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Human Thiopurine Methyltransferase: Molecular Cloning and Expression of T84 Colon Carcinoma Cell cDNA

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SUMMARY

Thiopurine methyltransferase (TPMT) catalyzes the *S*-methylation of thiopurine drugs such as 6-mercaptopurine. Levels of TPMT activity in human tissue are controlled by a common genetic polymorphism that is an important factor responsible for individual variation in thiopurine drug toxicity and therapeutic efficacy. Our goal was to purify, to obtain a partial amino acid sequence for, and to clone and express cDNA for human TPMT as a first step in determining the molecular basis for this genetic polymorphism. Human kidney TPMT was purified, the protein was subjected to limited proteolysis, and amino acid sequence information was obtained from the resultant peptide fragments. Primers based on the amino acid sequence information were used to amplify a unique sequence from human liver cDNA by use of the polymerase chain reaction. Because TPMT has been reported to be present in the colon, T84 human colon carcinoma cells were studied and were found to express TPMT activity with biochemical properties similar to those of the human kidney and liver enzymes. Oligonucleotide probes based on the human kidney TPMT amino acid sequence were then used to screen a T84 human colon carcinoma cell cDNA library. A 2.7-kilobase cDNA clone was isolated that contained an open reading frame of 735 nucleotides, which encoded a protein of 245 amino acids. The deduced amino acid sequence of the encoded protein included one 24- and two separate 12-amino acid sequences identical to those obtained by sequencing proteolytic fragments of purified human kidney TPMT. Transcripts were made *in vitro* from the open reading frame of the cDNA clone. These transcripts were translated in a rabbit reticulocyte lysate system, and the resulting translation product comigrated with human kidney TPMT in sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The T84 cell cDNA clone, truncated within the 3′ untranslated region at an *Sst*I restriction site, was then used to create an expression construct with the eukaryotic expression vector P91023(B), and this construct was used to transfect COS-1 cells. The transfected cells expressed a high level of TPMT enzymatic activity, and this activity displayed a pattern of inhibition by TPMT inhibitors identical to that of human kidney and T84 human colon carcinoma cell TPMT. Cloning of cDNA for this important drug-metabolizing enzyme may make it possible to define the molecular basis of the TPMT genetic polymorphism in humans.

TPMT (EC 2.1.1.67) is a cytoplasmic enzyme that catalyzes the Ado-Met-dependent *S*-methylation of aromatic and heterocyclic sulfhydryl compounds (1–3). Included among substrates for TPMT are the thiopurine drugs 6-MP and azathioprine (1, 2). The level of TPMT activity in human tissue is controlled by a common genetic polymorphism (4–7), and inherited variation in TPMT activity is an important factor responsible for individual differences in thiopurine drug toxicity and therapeutic efficacy (8–11). Phenotypic expression of the TPMT genetic polymorphism can be determined in an easily accessible human cell type, i.e., red blood cells (4), and this polymorphism also controls the level of enzyme activity in all other human tissues and cells that have been studied, including liver, kidney, and lymphocytes (12–14). Patients with genetically low or undetectable red blood cell TPMT activity are at greatly increased risk for the development of life-threatening thiopurine-induced myelosuppression when treated with azathioprine or 6-MP (8, 9, 11), whereas patients with high TPMT activity may be undertreated with standard doses of these drugs (10, 11).

Because of the clinical significance of the pharmacogenetic regulation of TPMT, understanding the molecular basis for the genetic polymorphism would be important both for defining the mechanism of inherited variation in this enzyme activity and helping to make it possible to develop clinically useful diagnostic tests. We set out to clone a cDNA for TPMT from human tissue as one step toward defining the molecular mech-

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ABBREVIATIONS: TPMT, thiopurine methyltransferase; PCR, polymerase chain reaction; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; Ado-Met, *S*-adenosyl-L-methionine; 6-MP, 6-mercaptopurine; 6-MMP, 6-methylmercaptopurine; DMEM, Dulbecco's modified Eagle's medium; PVDF, polyvinylidene difluoride; DMHBA, 3,4-dimethoxy-5-hydroxybenzoic acid.

anism for the genetic polymorphism. The strategy used included purifying human kidney TPMT, obtaining partial amino acid sequence of the enzyme after limited proteolysis, and using PCR to design oligonucleotide probes to screen human cDNA libraries. Use of this strategy made it possible to clone a cDNA from a T84 human colon carcinoma cell cDNA library. When expressed in COS-1 cells, this cDNA encoded a protein that catalyzed the TPMT enzyme reaction.

