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Human histamine *N*-methyltransferase pharmacogenetics: gene resequencing, promoter characterization, and functional studies of a common 5′-flanking region single nucleotide polymorphism (SNP)☆

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Abstract

Histamine *N*-methyltransferase (HNMT) catalyzes one of two major metabolic pathways for histamine. The levels of HNMT activity and immunoreactive protein in human tissues are regulated primarily by inheritance. Previous studies of *HNMT* identified two common single nucleotide polymorphisms (SNPs), including a functionally significant nonsynonymous coding SNP (cSNP), (C314T, Thr105Ile), but that polymorphism did not explain all of the phenotypic variation. In the present study, a genotype-to-phenotype strategy was used to search for additional genetic factors that might contribute to the regulation of human HNMT activity. Specifically, we began by resequencing the human *HNMT* gene using 90 ethnically anonymous DNA samples from the Coriell Cell Repository and identified a total of eight SNPs, including the two that had been reported previously. No new nonsynonymous cSNPs were observed, but three of the six novel SNPs were located in the 5′-flanking region (5′-FR) of the gene—including a third common polymorphism with a frequency of 0.367 (36.7%). That observation directed our attention to possible genetic effects on *HNMT* transcription. As a first step in testing that possibility, we created and studied a series of reporter gene constructs for the initial 1 kb of the *HNMT* 5′-FR. The core promoter and possible regulatory regions were identified and verified by electrophoresis mobility shift assays. We then studied the possible functional implications of the new common *HNMT* 5′-FR SNP. However, on the basis of reporter gene studies, that SNP appeared to have little effect on transcription. Phenotype–genotype correlation analysis performed with 112 human kidney biopsy samples that had been phenotyped for their level of HNMT activity confirmed that the common 5′-FR SNP was not associated with the level of HNMT activity *in vivo*. In summary, this series of experiments resulted in the identification of several novel *HNMT* polymorphisms, identification of the *HNMT* core promoter, and a comprehensive functional genomic study of a common *HNMT* 5′-FR SNP. These results represent an additional step in the definition of molecular genetic mechanisms involved in the regulation of this important autacoid-metabolizing enzyme in humans.

Keywords: Histamine; Histamine *N*-methyltransferase; HNMT; Methylation; Single nucleotide polymorphism; SNPs; Genotype–phenotype correlation analysis

1. Introduction

HNMT (EC 2.1.1.8) catalyzes one of two major metabolic pathways for histamine [1]. Histamine plays an important role in allergy and anaphylaxis, is involved in the regulation of gastric acid secretion, and is a neurotransmitter in the CNS [2–4]. N^{τ}-Methylation catalyzed by HNMT is the only process known to terminate the neurotransmitter actions of histamine in the brain and is the predominant pathway for histamine metabolism in bronchi

☆The *HNMT* resequencing data included in this paper have been deposited in the NIH-sponsored database PharmGKB with accession number PKB_N00000018.

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Abbreviations: HNMT, histamine *N*-methyltransferase; SNP, single nucleotide polymorphism; DMEM, Dulbecco's Modified Eagle's Medium; α-MEM, α-Minimal Essential Medium; DTT, dithiothreitol; PMSF, phenylmethylsulfonyl fluoride; PCR, polymerase chain reaction; ORF, open reading frame; EMS, electrophoresis mobility shift.

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[3,5]. Levels of HNMT enzymatic activity differ greatly among individuals, and that variation is regulated primarily by inheritance [6,7]. As initial steps toward pharmacogenetic studies, we previously cloned the human HNMT cDNA and gene [8,9] and performed phenotype–genotype correlation analyses with biopsy samples of an organ (the kidney) in which HNMT is highly expressed [10]. Those experiments resulted in the discovery of two common *HNMT* SNPs. One was a nonsynonymous coding SNP (cSNP) located in exon 4 (C314T, Thr105Ile) that was associated with decreased levels of enzyme activity and immunoreactive protein *in vivo* in kidney biopsy samples and *in vitro* after expression in COS-1 cells [10]. The other was a common 3′-UTR polymorphism that was linked to the exon 4 SNP but which had little independent effect on the phenotype [10]. The exon 4 polymorphism was subsequently reported to be a risk factor for asthma [11,12]. However, these two exon-based *HNMT* polymorphisms did not explain all variance in the level of enzyme activity [10], raising the possibility that additional molecular genetic mechanisms involved in *HNMT* regulation remained to be discovered. The present studies represent an attempt to systematically test that hypothesis.

The present experiments used a genotype-to-phenotype, rather than a phenotype-to-genotype strategy to study HNMT pharmacogenetics, focusing on the 5′-FR of the gene. Specifically, we resequenced *HNMT* using DNA samples from 90 ethnically anonymous subjects. Those experiments resulted in the identification of eight polymorphisms, three within the 5′-FR, including a third common SNP in addition to the common exon 4 and 3′-UTR SNPs that had been identified previously. A series of reporter gene constructs incorporating portions of the *HNMT* 5′-FR were then used to identify the core promoter for the gene. However, *in vitro* functional studies of the new common *HNMT* 5′-FR SNP failed to indicate that this polymorphism was of functional significance, a conclusion confirmed by genotype–phenotype correlation experiments performed with human kidney biopsy samples. In summary, this series of experiments has resulted in the identification of a series of *HNMT* polymorphisms, insights into the regulation of *HNMT* transcription in humans, and information with regard to the possible functional implications of a common *HNMT* 5′-FR SNP—all steps toward an understanding of molecular genetic mechanisms involved in the regulation of this important histamine-metabolizing enzyme in humans.

2. Materials and methods

2.1. Materials

HeLa and HepG2 cells were purchased from the American Type Culture Collection. DMEM and α-MEM were purchased from Gibco BRL. DTT and PMSF were obtained from the Sigma Chemical Co. Poly(deoxyinosine-deoxycytosine) [poly(dI-dC)] and [α-^{32}P]dCTP were purchased from the Amersham Corp. The Klenow DNA fragment and QuickSpin^{TM} columns were obtained from Bio-Rad.

2.2. *Human* HNMT *resequencing studies*

HNMT was resequenced using the 90-sample subset of Polymorphism Discovery Resource (PDR) DNA from the Coriell Cell Repository. These samples were ethnically anonymous, but the sample set had been structured to reflect the ethnic diversity present in the United States [13]. Written informed consent had been obtained from all donors for the use of their DNA for experimental purposes, and our studies were reviewed and approved by the Mayo Clinic Institutional Review Board (IRB). During the resequencing experiments, seven PCR amplifications were performed for each DNA sample. Those reactions amplified all six *HNMT* exons, plus splice junctions, as well as approximately 500 bp of the 5′-FR. A schematic representation of the structure of the human *HNMT* gene is shown in Fig. 1. M13 tags had been added to the 5′-ends of the primers to make it possible to use dye-primer sequencing chemistry to facilitate the identification of heterozygous bases [14]. The sequences of the primers used in the resequencing experiments, as well as those used in other experiments in this series, are listed in Table 1. The numbering scheme of primers involved designation of the "A" in the ATG translation initiation codon as +1, with positive numbers located 3′, and negative numbers located 5′ to that position. Intron-based primers were numbered from the initial 5′-nucleotide in the intron (positive numbers) or from the final 3′-nucleotide (negative numbers). Reactions were performed with 12.5 pmol of each primer, 5 μM dNTP, and 2.5 units of Ampli*Taq* Gold DNA polymerase (Perkin-Elmer). Amplification conditions involved 1 cycle at 94° for 12 min, followed by 35 cycles at 94° for 30 sec, 60° for 30 sec, and 72° for 3 min, with a final 10-min extension at 72°. With the exception of a single 5′-UTR amplicon, reaction products were sequenced in both directions. In that one case, a homopolymer was located near the 3′-end of the amplicon, so it could not be sequenced in the reverse direction. To exclude PCR-induced sequence artifacts, DNA samples in which SNP was observed only once among the 90 samples studied, were amplified independently and sequenced a second time.

