0026-895X/98/040708-10$3.00/0

MOLECULAR PHARMACOLOGY, **53:**708–717 (1998).

MOLECULAR PHARMACOLOGY

# Human Histamine *N*-Methyltransferase Pharmacogenetics: Common Genetic Polymorphisms that Alter Activity

CHARLES V. PREUSS, THOMAS C. WOOD, CAROL L. SZUMLANSKI, REBECCA B. RAFTOGIANIS, DIANE M. OTTERNESS, BLANKA GIRARD, MARY C. SCOTT,[1] and RICHARD M. WEINSHILBOUM

*Department of Pharmacology, Mayo Medical School, Mayo Clinic, Mayo Foundation, Rochester, Minnesota 55905*

Received September 16, 1997; Accepted January 7, 1998

This paper is available online at http://www.molpharm.org



## ABSTRACT

Histamine *N*-methyltransferase (HNMT) catalyzes a major pathway in histamine metabolism. Levels of HNMT activity in humans are regulated by inheritance. We set out to study the molecular basis for this genetic regulation. Northern blot analysis showed that HNMT is highly expressed in the kidney, so we determined levels of enzyme activity and thermal stability in 127 human renal biopsy samples. DNA was isolated from 12 kidney samples with widely different HNMT phenotypes, and exons of the *HNMT* gene were amplified with the polymerase chain reaction. In these 12 samples, we observed a C314T transition that resulted in a Thr105Ile change in encoded amino acid, as well as an A939G transition within the 3′-untranslated region. All remaining renal biopsy samples then were genotyped for these two variant sequences. Frequencies of the alleles encoding Thr105 and Ile105 in the 114 samples studied were 0.90 and 0.10, respectively, whereas frequencies for the nucleotide A939 and G alleles were 0.79 and 0.21, respectively. Kidney samples with the allele encoding Ile105 had significantly lower levels of HNMT activity and thermal stability than did those with the allele that encoded Thr105. These observations were confirmed by transient expression in COS-1 cells of constructs that contained all four alleles for these two polymorphisms. COS-1 cells transfected with the Ile105 allele had significantly lower HNMT activity and immunoreactive HNMT protein than did those transfected with the Thr105 allele. These observations will make it possible to test the hypothesis that genetic polymorphisms for HNMT may play a role in the pathophysiology of human disease.

HNMT (EC 2.1.1.8) is an Ado-Met-dependent cytosolic enzyme that catalyzes the $N^{\tau}$-methylation of histamine (Brown *et al*., 1959). Histamine plays an important role in allergy and anaphylaxis (Wasserman, 1983); it is involved in the regulation of gastric acid secretion (Loiselle and Wollin, 1993) and it is a neurotransmitter (Schwartz *et al*., 1991). *N*-Methylation catalyzed by HNMT and oxidative deamination catalyzed by diamine oxidase (EC 1.4.3.6) are the two major pathways for histamine biotransformation in mammals (Maslinski, 1975; Hough and Green, 1984). Because diamine oxidase is not expressed in the central nervous system (Burkard *et al*., 1963), *N*-methylation is the major process responsible for termination of the neurotransmitter actions of histamine in the brain (Schwartz *et al*., 1991); it is also the major pathway for the metabolism of histamine in bronchial epithelium (Okinaga *et al*., 1995). Previous biochemical genetic studies of HNMT activity in the human RBC demonstrated 5-fold individual variation in the level of this enzyme activity, predominantly as a result of a common genetic polymorphism (Scott *et al*., 1988; Price *et al*., 1993).

A cDNA for human kidney HNMT was cloned and expressed previously (Girard *et al*., 1994) as a step toward studies of molecular mechanisms involved in the genetic regulation of HNMT activity in humans. Subsequently, the gene for HNMT in humans also was cloned and localized to chromosome 2 (Aksoy *et al*., 1996). In the current study, our purpose was to use this information to determine the molecular basis for the HNMT genetic polymorphism in humans. We discovered two common polymorphisms, one of which altered encoded amino acid, that were correlated with level of HNMT activity in the human kidney and RBC. The discovery of common genetic polymorphisms for HNMT that are associated with altered levels of enzyme activity allows us to test the hypothesis that these polymorphisms might be related to a possible role for individual variation in histamine metabolism in the pathophysiology of diseases such as allergy, asthma, peptic ulcer disease, and neuropsychiatric illness.

This work was supported in part by National Institutes of Health Grants RO1-GM28157 (R.M.W.) and RO1-GM35720 (R.M.W.), by a supplement to Grant RO1-GM35720 supported by the Office of Research on Women's Health (R.M.W.), and by a National Research Scientist Award (National Institutes of Health) Individual Postdoctoral Fellowship (R.B.R.).

[1] Current affiliation: Department of Drug Metabolism, Schering-Plough Research Institute, Kenilworth, NJ 07033.

**ABBREVIATIONS:** HNMT, histamine *N*-methyltransferase; RBC, red blood cells; H/C, heated/control; PCR, polymerase chain reaction; UTR, untranslated region; COMT, catechol-*O*-methyltransferase; TPMT, thiopurine methyltransferase; Ado-Met, *S*-adenosyl-L-methionine; ORF, open reading frame.

## Materials and Methods

**Tissue acquisition and preparation.** Human renal tissue was obtained from 127 white subjects who underwent clinically indicated genitourinary surgery. Age, sex, and pathological diagnosis for these patients are given in Table 1. Blood samples for the preparation of RBC lysates were obtained from 32 of these patients. Renal tissue and blood samples were obtained under guidelines reviewed and approved by the Mayo Clinic Institutional Review Board. Both renal tissue samples and RBC lysates were stored frozen at −80°. Renal cortical cytosol preparations and RBC lysates were prepared as described previously (Pazmiño and Weinshilboum, 1978; Van Loon and Weinshilboum, 1990).

**HNMT activity and thermal stability.** HNMT activity was assayed in renal cytosol preparations and RBC lysates according to a modification of the method of Van Loon *et al*. (1985). This assay is based on the conversion of histamine to radioactively labeled $N^{\tau}$-methylhistamine with [*methyl*-$^{14}$C]Ado-Met or [*methyl*-$^{3}$H]Ado-Met as the methyl donor. Unless otherwise indicated, final concentrations of histamine and Ado-Met in the reaction were 37.5 and 12.5 $\mu$M, respectively, concentrations found to be optimal for measurement of the enzyme activity under these assay conditions (Boudíková-Girard *et al*., 1993; Girard *et al*., 1994). Blanks were samples that did not contain histamine. Enzyme thermal stability was measured as an H/C ratio and was calculated as the activity remaining in a heated sample divided by the activity in an unheated control sample. Renal cytosol was heated at 58° for 15 min, and RBC lysates were heated at 52° for 15 min to determine H/C ratios.

**Protein assay.** Protein concentrations were measured according to the method of Bradford (1976) with bovine serum albumin as a standard.

**HNMT antibodies and Western blot analyses.** Amino acid sequences located at both the amino and carboxyl termini of HNMT, corresponding to amino acids 1–20 and 264–283, respectively, were conjugated to keyhole limpet hemocyanin and were used to generate rabbit polyclonal antibodies (Cocalico Biologicals, Reamstown, PA). These two antisera, designed "N" for the antibody directed against the amino terminus and "C" for the antibody directed against the carboxyl terminus, respectively, were used to perform Western blot analyses with cytosolic proteins that had been separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Towbin *et al*., 1979). Bound antibody was detected by enhanced chemiluminescence with the ECL Western blotting system (Amersham, Arlington Heights, IL) as described previously (Aksoy *et al*., 1993). Western blot results were analyzed by densitometry performed with the Ambis optical system (Scanalytics, Billerica, MA).

