,** heart; lane **10,** brain; lane **11,** placenta; lane **12,** lung; lane, **13,** liver; lane **14,** skeletal muscle; lane **15,** kidney; lane **16,** pancreas; lane **17,** fetal brain; lane **18,** fetal lung; lane **19,** fetal liver; lane **20,** fetal kidney. The probes used for the upper and lower panels were GNMT cDNA and β-actin, respectively. The size markers were shown to the left margin of each panel.

in the human kidney after an extended period of autoradiography in the Northern blot assay. It is possible that the GNMT mRNA in human kidney was too low to be detected by Northern blot assay.

The GNMT gene expression is diminished in human HCC (Chen *et al.*, 1998). It is interesting to note that in Novikoff hepatoma, GNMT gene expression is also undetectable (Heady and Kerr, 1975). Therefore, the information regarding the genomic sequence and the chromosomal localization of GNMT will be useful for the study of the mechanism of GNMT gene control in normal and transformed hepatocytes.

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