2.3. HNMT *reporter gene constructs*

Luciferase reporter gene constructs were created to study the regulation of human *HNMT* transcription. Those constructs were named on the basis of their 5′- and 3′-ends relative to the "A" in the ATG translation initiation codon. For example, the construct designated (−1051, −50)

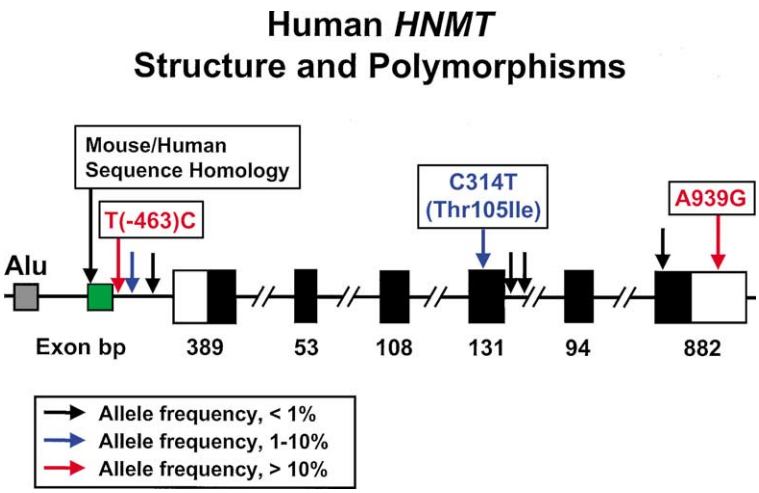

Fig. 1. Human *HNMT* structure and polymorphisms. Exons are represented as rectangles, with black rectangles indicating coding and white rectangles UTR sequences. The location of a region of high sequence homology with the mouse *Hnmt* gene is also shown, as is the location of an *Alu* repetitive sequence (shaded squares). Positions of SNPs are indicated by arrows, with colors representing frequencies.

Table 1
Primers used to resequence the human *HNMT* gene and to create reporter gene constructs and probes

Primer location	Primer name	Primer sequence
Human *HNMT* resequencing		
5′-FR	UF(−485)M13	5′-TGTAAAACGACGGCCAGTGCACAGAGGCAGATGACAGTCT-3′
Exon 1	E1R132M13	5′-CAGGAAACAGCTATGACCCTATTATGCCTGGCAGCTTCTTGTC-3′
5′-UTR	F(−33)M13	5′-TGTAAAACGACGGCCAGTGCTTCCTGCTCTGTCTTTCTCAGAAAACC-3′
Intron 1	I1R58M13	5′-CAGGAAACAGCTATGACCCACTGACGCACAGCCAGTCTGAGGC-3′
Intron 1	I1F(−64)M13	5′-TGTAAAACGACGGCCAGTGATAATCAGATTTCACAAAGCACCTAACAC-3′
Intron 2	I2R67M13	5′-CAGGAAACAGCTATGACCAAGGGAACAATITGAGTTAAGTC-3′
Intron 2	I2F(−83)M13	5′-TGTAAAACGACGGCCAGTGGGCAGATAATAAATCAGCTAAA-3′
Intron 3	I3R67M13	5′-CAGGAAACAGCTATGACCAAGGGAACAATTTGAGTTAAGTC-3′
Intron 3	I3F(−119)M13	5′-TGTAAAACGACGGCCAGTGAAAAACGTTCTTTCTATCTGTTTGTATATAA-3′
Intron 4	I4R191M13	5′-CAGGAAACAGCTATGACCTTGGAATGTTAAAGAGAATCTTAGTATAATA-3′
Intron 4	I4F(−73)M13	5′-TGTAAAACGACGGCCAGTAGGAGTATCTAGCCCAAGCAATA-3′
Intron 5	I5R71M13	5′-CAGGAAACAGCTATGACCTCAAACACAGGAATCCATGCATAC-3′
Intron 5	I5F(−117)M13	5′-TGTAAAACGACGGCCAGTCGCTGCACAAAGGACAAGATTATT-3′
3′-UTR	R975M13	5′-CAGGAAACAGCTATGACCTACATTAATGGATGAGTTTGTGATTTTAATA-3′
Kidney biopsy samples *HNMT* 5′-FR resequencing		
5′-FR	UF(−485)M13	5′-TGTAAAACGACGGCCAGTGCACAGAGGCAGATGACAGTCT-3′
5′-FR	UR(−140)M13	5′-CAGGAAACAGCTATGACCCATGCCACAAAGGCTGGCTTGTAG-3′
Reporter gene constructs		
5′-FR	UF(−1051)*Acc*65I	5′-*AAGAAGGGTACC*AAGGGACGGTTTAAGCAGCA-3′
5′-FR	UF(−745)*Acc*65I	5′-*AAGAAGGGTACC*CGCGCTTAGGGAAGGATTCTA-3′
5′-FR	UF(−493)*Acc*65I	5′-*AAGAAGGGTACC*GTAGAAAGCACAGAGGCAGATGAC-3′
5′-FR	UF(−395)*Acc*65I	5′-*AAGAAGGGTACC*GCCACTTFCGAACACACTTTCC-3′
5′-UTR	UR(−50)*Xho*I	5′-*AAGAAGCTCGAG*TCCTTCTCTTCCCAGTTCCCTCTTT-3′
5′-FR	UR(−401)*Xho*I	5′-*AAGAAGCTCGAG*CCAGGATAAAGAGGACAGCTACAC-3′
5′-UTR	UR(−117)*Xho*I	5′-*AAGAAGCTCGA*GATGCTAAGCTTGTTTGAATTTAAC-3′
5′-UTR	UR(−215)*Xho*I	5′-*AAGAAGCTCGAG*CCCCTATACGGTTGTTTTGT-3′
Probe (−294, −117)		
5′-FR	UF(−294)*Bam*HI	5′-*AAGAAGGGATCC*CAGGAATTTGAAAAGTTTTAGCAG-3′
5′-FR	UR(−117)*Bam*HI	5′-*AAGAAGGGATCC*ATGCTAAGCTTGTTTTGAATTTAAC-3′