TABLE 1
Age, gender, and pathological diagnosis for patients from whom the renal tissue used to perform these experiments was obtained

| | Human kidney samples | |
|---|---|---|
| | Women ($n$ = 49) | Men ($n$ = 78) |
| Age, (yr) (mean ± SD) | 61.9 ± 12.6 | 61.2 ± 12.3 |
| Range (yr) | 25–85 | 21–86 |
| Pathological diagnosis | | |
| Renal cell carcinoma | 38 | 56 |
| Ureteral transitional cell carcinoma | 1 | 3 |
| Renal pelvis transitional cell carcinoma | 1 | 8 |
| Pyelonephritis | 0 | 1 |
| Renal vein adventitial tumor | 1 | 0 |
| Angiomyolipoma | 2 | 0 |
| Renal oncocytoma | 2 | 3 |
| Leiomyosarcoma | 1 | 2 |
| Atherosclerosis of the renal artery | 0 | 1 |
| Adrenal cortical carcinoma | 1 | 0 |
| Renal cyst | 2 | 2 |
| Trauma | 0 | 1 |
| Adenocarcinoma of the prostate | 0 | 1 |

**Northern blot analyses.** Northern blot analyses were performed with Multiple Tissue Northern blots prepared by Clontech (Palo Alto, CA). Each lane contained ≈2 $\mu$g of poly(A)$^+$ RNA. The probe was the human HNMT cDNA ORF that had been labeled with [$\alpha$-$^{32}$P]dCTP through random priming (Feinberg and Vogelstein, 1983) with use of the Oligolabeling Kit (Pharmacia, Piscataway, NJ). Human $\beta$-actin cDNA also was used to probe the Northern blots to ensure approximately equal loading of each lane.

**HNMT PCR amplification and DNA sequencing.** Genomic DNA was isolated from 114 of the 127 human kidney biopsy samples with the QIAamp Tissue Kit (Qiagen, Chatsworth, CA). In the initial series of experiments, all exons encoding the *HNMT* ORF were amplified by use of the PCR with template DNA from 12 renal samples selected on the basis of very different renal HNMT phenotypes. Four of the samples selected had low HNMT activity and low thermal stability, 3 had high activity and high thermal stability, and 5 had intermediate activity. Sequences of the primers used to perform these amplifications, as well as those of all other primers described subsequently, are listed in Fig 1. PCR amplifications were performed in a Perkin-Elmer GeneAmp (Norwalk, CT) 2400 DNA Thermal Cycler with *Taq* DNA polymerase. Ampliwax Gem 100 beads were used to "hot start" the reactions. PCR mixtures were analyzed by electrophoresis on a 2% agarose gel, and another aliquot was diluted 10-fold and sequenced with the ABI PRISM Dye Primer Cycle Sequencing Kit with Amplitaq DNA polymerase, FS (Applied Biosystems, Foster City, CA). Dye primer cycle sequencing was chosen to analyze these initial 12 samples because heterozygous samples can be detected more reliably by using dye primer than with the more commonly used dye terminator method (Chadwick *et al*., 1996).

**Allele-specific restriction digestion.** Allele-specific restriction digestion was used to detect nucleotide polymorphisms discovered in the 12 initial renal samples as well as one additional variant sequence that had been reported previously. We detected two possible polymorphisms by sequencing the 12 initial renal samples. One of those variant sequences involved a C-to-T transition at nucleotide 314 within the ORF, and the other involved an A-to-G transition at nucleotide 939 in the 3′-UTR. Nucleotide numbers were assigned on the basis of their locations within the HNMT cDNA, with the A in the ATG translation initiation codon designated +1. Positive numbers were assigned to nucleotides located 3′, and negative numbers were assigned to those located 5′ to that initial position. The C314 allele could be differentiated from the T314 allele by *Eco*RV digestion of the exon 4 amplification product. The T314 allele was cleaved by *Eco*RV, whereas the alternative allele was not. Unfortunately, the A939G transition within the cDNA 3′-UTR did not alter a common restriction site. Therefore, we designed a primer, R912MUT, that would introduce an *Mfe*I restriction site within one of the alleles. Finally, there had been one previous report of an A595G sequence variant within *HNMT* exon 6 (Yamauchi *et al*., 1994). In this case, the G595 allele could be differentiated from that with A595 by *Tsp*45 I digestion of the exon 6 amplification product.

Whenever allele-specific restriction digestion assays were performed, control samples of known genotype were included with each assay to ensure that cleavage had occurred. However, because we had not observed the exon 6 variant sequence in any of our initial samples, the control for those experiments was created by site-directed mutagenesis performed with the overlap extension method of Ho *et al*. (1989). The primers used to perform the site-directed mutagenesis were I5F(−117) paired with R607MUT and F583MUT paired with R975, followed by an amplification performed with I5F(−117) paired with R975 (see Fig 1). The final amplification product was subcloned and sequenced to verify successful introduction of a G at nucleotide 595.

**COS-1 cell expression.** Four different HNMT expression constructs were synthesized for transient expression in COS-1 cells. Specifically, alleles that had the C314 sequence and encoded Thr105 were designated **1*, whereas the less frequent alleles with T314 that encoded Ile105 were designated **2*. Conversely, those alleles with

the A939 3′-UTR sequence were designated as *A*, whereas the less frequent alleles with G939 were designated *B*; the four possible alleles were designated **1A*, **1B*, **2A*, and **2B*. Each of these cDNA sequences either was already available in samples from our previous cDNA cloning studies, was obtained as an expressed sequence tag clone, or was created by overlap extension (Ho *et al*., 1989). We previously had cloned and expressed the *HNMT*2B* sequence (Girard *et al*., 1994); the *HNMT*1A* sequence was obtained in an expressed sequence tag clone, IMAGE CloneID 491112 (Lennon *et al*., 1996); and the **1B* and **2A* expression constructs were created by site-directed mutagenesis using **1A* and **2B* as starting material.

We had subcloned the **2B* sequence into pBluescript (Girard *et al*., 1994). That construct was used as template for the PCR performed with primers F(−33) and R1103, and the amplification product was subcloned into the eukaryotic expression vector pCR3.1 (InVitrogen, San Diego, CA). The *HNMT*1A* sequence was obtained from the American Type Culture Collection (Rockville, MD) in IMAGE CloneID 491112. The ORF of this clone was found to be truncated by 19 bp at the 5′-terminus; so it was necessary to use two sequential PCR amplifications to extend the 5′-end of the clone to the same length as that of the **2B* construct. Those reactions involved primer F1 paired with R1103, followed by an amplification performed with F(−33)L, also paired with R1103. This final amplification product also was subcloned into pCR3.1. Expression constructs for alleles **1B* and **2A* were created by overlap extension performed with the **1A* and **2B* constructs. Primer pairs for the initial reactions were F(−33) paired with R505 at the 5′-termini of both constructs, and F455 paired with R1103 at the 3′-termini. In both cases, overlap extension involved the use of primers F(−33) and R1103, and the final amplification products were subcloned into pCR3.1. All expression constructs were sequenced on both strands with dye terminator chemistry to ensure that no nucleotide mismatches had been introduced during the amplification reactions. The reactions primed with F(−33) and R1103 were performed with *Taq* DNA polymerase, whereas all other amplifications were performed with native *Pyrococcus furiosus* DNA polymerase. The final expression constructs contained the sequences for all four *HNMT* alleles that we had observed, **1A*, **1B*, **2A*, and **2B*.

COS-1 cells were transfected with 2 μg of DNA for each of the expression constructs as well as with expression vector that lacked insert by use of the DEAE-dextran method (Luthman and Magnusson, 1983) as described elsewhere (Wood *et al*., 1994). In all cases, the cells were also transfected with 1 μg of DNA from the pSV-β-galactosidase control vector (Promega, Madison, WI) to make it possible to correct the results for transfection efficiency. The COS-1 cell supernatant preparations were assayed for β-galactosidase activity with *O*-nitrophenyl-β-D-galactopyranoside as substrate as described by Rosenthal (1987).