Eighteen nucleotides of −21M13 F or M13 R sequence (underlined) were added to the 5′-ends of forward and reverse resequencing primers, respectively, to make it possible to use dye primer sequencing chemistry. Restriction sites for *Acc*65I, *Xho*I, or *Bam*HI attached to the 5′-ends of reporter gene construct primers are indicated by italics. In abbreviations for primer or probe names, I represents intron; F, forward; R, reverse; U, upstream; FR, flanking region; and UTR, untranslated region. With the exception of those that hybridized within introns, primers have been numbered from the "A" in the ATG translation initiation codon, with positive numbers in a 3′-, and negative numbers in a 5′-direction. Intron-based primers have been numbered from the initial nucleotide at the 5′-terminus of the intron (positive numbers) or the initial nucleotide at the 3′-terminus (negative numbers).

extended from 5′-FR nucleotide (−1051) to nucleotide (−50). Since an *Alu* repeat sequence was located approximately 1000 bp 5′-upstream from the translation initiation codon, the longest construct ended just 3′ of that *Alu*. Except for constructs (−395, −293) and (−395, −353), the PCR was used to amplify DNA fragments that were used to create the constructs. Primers used to perform those amplifications are also listed in Table 1. Forward and reverse primers had *Acc*65I and *Xho*I restriction sites attached to their 5′- and 3′-ends, respectively. Amplification products were digested overnight with *Acc*65I and *Xho*I, followed by purification with a QIAquick PCR Purification kit (Qiagen). Digested products were then cloned into the vector pGL-3 basic (Promega) upstream of the firefly luciferase ORF to create the reporter gene constructs. Each of the inserts had been sequenced on both strands to assure DNA sequence fidelity. Because the inserts used to create constructs (−395, −293) and (−395, −353) were less than 150 bp in length, those two inserts were generated by annealing sense and antisense oligonucleotides (Table 2). These oligonucleotides included restriction sites for *Acc*65I and *Xho*I. The annealed double-stranded DNA was digested with those restriction enzymes and was then cloned into pGL-3 basic. Constructs containing either *HNMT* wild-type or variant sequences for the common 5′-FR SNP, T(-463)C, were amplified using as template human genomic DNA samples homozygous for those two alleles. Primers UF(−1051), UF(−745), UF(−493), and UR(−50) (see Table 1) were used to create these constructs.

The luciferase reporter gene constructs were then used to transfect HepG2 and HeLa cells. Specifically, 2 μg of purified plasmid DNA was transfected into HepG2 or HeLa cells together with 200 ng of pRL-TK(Promega) DNA, or 20 ng of pRL-TK DNA, respectively. The *Renilla* luciferase activity expressed by pRL-TK was used as a control for transfection efficiency. Cells were also transfected with pGL-3 basic that lacked an insert. Plasmids were mixed with medium and Tfx TM-20 (Promega) prior to transfection, as suggested by the manufacturer. After incubation at 37° for 1–2 hr, 2 mL of DMEM or α-MEM with 15% fetal bovine serum was added to the plates. These cells were then incubated for an additional 48 hr and were harvested in passive lysis buffer (Promega). The cell lysates were used to assay luciferase activity. Each transfection was performed in triplicate, and all transfections were repeated at least twice to assure reproducibility. A dual luciferase assay (Promega) was used to measure luciferase activity in the transfected cell lysates. Ten microliters of cell lysate was mixed with 100 μL of luciferase assay buffer II (Promega) that contained firefly luciferase. Light units were measured in a Berthold Lumat LB 9501 luminometer (Berthold Technologies GmbH & Co. KG). *Renilla* luciferase activity was measured in the same tube after the addition of 100 μL of STOP&Glo Reagent (Promega). Results have been reported as the ratio of firefly luciferase light units to light units for *Renilla* luciferase.

2.4. HNMT *EMS assays*

The EMS assays utilized HepG2 cell lysates. The cell lysates were prepared using the method of Jiang and Eberhardt [15]. This method has been used widely to perform EMS assays [16,17]. Approximately 5×10^7 cells were washed twice with cold PBS and were harvested by scraping in the presence of 5 mL of cold PBS, followed by

Table 2
Oligonucleotides used to perform EMS assays and to create reporter gene constructs

Probe designation	Probe location	Probe sequence
Oligonucleotides used to create reporter gene constructs		
Oligo (−395, −293), sense	5′-FR	5′-AAGAAGGTACCGCCACTTCGAACAGTCACTTTCCCACTTGGAACTTACTTGTGGCTTTGCTGACAGGAAGTGATTTGAATACAGTTTGTTTTTATGCTTTCAACCTCTCGAGCTTCTT-3′
Oligo (−395, −293), antisense	5′-FR	5′-AAGAAGCTCGAGAGGTTGAAAGGTCATAAAAACAAACTGTATTCAAATCACTTCCTGTTTTGTCAGCAAAGCCACAAGTAAGTTCCAAGTGGGAAAGTGACTGTTCGAAGTGGCGGTACCCTTCTT-3′
Oligo (−395, −354), sense	5′-FR	5′-AAGAAGGGTACCGCCACTTCGAACAGTCACTTTCCCACTTGGAACTTACTTGTGCTCGAGCTTCTT-3′
Oligo (−395, −354), antisense	5′-FR	5′-AAGAAGCTCGAGCACAAGTAAGTTCCAAGTGGGAAAGTGACTGTTCGAAGTGGCGGTACCCTTCTT-3′
Oligonucleotides used to create probes for EMS assays		
Wild-type, sense	5′-FR	5′-GATCGCAGATGACAGTCTT***T***CGTTAAAGATTTCAC-3′
Wild-type, antisense	5′-FR	5′-GATCGTGAAATCTTTAACG***A***AAGACTGTCATCTGC-3′
Variant, sense	5′-FR	5′-GATCGCAGATGACAGTCTT***C***CGTTAAAGATTTCAC-3′
Variant, antisense	5′-FR	5′-GATCGTGAAATCTTTAACG***G***AAGACTGTCATCTGC-3′
Nonspecific, sense		5′-GATCTAGCTAGCTAGCTGACTGAC-3′
Nonspecific, antisense		5′-GATCGTCAGTCAGCTAGCTAGCTA-3′

The underlined sequence at the 5′-terminus of probes represents the 5′-overhang. Oligonucleotides used to create reporter gene constructs (−395, −354) and (−395, −293) contained *Acc*65I and *Xho*I restriction sites (underlined). The polymorphic nucleotide in the "wild type" and "variant" probes has been underlined, italicized and bold.

centrifugation at 800 *g* for 5 min at room temperature. The pellets were resuspended at room temperature in 3 packed cell volumes of 20 mM HEPES, pH 7.9, 20% glycerol, 0.5 M KCl, 0.5 mM EDTA, 0.5 mM PMSF, and 0.5 mM DTT. The cell suspensions were frozen on dry ice and thawed in a 37° water bath, followed by centrifugation at 14,000 *g* for 30 sec at 4°. The supernatant after this step was diluted with 4 vol. of 20 mM HEPES, pH 7.9, 20% glycerol, 0.2 mM EDTA, 0.5 mM PMSF, and 0.5 mM DTT. Protein concentrations were determined by the method of Bradford [18] with bovine serum albumin as a standard. The cell extracts were then stored at −80°.