**Data analysis.** The Genetics Computer Group (Madison, WI) software package Version 8.0 (Devereux et al., 1984) was used to analyze nucleotide sequences. Apparent $K_m$ values were calculated according to the method of Wilkinson (1961) with a computer program written by Cleland (1963). $T_{50}$ (temperature resulting in 50% inactivation) and $IC_{50}$ values were calculated with InPlot (GraphPAD Software, San Diego, CA). Linkage disequilibrium was tested with the EH Program by using the population-based sampling method described by Terwilliger and Ott (1994). Statistical comparisons of parametric data were performed by the use of Student's *t* test, analysis of variance, or the Pearson product moment correlation coefficient with the StatView program, Version 4.5 (Abacus Con-

| Primer Designation | Primer Location | Primer Sequence |
|---|---|---|
| **EXON SEQUENCE DETERMINATION** | | |
| -21M13F | ----- | TGTAAAACGACGGCCAGT |
| M13R | ----- | CAGGAAACAGCTATGACC |
| [ F(-33) | 5′-UTR | GCTTCCTGCTCTGTCTTTCTCAGAAAACC |
| I1R58 ] | INTRON 1 | CACTGACGCACAGCCAGTCTGAGGC |
| [ I1F(-64) | INTRON 1 | GATAATCAGATTTCACAAAGCACCTAACAC |
| I2R67 ] | INTRON 2 | CAATATTGTCATCACACAGCATAATGGTTAAAGTGAAATATG |
| [ I2F(-83) | INTRON 2 | GGGCAGATAATAAATCAGCTAAA |
| I3R67 ] | INTRON 3 | AAGGGAACAATTTGAGTTAAGTC |
| [ I3F(-119) | INTRON 3 | GAAAAACGTTCTTTCTATCTGTTTGTATATAA |
| I4R191 ] | INTRON 4 | TTGGAATGTTAAAGAGAATCTTAGTATAATA |
| [ I4F(-73) | INTRON 4 | AGGAGTATCTAGCCCAAGCAATA |
| I5R71 ] | INTRON 5 | TCAAACACAGGAATCCATGCATAC |
| [ I5F(-117) | INTRON 5 | GCTGCACAAAGGACAAGATTATT |
| R975 ] | 3′-UTR | TACATTAATGGATGAGTTTGTGATTTTAATA |
| **SITE DIRECTED MUTAGENESIS** | | |
| F(-33)L* | 5′-UTR | GCTTCCTGCTCTGTCTTTCTCAGAAAACCAAATATGGCATCTTCCATGA |
| F1* | EXON 1 | ATGGCATCTTCCATGAGGAGCTTGTTTTCTGACCAC |
| F455* | EXON 5 | CAGCTACCCTGAAATTCTTCCATAGTCTCTTAGG |
| R505* | EXON 5 | GCATCTTAGCATTGGTACCTAAGAGACTATGG |
| F583MUT* | EXON 6 | CTCTGCCAGTATGTCACATCAGATG |
| R607MUT* | EXON 6 | CATCTGATGTGAATACTGGCAGAG |
| R912MUT* | 3′-UTR | ATTTTGAACAACTGAATCACTCAATT |
| R1103* | 3′-UTR | GCCTGCATGTGGTCTCTCTACCTGGAGG |

**Fig. 1.** Sequences of primers used to perform PCR amplifications. Eighteen nucleotides of −21M13F or M13R sequences were added to the 5′-ends of the forward and reverse primers, respectively, that were used to determine exon sequences. The M13 sequences were added to make it possible to use dye primer DNA sequencing chemistry (see Materials and Methods). *I*, Intron. *F*, Forward. *R*, Reverse. *MUT*, Variant sequence introduced into primers to perform site-directed mutagenesis. *Underlined*, nucleotides that were altered in the MUT primers. ∗, Primers that did not contain the M13 sequences. *Brackets*, primer pairs that were used to amplify exons of the *HNMT* gene.

cepts, Berkeley, CA). Statistical comparisons of nonparametric data were performed by use of the Spearman rank correlation coefficient or the Mann-Whitney *U* test with the Multi-Function Statistics Library, Version 3.1 (Northwest Analytical, Portland, OR).

## Results

The approach used to study the molecular basis for the genetic regulation of HNMT in humans began with a determination of level of HNMT activity and thermal stability [a sensitive measure of variation in amino acid sequence (Weinshilboum, 1981)] in a large number of human kidney samples, a tissue in which we confirmed by Northern blot analysis that HNMT was highly expressed. DNA then was isolated from selected renal samples with widely differing HNMT phenotypes, and all exons encoding the HNMT cDNA ORF were sequenced. Two variant nucleotide sequences were detected, one of which altered encoded amino acid. All of the kidney samples were then genotyped for these two polymorphisms, and a significant relationship was demonstrated between *HNMT* genotype and HNMT phenotype. Finally, sequences of cDNAs encoded by the four *HNMT* alleles detected in the course of the study were expressed in COS-1 cells. The results of those experiments confirmed the relationship between *HNMT* genotype and levels of both HNMT activity and thermal stability.

**Northern blot analysis.** HNMT mRNA could be detected in most of the tissues studied by Northern blot analysis, but kidney and liver showed particularly high levels of expression (Fig. 2). However, there also was significant expression in spleen, prostate, ovary, colon, and spinal cord. The possible functional implications of this widespread tissue expression remain to be determined. Because HNMT enzymatic activity in the kidney also is high relative to that in other tissues (Brown *et al*., 1959) and because optimal conditions for the assay of HNMT activity in human renal cytosol had been determined (Boudíková-Girard *et al*., 1993; Girard *et al*., 1994), the kidney was chosen as the tissue in which to study possible HNMT genotype/phenotype correlations.

**HNMT phenotype in renal tissue.** HNMT activity and thermal stability were determined in 127 renal biopsy samples obtained during clinically indicated surgery (Table 1). DNA was available from only 114 of these samples, and blood was obtained from 32 of the patients. The relationship between level of human kidney HNMT activity and thermal stability (expressed as an H/C ratio) in the 114 samples that subsequently were genotyped is depicted graphically in Fig. 3A. The mean level of HNMT activity in all 127 of the samples phenotyped was 31.6 ± 8.6 nmol/hr/mg of protein (mean ± standard deviation), with a range within ±3 standard deviations that varied 6-fold, from 9.1 to 59.0 nmol/hr/mg of protein. Levels of HNMT activity did not differ significantly between women and men (32.9 ± 9.4 for women and 30.9 ± 8.1 for men, mean ± standard deviation; $p = 0.164$), and there was no significant correlation of activity with either patient age ($r_s = -0.01, p > 0.8$) or the time that the renal tissue had been stored frozen ($r_s = -0.12, p = 0.15$). H/C ratios, like levels of activity, also failed to show significant differences between the sexes [0.50 ± 0.06 for women and 0.50 ± 0.05 for men ($p = 0.6152$ by Mann-Whitney *U* test)] or a significant correlation with patient age ($r_s = 0.05$,

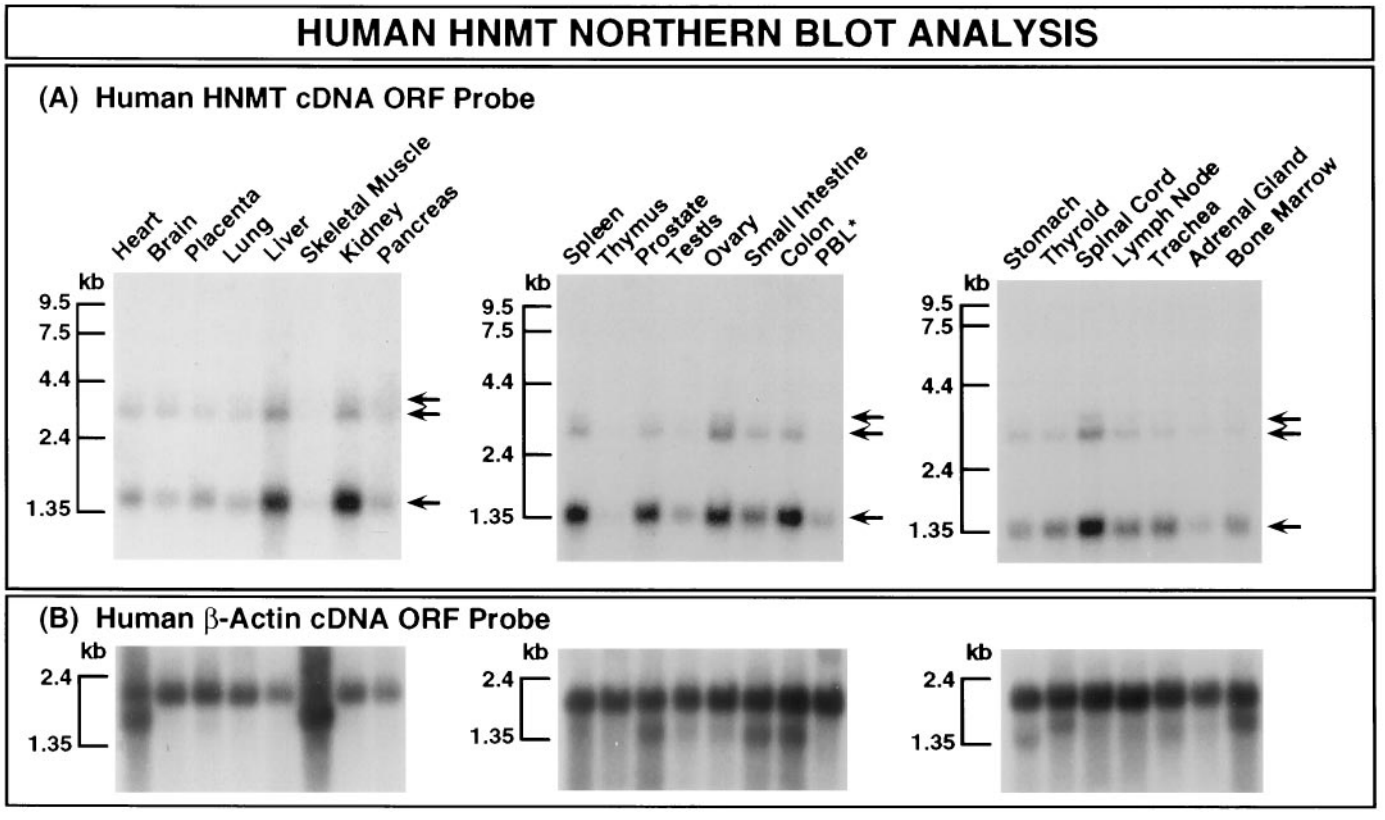


**Fig. 2.** HNMT Northern blot analysis. Each *lane* contained ≈2 μg of poly(A)$^+$ RNA. A, The human HNMT cDNA ORF was used as a probe. *Arrows*, HNMT mRNA species of different lengths. B, Human β-actin was used as a probe to ensure approximately equal loading of each lane. *PBL*, peripheral blood leukocyte.

$p = 0.5$). It should be noted that samples with the lowest H/C ratios also had the lowest levels of enzyme activity (Fig. 3A), a phenomenon that we have observed previously during studies of another genetically polymorphic human methyltransferase enzyme, COMT (EC 2.1.1.6) (Scanlon *et al.*, 1979; Boudíková *et al.*, 1990; Lachman *et al.*, 1996). Enzyme thermal stability is commonly measured in biochemical genetic experiments (Weinshilboum, 1981), but caution must always be exercised in the interpretation of these data. However, it is unlikely that the relationship between level of activity and thermal stability shown in Fig. 3A is an artifact due to enzyme "inactivation" because level of activity also correlated with levels of immunoreactive HNMT protein both for these tissue biopsy samples, and, as will be described subsequently, after the transient expression of different HNMT alleles in COS-1 cells.

**HNMT polymorphism detection.** DNA from 4 kidney samples with low activity and low thermal stability, 3 samples with high activity and high thermal stability, and 5 samples with intermediate activity (Fig. 3A, *circled*) were selected for the initial studies of possible phenotype/genotype correlation. However, as a first step, Western blot analyses of cytosol preparations from these 12 samples were performed with two different anti-HNMT antibodies: one directed against the amino terminus (antibody-N) and the other directed against the carboxyl terminus (antibody-C). There was a highly significant correlation between HNMT enzymatic activity and immunoreactive protein in these 12 renal samples for both antibodies (Fig. 4). These observations will be referred to when the results of the COS-1 cell expression experiments are described.

DNA then was isolated from these same 12 samples and was used to determine the sequences of *HNMT* exons that encoded the cDNA ORF. Those studies demonstrated the presence of a C-to-T transition at nucleotide 314 within exon 4 that resulted in a Thr105Ile change in encoded amino acid.

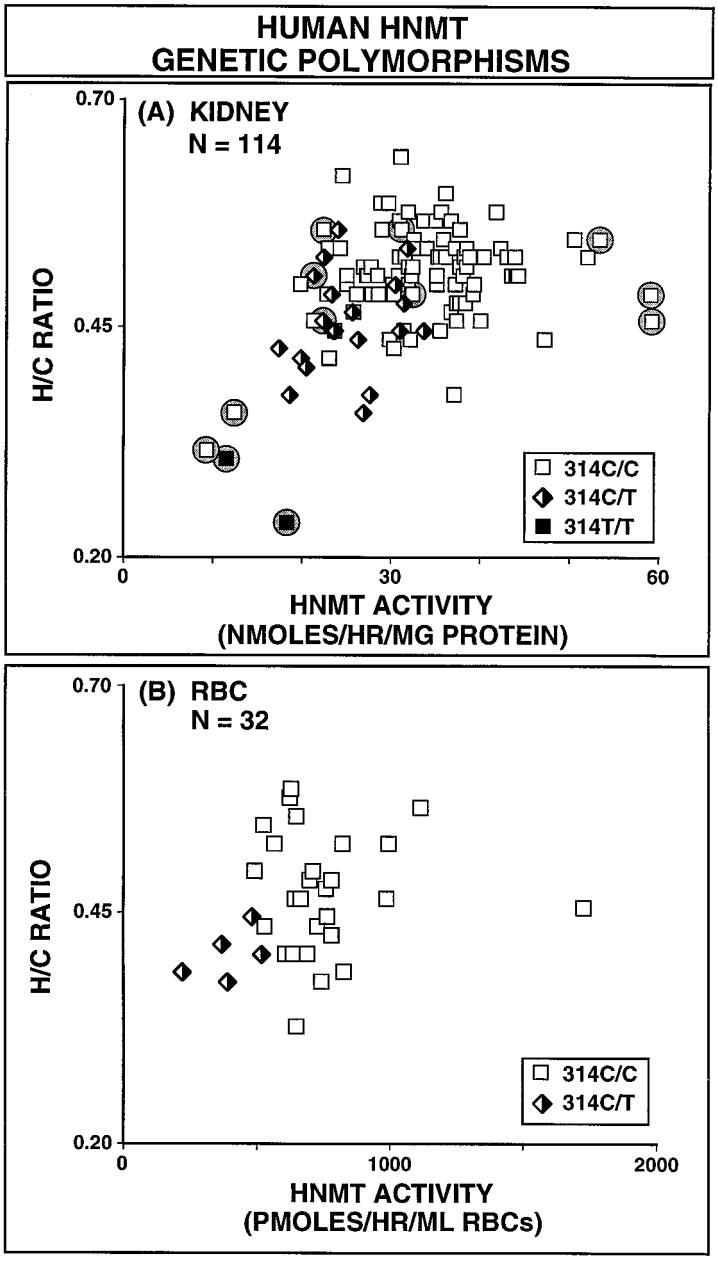


**Fig. 3.** Human kidney and RBC HNMT activity and thermal stability and HNMT genotype/phenotype correlations for the nucleotide 314, codon 105 genetic polymorphism. The relationship among *HNMT* nucleotide 314 genotype, enzyme activity, and thermal stability is shown for (A) 114 renal biopsy samples and (B) 32 RBC lysate samples. *Circled*, samples used to perform the initial DNA sequencing experiments in which the entire ORF of the *HNMT* gene was sequenced.

Two of the 4 samples with both low HNMT activity and low thermal stability were homozygous for the T314 allele. That same variant sequence at nucleotide 314 had been noted previously in HNMT cDNA and gene sequences (Girard *et al*., 1994; Aksoy *et al*., 1996). An A595G transition within exon 6 that would result in a change of encoded amino acid from Ile199Val also had been reported in one cDNA sequence from a Japanese subject (Yamauchi *et al*., 1994). We did not observe that variant sequence in either the initial 12 samples or, as described subsequently, in any of the total of 114 renal samples studied. However, we did observe an A939G transition within the 3′-UTR of the HNMT cDNA in 5 of the initial 12 samples. As the next step in the analysis, we used allele-specific restriction digestion assays for the exon 4 and the 3′-UTR variant sequences that we had observed, as well as the exon 6 variant reported by Yamauchi *et al*. (1994) that we had failed to observe in the initial 12 samples, to determine *HNMT* genotypes for DNA isolated from all available renal samples.