Double-stranded DNA generated from the single-stranded oligonucleotides listed in Table 2 was used to perform the EMS assays. Each oligonucleotide was designed to have GATC overhangs at the 5′-end. Sense and anti-sense oligonucleotides (Table 2) were annealed at 80° for 5 min, followed by incubation at 37° for 1 hr. Since the DNA segment that extended from nucleotide (−294) to (−117) was more than 150 bp in length, it was generated by PCR amplification with forward primer UF(−294) and reverse primer UR(−117) (Table 1). Both primers had *Bam*HI restriction sites at their 5′-ends. The amplicon was then digested overnight with *Bam*HI to leave 5′-overhangs. Then 0.5 μg of double-stranded probe DNA was 3′-end labeled with [α-^{32}P]dCTP (3000 μCi/mmol) using 1 unit of the Klenow fragment from *Escherichia coli* DNA polymerase. The probes were purified by passage through G-25 Sephadex columns (Pharmacia). DNA-protein binding was performed in a 20-μL reaction volume in the presence of 20 mM HEPES, pH 7.9, 20% glycerol, 100 mM KCl, 0.2 mM EDTA, 0.5 mM PMSF, 0.5 mM DTT, 300 μg/mL of bovine serum albumin, and 60 μg/mL of poly(dI-dC). Binding reactions were initiated by adding approximately 20,000 cpm of labeled DNA probe to 20–50 μg of cellular extract protein. Electrophoresis was then performed with 5% acrylamide gels. The gels were dried, and autoradiography was performed. Competition experiments were carried out in the same fashion except that 10-, 100-, and 250-fold excess non-radioactive probe was added during the binding reaction.

2.5. *Human renal biopsy* HNMT *5′-FR resequencing studies*

DNA was isolated from 112 human kidney biopsy samples that had been phenotyped previously for the level of HNMT enzyme activity and for thermal stability [10]. These anonymized samples had been obtained during clinically-indicated surgical procedures. The clinical characteristics of the patients from whom the biopsies were obtained have been described previously [10]. The use of these samples for experimental purposes was reviewed and approved by the Mayo IRB. Primers UF(−485)M13 and UR(−140)M13 (Table 1) were used to amplify a segment of the gene that contained the common *HNMT* 5′-FR SNP, and dye primer sequencing chemistry was used to determine the DNA sequence.

2.6. *HNMT enzyme assay*

HNMT enzyme activity was measured in renal biopsy samples and in HepG2 and HeLa cells with a modification of the radiochemical assay described originally by Van Loon *et al*. [19]. That assay utilized [^{14}C-*methyl*]-*S*-adenosyl-L-methionine (Dupont-NEN) as a methyl donor and histamine as a methyl acceptor substrate. The assay procedure used for renal biopsy preparations has been described in detail previously [10], and those same assay conditions were used to measure the enzyme activity in HepG2 and HeLa cytosol preparations.

2.7. *Data analysis*

The University of Wisconsin Genetics Computer Group software package, Version 9.0, was used to analyze nucleotide sequences [20]. The University of Washington software programs PolyPhred 3.0 and Consed 8.0 [21,22] were used to analyze the DNA resequencing data and to identify SNPs. Statistical comparisons among values for the luciferase activity after transfection with the reporter gene constructs as well as values for enzyme activity levels among different *HNMT* genotypes in the human kidney biopsy samples were performed by the use of ANOVA. Linkage among *HNMT* polymorphisms was analyzed by calculating D′ values. D′ is a quantitative method for reporting linkage data that is independent of allele frequency [23,24].

3. Results

The initial step in this series of experiments involved resequencing the human *HNMT* gene using DNA samples from 90 ethnically diverse subjects. When those studies resulted in the identification of three 5′-FR SNPs, including one common polymorphism with an allele frequency of 0.367 (36.7%), we proceeded to systematically study the role of the 5′-FR in the regulation of *HNMT* transcription. After localizing the *HNMT* core promoter by using reporter gene constructs and EMS assays, the possible effects of the common 5′-FR SNP on reporter gene function were determined. When those *in vitro* studies failed to provide evidence that this common SNP altered transcription, we also performed genotype–phenotype correlation analysis with 112 human renal biopsy samples, studies which confirmed the results that we had obtained during the reporter gene studies.

3.1. *Human* HNMT *resequencing*

The human *HNMT* gene was resequenced using the 90-sample subset of Coriell Cell Repository PDR DNA sam-

Table 3
Human *HNMT* genetic polymorphisms

Location	Nucleotide	Nucleotide change	Amino acid change	Frequency of variant allele
5′-FR	−463	T → C		0.367
5′-FR	−431	G → A		0.022
5′-FR	−376	T → C		0.006
Exon 4	314	C → T	Thr → Ile	0.094
Intron4	I4(49)	A → G		0.006
Intron 4	I4(106)	T → C		0.006
Exon 6	593	T → C		0.006
3′-UTR	939	A → G		0.302

Allele frequencies were calculated on the basis of 90 samples (180 alleles). SNPs located within exons have been boxed.

ples [13]. All 6 exons, all exon–intron splice junctions, and approximately 500 bp of the 5′-FR were resequenced. A total of 500,000 bp of DNA sequence was analyzed, 87.5% of which was sequenced on both strands. The presence of homopolymer sequence prevented the remaining DNA from being sequenced on both strands. Eight SNPs, but no insertion/deletion events, were observed in these 90 DNA samples (Table 3). Three of the SNPs were located in the 5′-FR, two in coding exons, two in introns, and one in the 3′-UTR (Table 3). Only three of the SNPs had allele frequencies greater than 0.05 (5%). Two of those common polymorphisms, the exon 4 nonsynonymous cSNP (C314T) and the 3′-UTR SNP (A939G), had been reported previously [10]. The third common SNP, T(−463)C, was located in the 5′-FR, and had a frequency in these samples of 0.367 (Table 3). Our *HNMT* resequencing data have been deposited in the NIH-sponsored database PharmGKB (http://www.PharmGKB.org).

We then compared the SNPs that we observed during our resequencing studies with those available in public databases. Thirty-four human *HNMT* polymorphisms had been deposited in the SNP database (http://www.ncbi.nlm.nih.gov/SNP/), including 31 located in introns, 2 in the 3′-UTR, and 1 in exon 4. The 31 intron-based and one of the 3′-UTR SNPs in public databases were not located in regions of the gene that we had sequenced. Except for the two SNPs at nucleotide 314 in exon 4 and at nucleotide 939 in the 3′-UTR that we had reported previously [10], the other six SNPs identified during our resequencing studies—including the common SNP at nucleotide (−463) in the 5′-FR—were not represented in any of those public databases. We also used the HNMT cDNA ORF to search the EST database (http://www.ncbi.nlm.nih.gov/blast/) and analyzed the 101 human HNMT EST sequences in that database for the presence of polymorphisms. Only the initial 500 bp of each EST sequence were used during this analysis to assure high sequence quality. Except for the two polymorphisms that we had reported previously in exon 4 and in the 3′-UTR, no other SNPs were present in these HNMT EST sequences.