***HNMT* allele frequencies.** Allele-specific restriction digestion was used to determine *HNMT* genotype in the 102 additional renal samples for which DNA was available. The T314 sequence for the exon 4 polymorphism could be cleaved by *Eco*RV digestion, whereas the C314 sequence was not digested. The variant sequence within the 3′-UTR at nucleotide 939 did not alter a common restriction site, so the sequence of the primer used to perform that PCR amplification was altered to introduce an *Mfe*I site if G939 was present. *Mfe*I digestion then was used to differentiate the two nucleotides at position 939. Finally, the exon 6 amplification product could be cleaved by *Tsp*45 I restriction digestion if the G595 variant sequence that had been reported by Yamauchi *et al*. (1994) was present; therefore, *Tsp*45 I restriction digestion was used to search for that variant sequence. When these three allele-specific restriction digestion assays were performed with DNA from the 102 remaining renal DNA samples available, 2 of the 114 samples studied were homozygous for the T314 variant that encoded Ile105, and 20 samples were heterozygous for that allele. That distribution of genotypes fits the predictions of the Hardy-Weinberg theorem for the observed allele frequencies of 0.90 for C314 and 0.10 for the less common T314 sequence. Five of the 114 samples studied were homozygous for G939, and 38 were heterozygous for that allele. Those values also approximated the predictions of the Hardy-Weinberg theorem based on the observed frequencies of 0.79 for A939 and 0.21 for the less common G939 allele. Therefore, both the exon 4 and 3′-UTR variant sequences represented common polymorphisms in this population sample (Cavalli-Sforza and Bodmer, 1971). None of these 114 samples contained the exon 6 variant sequence reported previously in one sample from a Japanese subject (Yamauchi *et al*., 1994).

**HNMT genotype/phenotype correlation.** When genotype for the exon 4, codon 105 polymorphism was plotted on the scattergram shown in Fig. 3A, that genotype was correlated with HNMT phenotype in these human renal tissue

TABLE 2
*HNMT* genotype at nucleotides 314 and 939 and their relationship to phenotype

| Tissue | Genotype | N | Renal activity | H/C ratio |
|---|---|---|---|---|
| Kidney | *314C/C* | 92 | 33.6 ± 0.8[a] | 0.51 ± 0.005[a] |
| | *314C/T* | 20 | 24.9 ± 1.0 | 0.45 ± 0.012 |
| | *314T/T* | 2 | 14.8 | 0.27 |
| RBC | *314C/C* | 27 | 744 ± 46[b] | 0.47 ± 0.013[b] |
| | *314C/T* | 5 | 385 ± 51 | 0.41 ± 0.012 |
| Kidney | *939A/A* | 71 | 34.3 ± 0.9[c] | 0.51 ± 0.006[c] |
| | *939A/G* | 38 | 28.4 ± 1.2[c] | 0.48 ± 0.009 |
| | *939G/G* | 5 | 20.2 ± 2.5[c] | 0.42 ± 0.68 |
| RBC | *939A/A* | 21 | 740 ± 58 | 0.48 ± 0.016 |
| | *939A/G* | 10 | 609 ± 71 | 0.44 ± 0.013 |
| | *939G/G* | 1 | 378 | 0.38 |

Values for HNMT activities and H/C ratios are mean ± standard error. Renal activity is given in nmol/hr/mg of protein, and RBC activity is given in pmol/hr/ml of RBCs.
[a] $p < 0.0001$ compared with values for heterozygous renal samples.
[b] $p = 0.03$ compared with values for heterozygous RBC samples.
[c] $p < 0.05$ compared with the other two genotypes for that polymorphism within the renal samples.

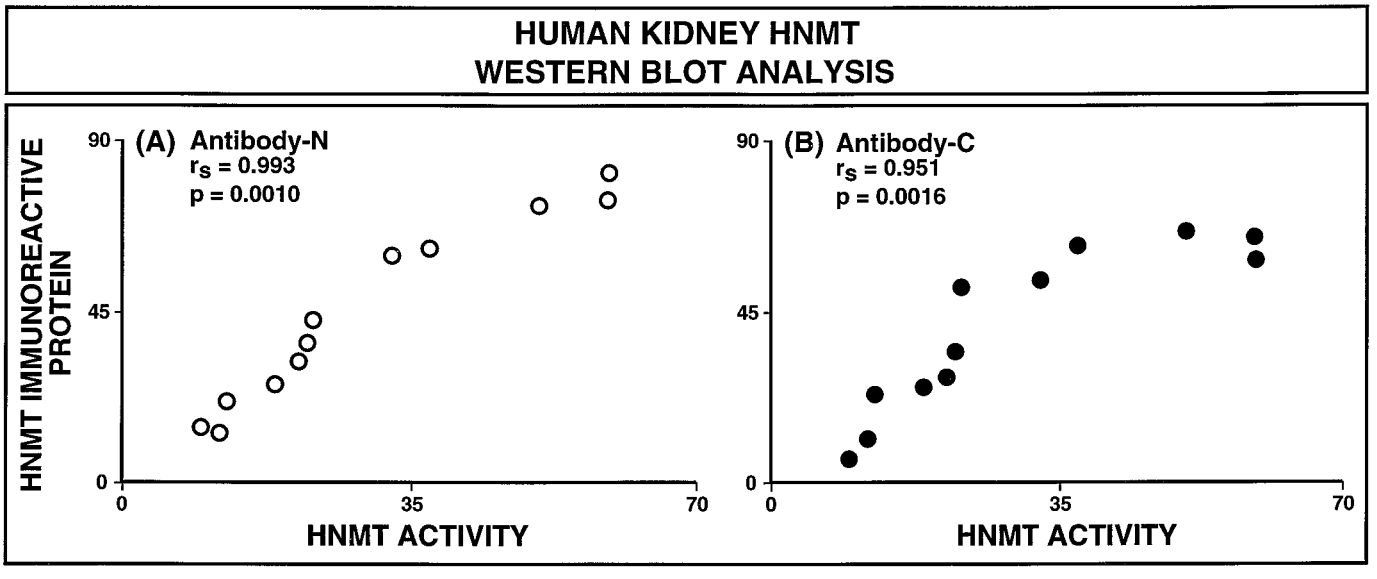


**Fig. 4.** HNMT Western blot analysis of 12 selected human renal samples. A, Correlation of HNMT enzymatic activity and immunoreactive protein after Western blot analysis performed with HNMT antibody-N, directed against the amino terminus of the protein, for the 12 *circled* samples in Fig. 2A. B, Correlation of HNMT enzymatic activity and immunoreactive protein for the same 12 renal samples when the Western blots were probed with HNMT antibody-C, directed against the carboxyl terminus of the protein.

samples. Specifically, the two samples with the lowest H/C ratios, both of which were also among the samples with the lowest levels of activity, were homozygous for the T314 allele that encoded Ile105. Furthermore, heterozygous samples clustered at a lower average level of activity than did those homozygous for C314, with the allele encoding the more common Thr105 variant (Fig. 3A). Although RBC lysates were available for less than one third of these patients, a similar genotype/phenotype relationship was present in the RBC lysates (Fig. 3B). The genotype/phenotype correlation depicted graphically in Fig. 3 was confirmed by statistical analysis of the results (Table 2). The data listed in Table 2 showed a statistically significant decrease in both HNMT activity and thermal stability in samples with the allele that encodes Ile105. However, the genetic polymorphism within exon 4 did not entirely account for HNMT phenotypic variance because there was overlap among phenotypes for this polymorphism (Fig. 3).