We also used our resequencing data to calculate two standard measures of nucleotide diversity: π, average heterozygosity per site, and θ, the population mutation parameter [25,26]. Those results were compared with data reported recently by Stephens *et al*. [27] for a large number of human genes which that group had resequenced. For the 292 autosomal genes resequenced by Stephens *et al*. [27], π averaged 0.058% and the average value for θ was 0.096%. When we calculated those same parameters for *HNMT*, our results were very similar, with π equal to 0.045% and with a θ value of 0.054%.

In summary, we identified eight polymorphisms during our *HNMT* resequencing studies, but no nonsynonymous cSNPs were observed beyond the exon 4 polymorphism that we had identified previously [10]. However, there were three novel 5′-FR SNPs, including a common nucleotide T(−463)C SNP with an allele frequency of 0.367 (Table 3). That observation raised the possibility that this common polymorphism within the 5′-FR might contribute to individual variation in *HNMT* expression. However, to pursue that possibility, it was first necessary to define areas of gene sequence involved in the regulation of *HNMT* transcription.

3.2. HNMT *regulatory domains and core promoter*

The human *HNMT* gene contains no TATA box, no CAAT, and no initiator (Inr) sequences [9]. In addition, there is currently no information available with regard to the regulation of *HNMT* transcription in humans. As a step toward the identification of areas within the *HNMT* 5′-FR that might participate in transcription regulation, we had previously cloned the mouse *Hnmt* gene [28] and compared 5′-FRs in the two species to identify areas of high sequence homology. That comparison resulted in the identification of two regions of high cross-species sequence homology, one in the 5′-UTR of the human gene and a second region in the 5′-FR that displayed approximately 70% sequence identity between the two species (Fig. 1) [28]. During the present experiments, we created a series of 5′-FR constructs with 5′-serial deletions to determine the location of the *HNMT* core promoter, possible 5′-FR regulatory sequences, and a possible function for the high sequence homology region between the mouse and human genes (Fig. 2A). Since HNMT is highly expressed in the liver [1], we used HepG2 cells to express these reporter gene constructs. We also used HeLa cells. Prior to performing transfections, HNMT enzyme activity was measured in both cell lines. Both lines expressed HNMT, but the basal level of activity was approximately an order of magnitude higher in HepG2 than in HeLa cells (data not shown).

The ability of the human *HNMT* 5′-FR to drive luciferase expression in these two human cell lines is depicted graphically in Fig. 2. In both lines, luciferase expression was normalized to that observed after transfection with the longest construct (−1051, −50). Patterns of expression were very similar in HepG2 and HeLa cells (Fig. 2), but

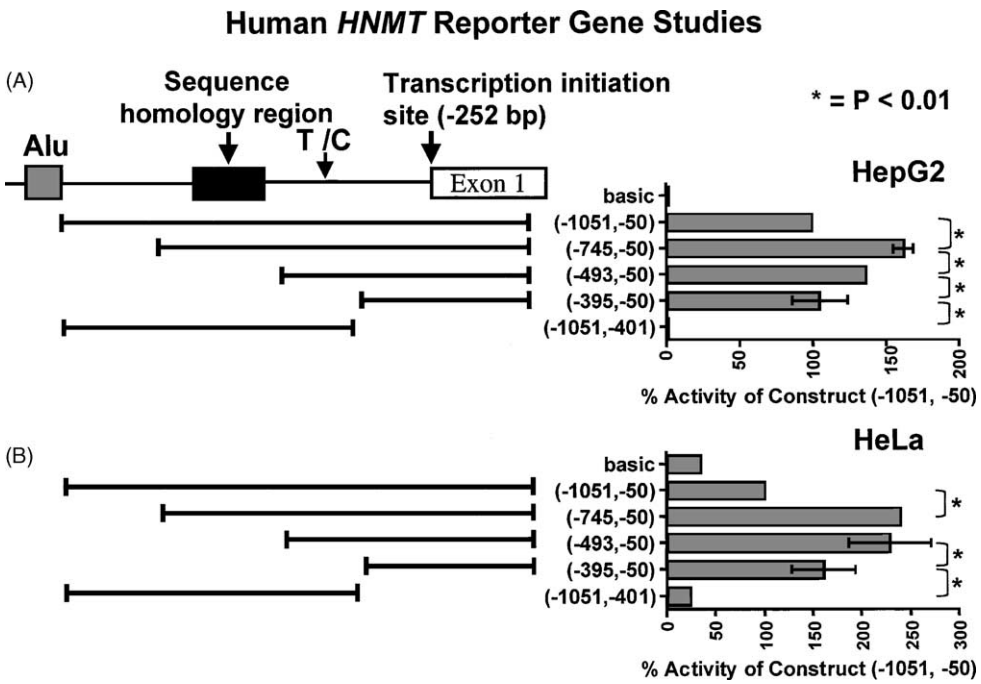

Fig. 2. *HNMT* 5′-FR reporter gene construct data. Data for luciferase reporter gene constructs created by sequential 5′-deletions are shown. Locations of an *Alu* sequence and an area of high sequence homology between the mouse and human genes are also shown, as is the location of the site of transcription initiation. The upper (A) panel shows data for HepG2, and the lower (B) panel shows data for HeLa cells. Luciferase activity for the longest construct (−1051, −50) transfected into each cell line was defined as 100%, and activities after transfection with other constructs were expressed relative to that of the longest construct. Results are means ± SD for 8 independent transfections. Key: (∗) $P < 0.01$, with brackets showing comparisons for the statistical analyses.

there was much higher luciferase activity in HepG2 cells—approximately 2 orders of magnitude higher than that observed in HeLa cells. Constructs that included the *HNMT* 5′-FR showed striking increases in luciferase activity in both cell lines over that observed in the presence of the "basic" construct. Furthermore, data from both cell lines provided evidence that a negative regulatory sequence was present between 5′-FR nucleotides (−1051) and (−745) since luciferase activity increased significantly after deletion of that portion of the construct (Fig. 2). Results obtained with both cell lines also indicated that a positive regulatory sequence was located between nucleotides (−493) and (−395), since deletion of that area resulted in a significant decrease in luciferase expression (Fig. 2). Deletion of the area of high sequence homology between mouse and human genes, the area located between nucleotides (−745) and (−493), had a significant effect—a decrease—on luciferase expression in HepG2, but not in HeLa cells. Finally, the core promoter for the gene appeared to be located approximately (−395) to (−50) bp upstream from the ATG translation initiation codon since, after deletion of that region, promoter activity decreased nearly to the level seen with the "basic" construct (Fig. 2).