When a similar analysis was performed for nucleotide 939 within the 3′-UTR, that polymorphism was less clearly correlated with HNMT phenotype than was the nucleotide 314 polymorphism (Fig. 5, A and B). However, statistical analysis of the relationship between *HNMT* genotype for the 3′-UTR polymorphism and HNMT phenotype in renal samples and RBC lysates showed that the G939 allele also was associated with a statistically significant decrease of both of these variables in renal tissue, but there were no statistically significant differences for the smaller number of RBC lysates (Table 2). An analysis of possible linkage disequilibrium between the exon 4 and 3′-UTR polymorphisms then was performed (Terwilliger and Ott, 1994). The hypothesis of no association between the two polymorphisms was rejected, but the hypothesis of association was supported with $\chi^2 = 62$ ($p < 0.0001$). Therefore, the analysis demonstrated linkage disequilibrium between the two polymorphisms, making it dif-

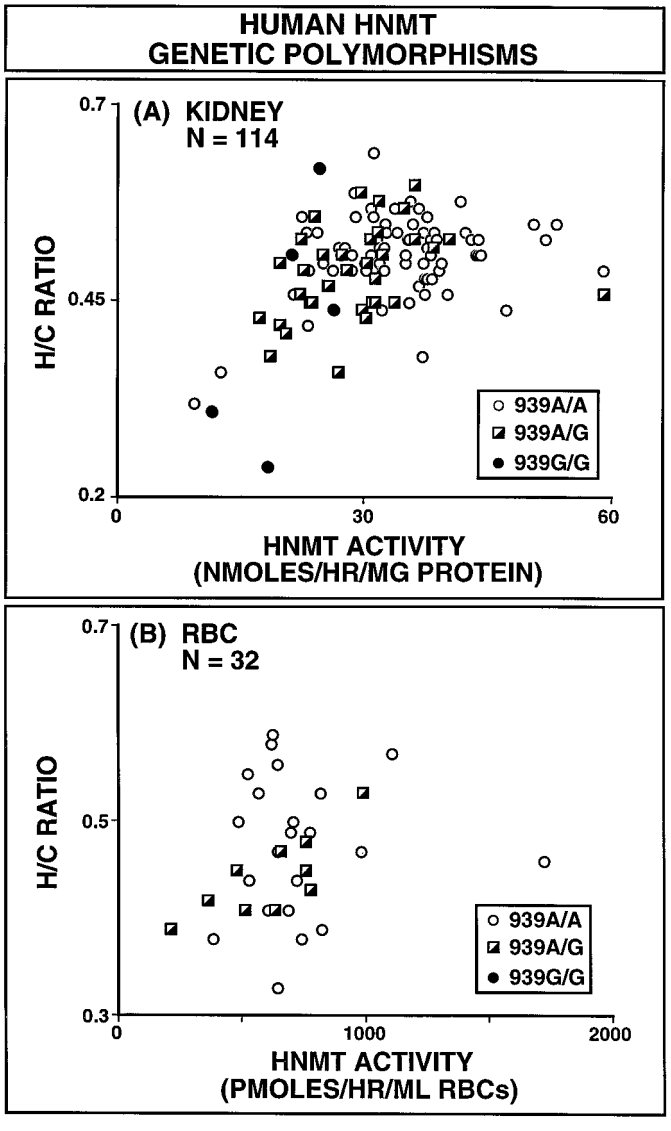


**Fig. 5.** HNMT genotype/phenotype correlations for the nucleotide 939 3′-UTR genetic polymorphism. The relationship among *HNMT* genotype, enzyme activity, and thermal stability is shown for (A) 114 renal biopsy samples and (B) 32 RBC lysate samples.

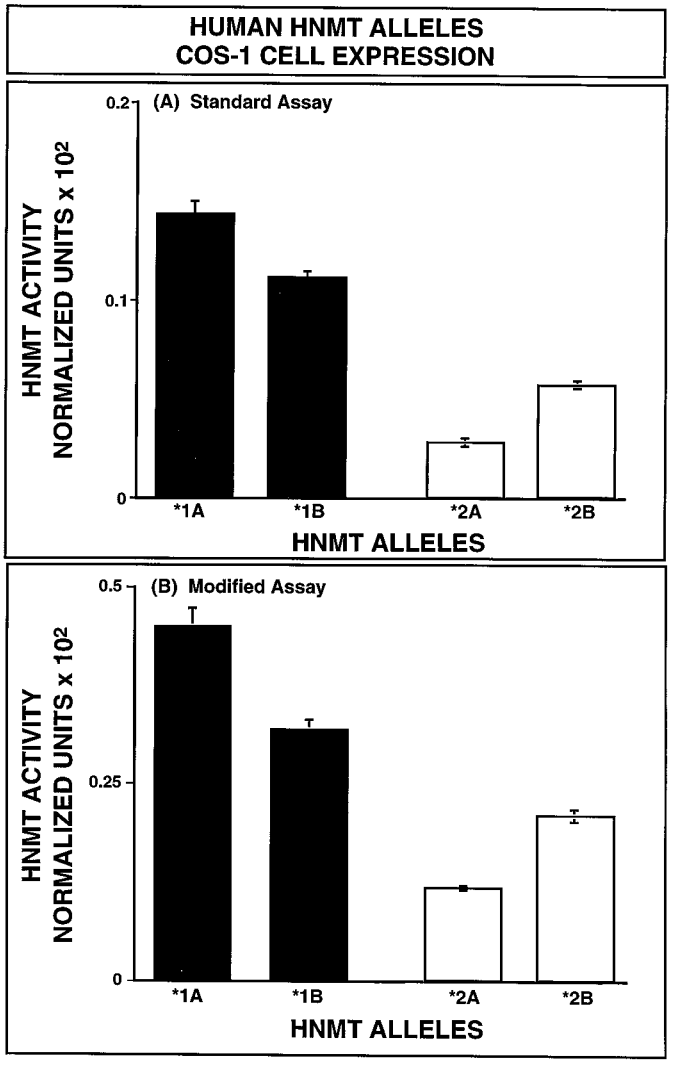


**Fig. 6.** Recombinant human HNMT level of activity. A, Average levels of HNMT activity after transient expression in COS-1 cells are shown for each of the four HNMT alleles (mean ± standard error, six experiments). All values have been corrected for transfection efficiency. Values for the four recombinant proteins encoded by the four *HNMT* alleles were significantly different from each other ($p < 0.001$ by analysis of variance). B, The same preparations were assayed with higher histamine and Ado-Met concentrations than those used in the experiments shown in A (see text for details). Values for the four alleles were significantly different from each other ($p < 0.001$).

ficult to determine in tissue samples whether the 3′-UTR polymorphism had any effect on HNMT phenotype independent of that due to the codon 105 polymorphism that altered the encoded amino acid.

**COS-1 cell expression.** The four *HNMT* alleles identified during these studies then were transiently expressed in COS-1 cells. Transfection with both constructs that encoded Thr105, alleles **1A* and **1B*, resulted in significantly higher levels of enzyme activity than did transfection with the two alleles that encoded Ile105, **2A* and **2B* (Fig. 6A). However, the effect of the nucleotide 939 polymorphism was variable, with slightly decreased activity for G939 in the presence of Thr105 and slightly increased activity for G939 in the presence of Ile105 (Fig. 6A). These initial assays were performed under optimal conditions for the measurement of HNMT activity in human renal preparations (Boudíková-Girard *et al*., 1993; Girard *et al*., 1994). That fact will become significant in the context of the subsequent results of substrate kinetic experiments performed with these recombinant enzymes.

TABLE 3

Recombinant human HNMT substrate kinetics and thermal inactivation

Apparent $K_m$ values and temperatures resulting in 50% inactivation after a 15-min preincubation ($T_{50}$) are listed.

| Allele | $K_m$ | | $T_{50}$ | |
|---|---|---|---|---|
| | Histamine | Ado-Met | Standard assay | Modified assay |
| | *μM* | | *degrees* | |
| *HNMT*1A* | 25.3 ± 0.25 | 10.0 ± 0.21 | 57.2 ± 0.13 | 55.9 ± 0.03 |
| *HNMT*1B* | 23.4 ± 0.28 | 8.2 ± 0.14 | 56.9 ± 0.03 | 56.1 ± 0.03 |
| *HNMT*2A* | 35.0 ± 2.07 | 11.7 ± 1.05 | 54.6 ± 0.10 | 52.3 ± 0.30 |
| *HNMT*2B* | 35.5 ± 1.55 | 13.0 ± 0.52 | 54.2 ± 0.26 | 50.9 ± 0.46 |

All values are mean ± standard error for three determinations.

$K_m$ values for histamine for both of the proteins encoded by **1* constructs were significantly different from those encoded by **2* constructs ($p < 0.01$ by ANOVA).

$T_{50}$ values for proteins encoded by **1* constructs differed significantly from those for proteins encoded by **2* constructs ($p < 0.01$ by ANOVA).

Please see text for an explanation of the "standard" and "modified" assays.

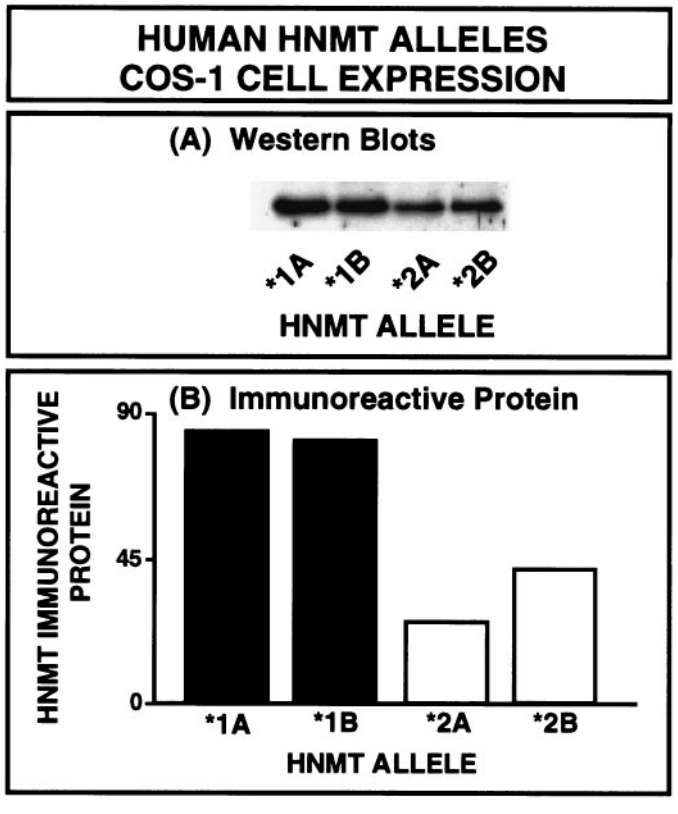


**Fig. 7.** Recombinant human HNMT immunoreactive protein. A, Western blot analysis of recombinant proteins for each of the four *HNMT* alleles after transient expression in COS-1 cells. The blots were probed with antibody-N, directed against the amino terminus of the proteins. B, Average values for densitometric analysis of duplicate Western blots for each of the four *HNMT* alleles after transient expression in COS-1 cells.

The recombinant HNMT proteins were used to perform substrate kinetic and thermal inactivation experiments. Six concentrations of histamine that ranged from 2.3 to 75.0 μM were used to perform substrate kinetic studies, and double inverse plots were constructed. Apparent $K_m$ values of all four constructs for histamine were similar, but those for constructs that encoded Ile105 were slightly but significantly higher than were those for constructs that encoded Thr105 (Table 3). Furthermore, all of these apparent $K_m$ values for histamine were significantly higher than were those measured in human renal preparations (Girard *et al*., 1994). A similar situation was found when Ado-Met was the varied cosubstrate. Apparent $K_m$ values for Ado-Met of proteins encoded by the four constructs were similar (Table 3) but slightly higher for constructs that encoded Ile105 than for those that encoded Thr105. Apparent $K_m$ values of the recombinant proteins for Ado-Met also were higher than those reported when the activity was measured in human kidney preparations (Girard *et al*., 1994). Therefore, the activities of the recombinant proteins were measured again at higher concentrations of histamine (300 μM) and Ado-Met (50 μM) to ensure saturation of the enzymes with respect to both cosubstrates. The results obtained with this "modified assay" were qualitatively identical to those obtained initially (Fig. 6B). No detectable endogenous HNMT activity was present in the COS-1 cells under either set of assay conditions. Furthermore, when either the original assay or the modified assay was used to measure the temperature at which these recombinant enzymes were 50% inactivated, results with both assay conditions demonstrated that constructs that encoded Ile105 were significantly more thermolabile than were those that encoded Thr105 (Table 3). Because differences in kinetic properties did not seem to explain the differences in activity among the recombinant HNMT proteins, it was possible that differences in protein quantity might do so, just as had been found for the renal biopsy samples (Fig. 4). When Western blots were performed with the recombinant enzymes, the results were similar to those obtained by the measurement of enzyme activity and showed higher HNMT immunoreactive protein levels in preparations obtained from cells transfected with the alleles that encoded Thr105 than after transfection with alleles that encoded Ile105 (Fig. 7). All of these results demonstrated that the genotype/phenotype correlation for HNMT present in human renal tissue could also be demonstrated during transient expression in COS-1 cells.

## Discussion

The physiology and pharmacology of histamine were first described early in this century by Sir Henry Dale and his

coworkers (Dale and Laidlaw, 1910). Histamine now is known to be a neurotransmitter (Hough and Green, 1984; Schwartz *et al.*, 1991); it plays an important role in allergic responses (Wasserman, 1983) and it is involved in the regulation of gastric acid secretion (Loiselle and Wollin, 1993). One of the two major metabolic pathways for histamine, and the only mechanism for the termination of its neurotransmitter actions, is $N^{\tau}$-methylation catalyzed by HNMT (Schwartz *et al.*, 1991). Therefore, differences among individuals in the methylation of histamine could potentially have implications for the pathophysiology of asthma, peptic ulcer disease, and neuropsychiatric illness. Previous biochemical genetic studies demonstrated that individual variation in HNMT activity in an easily accessible human cell type, the RBC, was regulated by a common genetic polymorphism (Scott *et al.*, 1988; Price *et al.*, 1993). The current study was performed in an attempt to determine the molecular basis for the effects of inheritance on HNMT activity in humans. We discovered two common *HNMT* polymorphisms, including a C314T transition within exon 4 that resulted in a change in encoded amino acid from Thr105 to Ile, that were associated with decreased enzyme activity, immunoreactive protein, and thermal stability in renal biopsy samples, observations that we confirmed with transient expression experiments performed with COS-1 cells.

These observations for HNMT are similar to those made during studies of genetic polymorphisms for other cytosolic methyltransferase enzymes in humans. For example, levels of activity for both the catecholamine-metabolizing enzyme COMT and the thiopurine drug-metabolizing enzyme TPMT (EC 2.1.1.67) in humans are regulated by common genetic polymorphisms that alter encoded amino acids (Lachman *et al.*, 1996; Szumlanski *et al.*, 1996). In the case of TPMT, those differences in amino acid sequence are, as we report here for HNMT, correlated with levels of immunoreactive protein (Szumlanski *et al.*, 1996). The underlying mechanism responsible for these observations could involve amino acid sequence-dependent differences in protein stability or differences in rates of protein degradation. That possible mechanism for the HNMT polymorphism will have to be explored in the course of future studies. In the cases of both COMT and TPMT, inherited variations in levels of enzyme activity also are correlated with large variations in function (i.e., the metabolism of drugs that are substrates for these two methyltransferase enzymes) (Campbell *et al.*, 1984; Lennard *et al.*, 1987). In summary, the results of the current study provide at least a partial molecular explanation for the genetic regulation of HNMT in humans. It will now be possible to study the influence of inherited variation in HNMT activity on the pathophysiology of diseases in which histamine may play a role, diseases such as asthma, allergy, peptic ulcer disease, and neuropsychiatric disorders.

**Acknowledgments**

We thank Luanne Wussow for her assistance with preparation of the manuscript.

**References**

Aksoy S, Klener J, and Weinshilboum RM (1993) Catechol O-methyltransferase pharmacogenetics: photoaffinity labeling and western blot analysis of human liver samples. *Pharmacogenetics* **3:**116–122.