In an attempt to further refine our localization of the *HNMT* core promoter, an additional series of constructs was created with 3′-deletions (Fig. 3). In HepG2 cells, luciferase activity with the (−395, −117) construct was

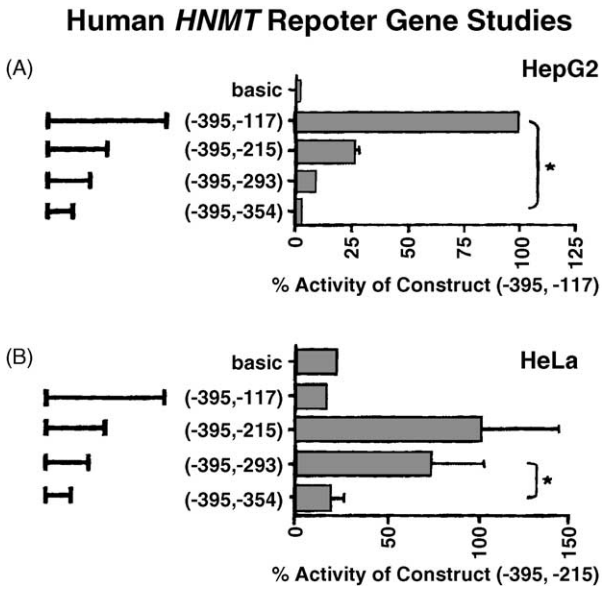

Fig. 3. *HNMT* core promoter region reporter gene construct data. Data for luciferase reporter gene constructs created by sequential 3′-deletions are shown. The upper (A) panel shows data for HepG2, and the lower (B) panel shows data for HeLa cells. Constructs with the highest activity in each cell line were defined as 100%, and activities after transfection with other constructs are expressed relative to that of the construct with the highest activity. Results are means ± SD for 6 independent transfections. Key: (∗) $P < 0.01$, with brackets showing comparisons for the statistical analyses.

more than 10-fold higher than for the (−395, −293) construct (Fig. 3A). These results demonstrated that, in HepG2 cells, the core promoter was located approximately (−294) to (−117) bp upstream from the "A" in the initial ORF codon [9]. In HeLa cells, deletion of the area from (−215) to (−294) did not decrease activity significantly, in part because of larger variance as a result of the lower level of luciferase expression in this cell line. However, luciferase activity in the HeLa cells did decrease to basal levels after deletion of the region from (−355) to (−293) (Fig. 3B), indicating that this region contained the *HNMT* core promoter.

In an attempt to confirm and extend these results obtained with reporter gene constructs, EMS assays were performed with probes representing the *HNMT* putative core promoter region for HepG2 cells. We performed the EMS assays only with HepG2 cells because that cell line had displayed high constitutive levels of HNMT activity as well as high levels of reporter gene activity after transfection with the *HNMT* 5′-FR constructs. The initial EMS assays were designed to determine whether the *HNMT* core promoter sequence identified by the reporter gene constructs was capable of binding proteins present in a cell preparation from HepG2 cells. The probe that we used extended from 5′-FR nucleotide (−294) to (−117). The EMS results indicated that multiple proteins bound to the probe (Fig. 4). Furthermore, this binding was competed by a non-radioactive probe in a concentration-dependent fashion (Fig. 4). These observations supported the results of the HepG2 reporter gene experiments and were compatible with the presence of a core promoter in this portion of the gene. Sequence analysis of this region of *HNMT*, performed with Transfac version 4.0, revealed several sequences that could potentially bind transcription factors, including AP1, Oct1, and TFIID.

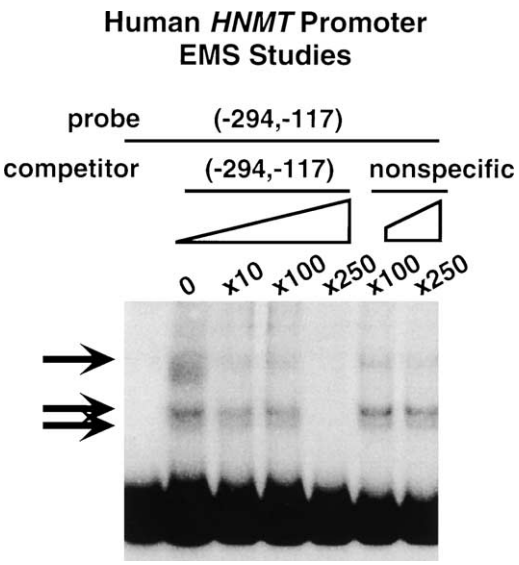

Fig. 4. *HNMT* promoter EMS assays. EMS assays were performed with probe (−294, −117) and HepG2 cell extracts. DNA–protein complexes are indicated by arrows. Competition studies were performed in the presence of increasing concentrations of nonradioactive specific and nonspecific probes (see Table 2).

3.3. 5′-FR SNP functional studies

Our initial reporter gene construct experiments (Fig. 2) had demonstrated that an area of the *HNMT* 5′-FR located approximately 500 bp 5′-upstream from the translation initiation codon was capable of driving reporter gene expression. Since we had observed a common SNP (frequency 0.367) within this region at nucleotide (−463), we next attempted to determine whether that SNP might alter *HNMT* transcription. Specifically, reporter gene constructs were created that included both the wild-type and variant sequences for this SNP, and those constructs were compared with regard to their ability to drive luciferase expression in both HepG2 and HeLa cells (Fig. 5). With the exception of the (−745, −50) constructs, no significant differences were observed between wild-type and variant sequences. The (−745, −50) constructs had significantly lower activity in both cell lines for the variant sequence. However, the magnitude of that effect, although statistically significant, was not striking (Fig. 5).

We also performed EMS assays with HepG2 cells using a probe that contained either the wild-type or variant allele sequences (see Table 2 for probe sequence). Once again, we elected to perform these studies only with HepG2 cells because of the higher levels of both basal HNMT activity and luciferase activity for reporter gene constructs in this cell line. The probes that we used formed complexes when incubated with HepG2 cell extract (Fig. 6). However, that binding appeared to be nonspecific since excess of both sequence-specific and nonspecific probes was able to compete with radioactively labeled probe (Fig. 6). Therefore, we concluded that, under these conditions, the common 5′-FR SNP did not appear to be critical for the binding of proteins present in the HepG2 cell preparation, compatible with results that we had obtained with the reporter constructs. However, even though the common 5′-FR SNP at nucleotide (−463) did not appear to have significant direct functional effects on transcription based on these *in vitro* studies, the possibility still existed that it might be linked with *HNMT* regulatory regions located at a distance from the site of transcription initiation. To determine whether the SNP at *HNMT* nucleotide (−463) might be associated with the level of human HNMT enzyme activity *in vivo*, we also performed genotype–phenotype correlation studies with human renal biopsy samples.