Aksoy S, Raftogianis R, and Weinshilboum R (1996) Human histamine N-methyltransferase gene: structural characterization and chromosomal localization. *Biochem Biophys Res Commun* **219:**548–554.

Boudíková B, Szumlanski C, Maidak B, and Weinshilboum R (1990) Human liver catechol O-methyltransferase pharmacogenetics. *Clin Pharmacol Ther* **48:**381–389.

Boudíková-Girard B, Scott MC, and Weinshilboum R (1993) Histamine N-methyltransferase: inhibition by monoamine oxidase inhibitors. *Agents Actions* **40:**1–10.

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72:**248–254.

Brown DD, Tomchick R, and Axelrod J (1959) The distribution and properties of a histamine-methylating enzyme. *J Biol Chem* **234:**2948–2950.

Burkard WP, Gey KF, and Pletscher A (1963) Diamine oxidase in the brain of vertebrates. *J Neurochem* **10:**183–186.

Campbell NRC, Dunnette JH, Mwaluko G, Van Loon J, and Weinshilboum RM (1984) Platelet phenol sulfotransferase and erythrocyte catechol-O-methyltransferase activities: correlation with methyldopa metabolism in man. *Clin Pharmacol Ther* **35:**55–63.

Cavalli-Sforza LL and Bodmer WF (1971) *The Genetics of Human Populations*. W. H. Freeman, San Francisco.

Chadwick RB, Conrad MP, McGinnis MD, Johnston-Dow L, Spurgeon SL, and Kronick MN (1996) Heterozygote and mutation detection by direct automated fluorescent DNA sequencing using a mutant *Taq* DNA polymerase. *Biotechniques* **20:**676–683.

Cleland WW (1963) Computer programmes for processing enzyme kinetic data. *Nature (Lond)* **198:**463–465.

Dale HH and Laidlaw PP (1910) The physiological action of $\beta$-iminazolyethylamine. *J Physiol* **41:**318–344.

Devereux J, Haeberli P, and Smithies O (1984) A comprehensive set of sequence analysis programs for the VAX. *Nucleic Acids Res* **12:**387–395.

Feinberg AP and Vogelstein B (1983) A technique for radiolabelling DNA restriction endonuclease fragments to high specific activity. *Anal Biochem* **132:**6–13.

Girard B, Otterness DM, Wood TC, Honchel R, Wieben ED, and Weinshilboum RM (1994) Human histamine *N*-methyltransferase pharmacogenetics: cloning and expression of kidney cDNA. *Mol Pharmacol* **45:**461–468.

Ho SN, Hunt HD, Horton RM, Pullen JK, and Pease LR (1989) Site-directed mutagenesis by overlap extension using polymerase chain reaction. *Gene* **77:**51–59.

Hough LB and Green JP (1984) Histamine and its receptors in the nervous system. *Handb Neurochem* **6:**145–211.

Lachman HM, Papolos DF, Saito T, Yu Y-M, Szumlanski CL, and Weinshilboum RM (1996) Human catechol O-methyltransferase pharmacogenetics: description of a functional polymorphism and its potential application to neuropsychiatric disorders. *Pharmacogenetics* **6:**243–250.

Lennard L, Van Loon JA, Lilleyman JS, and Weinshilboum RM (1987) Thiopurine pharmacogenetics in leukemia: correlation of erythrocyte thiopurine methyltransferase activity and 6-thioguanine nucleotide concentrations. *Clin Pharmacol Ther* **41:**18–25.

Lennon G, Auffray C, Polymeropoulos M, and Soares MB (1996) The IMAGE Consortium: an integrated molecular analysis of genomes and their expression. *Genomics* **33:**151–152.

Loiselle J and Wollin W (1993) Mucosal histamine elimination and its effect on acid secretion in rabbit gastric mucosa. *Gastroenterology* **104:**1013–1020.

Luthman H and Magnusson G (1983) High efficiency polyoma DNA transfection of chloroquine treated cells. *Nucleic Acids Res* **11:**1295–1308.

Maslinski C (1975) Histamine and its metabolism in mammals. Part II. Catabolism of histamine and histamine liberation. *Agents Actions* **5:**183–225.

Okinaga S, Ohrui T, Nakazawa H, Yamauchi K, Sakurai E, Watanabe T, Sekizawa K, and Sasaki H (1995) The role of HMT (histamine N-methyltransferase) in airways: a review. *Methods Fundam Exp Clin Pharmacol* **17**(Suppl. C)**:**16–20.

Pazmiño PA and Weinshilboum RM (1978) Human erythrocyte phenol-O-methyltransferase: radiochemical microassay and biochemical properties. *Clin Chim Acta* **89:**317–329.

Price RA, Scott MC, and Weinshilboum RM (1993) Genetic segregation analysis of red blood cell (RBC) histamine N-methyltransferase (HNMT) activity. *Genet Epidemiol* **10:**123–131.

Rosenthal N (1987) Identification of regulatory elements of cloned genes with functional assays. *Methods Enzymol* **152:**704–720.

Scanlon PD, Raymond FA, and Weinshilboum RM (1979) Catechol-O-methyltransferase: thermolabile enzyme in erythrocytes of subjects homozygous for the allele for low activity. *Science (Washington DC)* **203:**63–65.

Schwartz JC, Arrang MM, Garbarg M, Pollaed H, and Ruat M (1991) Histaminergic transmission in the mammalian brain. *Physiol Rev* **71:**1–51.

Scott MC, Van Loon JA, and Weinshilboum RM (1988) Pharmacogenetics of N-methylation: heritability of human erythrocyte histamine N-methyltransferase activity. *Clin Pharmacol Ther* **43:**256–262.

Szumlanski C, Otterness D, Her C, Lee D, Brandriff B, Kelsell D, Spurr N, Lennard L, Wieben E, and Weinshilboum R (1996) Thiopurine methyltransferase pharmacogenetics: human gene cloning and characterization of a common polymorphism. *DNA Cell Biol* **15:**17–30.

Terwilliger JD and Ott J (1994) *Handbook of Human Genetic Linkage*. The Johns Hopkins University Press, Baltimore.

Towbin H, Staehelin T, and Gordon J (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci USA* **76:**4350–4354.

Van Loon JA and Weinshilboum RM (1990) Thiopurine methyltransferase isozymes in human renal tissue. *Drug Metab Dispos* **18:**632–638.

Van Loon JA, Pazmiño PA, and Weinshilboum RM (1985) Human erythrocyte histamine N-methyltransferase: radiochemical microassay and biochemical properties. *Clin Chim Acta* **149:**237–251.

Wasserman SI (1983) Mediators of immediate hypersensitivity. *J Allergy Clin Immunol* **72:**101–115.

Weinshilboum RM (1981) Enzyme thermal stability and population genetic studies:

application to erythrocyte catechol-O-methyltransferase and plasma dopamine β-hydroxylase, in *Genetic Strategies in Psychobiology and Psychiatry* (Gershon ES, Matthysse S, Breakefield XO, and Ciaranello RD, eds), pp 79–94, Boxwood Press, Pacific Grove, CA.

Wilkinson GN (1961) Statistical estimations in enzyme kinetics. *Biochem J* **80:**324–332.

Wood TC, Aksoy IA, Aksoy S, and Weinshilboum RM (1994) Human liver thermolabile phenol sulfotransferase: cDNA cloning, expression and characterization. *Biochem Biophys Res Commun* **198:**1119–1127.

Yamauchi K, Sekizawa K, Suzuki H, Nakazawa H, Ohkawara Y, Katayose D, Ohtsu H, Tamura G, Shibahara S, Takemura M, Maeyama K, Watanabe T, Sasaki H, Shirata K, and Takishima T (1994) Structure and function of human histamine N-methyltransferase: critical enzyme in histamine metabolism in airway. *Am J Physiol* **267:**L342–L349.

**Send reprint requests to:** Dr. Richard Weinshilboum, Department of Pharmacology, Mayo Medical School, Mayo Clinic, Mayo Foundation, 200 First Street S.W., Rochester, MN 55905. E-mail: weinshilboum.richard@mayo.edu