3.4. Genotype–phenotype correlation analysis

Genotype–phenotype correlation studies had been performed previously with 114 human kidney biopsy samples. Those studies resulted in the identification of the common *HNMT* exon 4 polymorphism at codon 105 that contributes to the regulation of the level of HNMT enzyme activity

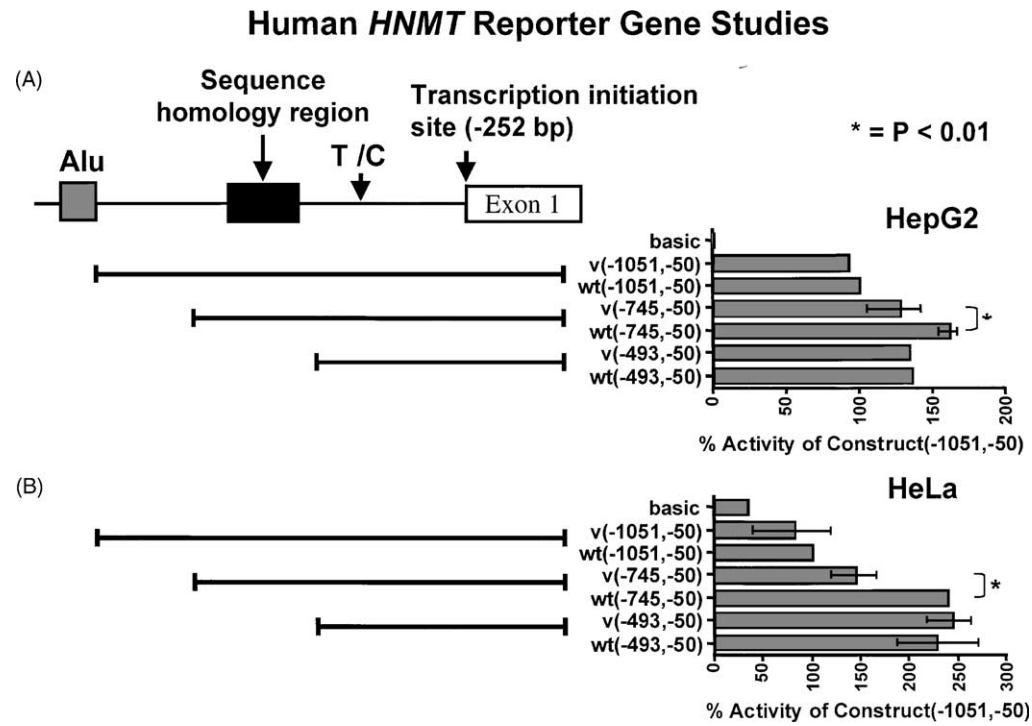

Fig. 5. *HNMT* 5′-FR reporter gene construct data for 5′-FR wild-type and variant allele sequences. Data for luciferase reporter gene constructs containing wild-type (wt) and variant allele (v) sequences for the 5′-FR SNP at nucleotide (−463) are shown. The upper (A) panel shows data for HepG2, and the lower (B) panel shows data for HeLa cells. Luciferase activity for the longest wild-type construct (−1051, −50) was defined as 100%, and activities after transfection with the other constructs were expressed relative to that of the longest construct. Results are means ± SD for 6 independent transfections. Key: (∗) $P < 0.01$, with brackets showing comparisons for the statistical analyses.

[10]. We set out to use those same tissue samples to test the possible association of the newly discovered common 5′-FR SNP with the level of HNMT activity. DNA from 112 of the original 114 kidney samples was still available for study. As a first step, the PCR was used to amplify the portion of the *HNMT* 5′-FR that contained the SNP using these 112 DNA samples as templates, and amplicons were sequenced to determine genotype at polymorphic nucleotide (−463). The variant allele frequency in these samples was 0.38, virtually identical to the frequency that we

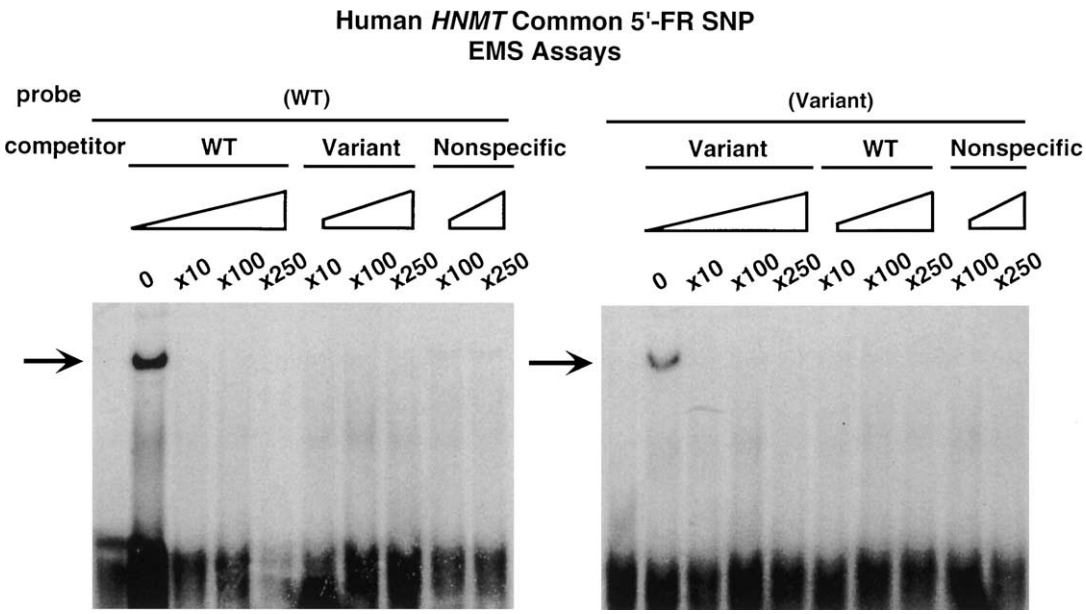

Fig. 6. *HNMT* 5′-FR SNP EMS assays. EMS assays were performed using probes containing wild-type (WT) or variant sequence for the common *HNMT* 5′-FR SNP at nucleotide (−463) and HepG2 cell extracts. Competition studies were performed with increasing concentrations of nonradioactive probes that contained either the WT or variant 5′-FR sequence. Competition studies were also performed with a nonspecific probe. See Table 2 for probe sequences.

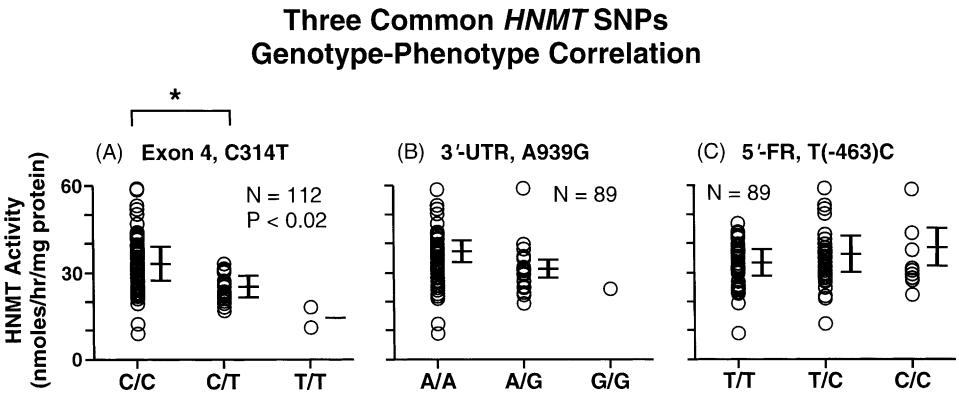

Fig. 7. *HNMT* genotype–phenotype correlation analyses for three common SNPs. Data for the *HNMT* (A) exon 4, (B) 3′-UTR, and (C) 5′-FR SNPs are shown. Relationships are depicted between *HNMT* genotype and enzyme activity in 112 renal biopsy samples for the exon 4 SNP, as well as 89 samples homozygous for the wild-type exon 4 allele for the common SNPs located in the 3′-UTR and 5′-FR. Key: (∗) $P < 0.02$ for the level of enzyme activity between genotypes. Mean activities ± SEM are indicated.

observed during our resequencing experiments with the PDR samples (Table 3). Specifically, DNA from 44 of the 112 samples was homozygous wild type (T/T), 50 samples were heterozygous (T/C), and 18 were homozygous for the variant allele (C/C). These genotype frequencies conformed to the predictions of the Hardy-Weinberg theorem ($P < 0.05$, by χ^2 analysis).

A genotype–phenotype correlation study had already been performed with these samples for the other two common *HNMT* SNPs, those in exon 4 and in the 3′-UTR [10]. We now attempted to determine the possible relationship between the new common 5′-FR SNP and the phenotype. Fig. 7 shows a scatter plot of the relationship between *HNMT* genotype and enzyme activity for each of these three common SNPs. There was, as anticipated, a significant relationship between enzyme activity and genotype for the exon 4 SNP ($P < 0.02$). Because this SNP influenced the level of activity, analysis of the possible effects of the 3′-UTR and 5′-FR SNPs was conducted only for samples homozygous for the wild-type exon 4 polymorphism (Fig. 7). By performing the analysis in that fashion, we were able to study these two SNPs in isolation, without the confounding effects of the exon 4 polymorphism. That analysis showed that neither the 5′-FR nor the 3′-UTR SNP was significantly associated with phenotype in samples homozygous for the wild-type exon 4 allele. These *in vivo* results confirmed the data that we had obtained with reporter gene constructs and indicated that the common polymorphism in the *HNMT* 5′-FR at nucleotide (−463) did not appear to be associated with significant functional consequences, at least with regard to the level of enzyme activity.

As a final step in our analysis, we also studied linkage among these three common *HNMT* SNPs using the 112 DNA samples from renal biopsies. Allele frequencies for the 5′-FR, exon 4, and 3′-UTR variant alleles in these 112 samples (224 alleles) were 0.38, 0.10, and 0.19, respectively. We then calculated pairwise D′ values, a measure of linkage that is independent of allele frequency [23,24]. D′ values can vary from +1.0 for two polymorphisms that are always linked to −1.0 for two that never occur together. We confirmed, as previously reported [10], that the exon 4 SNP was positively linked to the polymorphism in the 3′-UTR, with a D′ value of 0.88 ($P < 0.0001$). The exon 4 SNP was also positively linked to the 5′-FR SNP with a D′ value of 0.78 ($P < 0.001$). Since the exon 4 polymorphism was linked to both of the two other common *HNMT* SNPs, it was reasonable to anticipate that those SNPs might also be linked, and that is what we observed. The D′ value for the 5′-FR and 3′-UTR SNPs was 0.43 ($P < 0.01$).

4. Discussion

HNMT catalyzes the N^{τ}-methylation of histamine [1]. Levels of HNMT activity in human tissues are regulated primarily by inheritance [6,7]. We previously had identified a functionally significant nonsynonymous cSNP in *HNMT* exon 4 [10]. The presence of the variant allele for that SNP was associated with decreased levels of both HNMT enzyme activity and immunoreactive protein [10], and it represented a risk factor for asthma, with an odds ratio of approximately 2.0 [11,12]. However, the *HNMT* exon 4 polymorphism explained only a portion of the variance in the level of enzyme activity [10], raising the possibility that additional molecular genetic mechanisms might be involved in the regulation of this important methyl conjugating enzyme in humans. To test that hypothesis, in the present experiments we adopted a genotype-to-phenotype experimental strategy. The initial step in that strategy involved resequencing *HNMT* using 90 PDR human DNA samples. Those studies resulted in the identification of 8 polymorphisms located in different regions of the gene (Fig. 1, Table 3). However, only 3 SNPs, including a novel common polymorphism located in the 5′-FR, had allele frequencies of greater than 0.05 (5%). The other two common polymorphisms were the exon 4 and 3′-UTR SNPs that we had identified and characterized pre-

viously [10], thus serving to focus our attention on the 5′-FR of the gene. However, before we could study the possible functional implications of the new 5′-FR SNP, we first had to define gene sequences and areas involved in the regulation of *HNMT* transcription.

That process began by the use of 5′-FR reporter gene constructs transfected into HepG2 and HeLa cells to identify the core promoter and regulatory sequences for the gene (Figs. 2 and 3). HepG2 cells displayed higher levels of reporter gene activity than did HeLa cells transfected with the same constructs. That observation suggested that *HNMT* transcription might differ between HeLa and HepG2 cells, a conclusion compatible with the basal levels of HNMT activity that we observed in these two cell lines. Sequence analysis of the initial 1 kb upstream from the ''A'' of the *HNMT* translation initiation codon failed to show any obvious binding sites for common xenobiotic inducers (e.g. the Ah receptor complex), nor did that area contain canonical hormone response elements. Those observations were compatible with the lack of gender-dependent differences in levels of HNMT activity in human tissues such as liver, lung, colon, kidney, or red blood cells [6,10,29]. At that point, we moved on to systematically test the possible functional implications of the third common *HNMT* SNP that we had discovered during the resequencing studies, the 5′-FR polymorphism at nucleotide (−463). However, neither the results obtained with reporter gene constructs (Fig. 5) nor those obtained during the EMS assays (Fig. 6) supported a functional effect of the variant allele on *HNMT* transcription. Finally, results obtained during these *in vitro* assays were confirmed and extended by studies of a possible relationship between *HNMT* genotype at the 5′-FR nucleotide (−463) and the level of enzyme activity in human renal biopsy tissue (Fig. 7C). Those results also made it much less likely that the 5′-FR SNP might be linked to functionally significant polymorphisms, other than the exon 4 variant at ORF nucleotide 314 (Fig. 7A). Therefore, of the three common *HNMT* genetic polymorphisms with allele frequencies of 0.05 or greater, only the exon 4 SNP at nucleotide 314 has, thus far, been shown to have functional consequences [10].

In summary, we have utilized a genotype-to-phenotype strategy to perform pharmacogenetic studies of *HNMT* in humans. Gene resequencing experiments resulted in the identification of six novel *HNMT* SNPs, one of which, the 5′-FR SNP at nucleotide (−463), was common (Fig. 1, Table 3). To evaluate the possible functional implications of that SNP, we first had to study *HNMT* transcription regulation and to define the core promoter for the gene. We then systematically explored the possible functional effects of the common *HNMT* 5′-FR SNP. Although we failed to obtain evidence that this polymorphism was of functional significance, the experimental strategy that we applied represents a growing trend that involves the extension of pharmacogenetic studies ''beyond the ORF'' to include systematic studies of 5′-FRs for possible effects on transcription and of introns to study possible effects of polymorphisms on RNA splicing. The need for high throughput assays to apply to this type of functional genomics is evident. Finally, these studies represent an additional step forward in our understanding of the molecular mechanisms involved in the regulation of an important autacoid-metabolizing enzyme in humans, HNMT.

Acknowledgments

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