Materials and Methods

Tissue and cell acquisition and preparation. Renal tissue was obtained either during autopsy or from patients undergoing clinically indicated nephrectomies. Hepatic tissue was obtained from patients who underwent clinically indicated partial hepatectomies for the removal of primary or metastatic hepatic tumors. All tissue was obtained under guidelines approved by the Mayo Clinic Institutional Review Board. Macroscopically normal tissue was stored at −80°, conditions under which TPMT activity is stable (14–16). Before enzyme purification, renal tissue was homogenized in 5 mM potassium phosphate buffer, pH 7.5, with a Polytron homogenizer, and a 100,000 × *g* supernatant was prepared as described previously (2). T84 human colon carcinoma cells (American Type Culture Collection, Rockville, MD) were grown to confluence in an equal-volume mixture of DMEM and Ham's F12 medium with 5% fetal calf serum. The cells were then harvested, cell pellets were washed in phosphate-buffered saline, and the pellets were homogenized for 30 sec in 1 ml of 5 mM potassium phosphate buffer, pH 7.5, with a Polytron homogenizer. Homogenates of the cells were centrifuged at 100,000 × *g* for 1 hr at 4°, and the supernatant was used to assay TPMT activity and to determine selected biochemical properties of the enzyme.

TPMT assay. TPMT activity in renal tissue was measured by the method of Weinshilboum *et al.* (17), as modified by Woodson and Weinshilboum (2), to assay the enzyme in human kidney preparations. The assay is based on the conversion of 6-MP to radioactively labeled 6-MMP, with [*methyl*-^{14}C]Ado-Met as the methyl donor. Assay conditions for the measurement of TPMT in T84 human colon carcinoma cells and in COS-1 cells were identical to those used to measure the enzyme activity in human renal tissue. One unit of TPMT activity represented the formation of 1 nmol of 6-MMP/hr of incubation at 37°.

Protein assay. Protein concentrations were measured by the method of Bradford (18), with bovine serum albumin as a standard.

TPMT purification. Human renal tissue contains two isoforms of TPMT, peak I and peak II, that can be separated by DEAE ion exchange chromatography (16). The two isoforms were separated by DEAE ion exchange chromatography, and the peak I enzyme was then purified by gel filtration chromatography performed with Sephadex G-100 Superfine as described by Van Loon and Weinshilboum (16). In some experiments, hydroxylapatite chromatography performed as described by Van Loon *et al.* (19) was added as a third purification step.

Limited proteolysis. Human kidney TPMT peak I that had been partially purified through either the gel filtration or the hydroxylapatite chromatography step was subjected to limited proteolysis before amino acid sequencing. When enzymatic proteolysis was performed by the method of Cleveland *et al.* (20), 50–100 μg of partially purified TPMT were placed on a 10% polyacrylamide-SDS slab gel, electrophoresis was performed as described by Laemmli (21), proteins were visualized with Coomassie Blue, TPMT protein was excised in gel slices, and slices were placed in the wells of a 15% polyacrylamide-SDS gel together with proteolytic enzyme. The location of TPMT on the initial gel was verified by electrophoresis of enzyme photoaffinity labeled with [*methyl*-^{3}H]Ado-Met, as described by Van Loon *et al.* (19). The proteases used in these experiments included *Staphylococcus aureus* V8 protease (0.03 μg/well) and papain (0.2 μg/well). Proteolysis times before electrophoresis were 30 min for *S. aureus* V8 protease and 12 hr for papain. The peptide fragments generated during proteolysis were separated by SDS-PAGE and were transferred by the method of Towbin *et al.* (22) to PVDF membranes for 3 hr at 80 V, in 192 mM glycine, 25 mM Tris, pH 8.3, and 15% methanol. The PVDF membranes were stained with 0.5% Coomassie Blue in 40% methanol, followed by destaining with 50% methanol.

Proteolysis was also performed by cyanogen bromide cleavage (23). In those experiments, 100–200 μg of partially purified TPMT were subjected to SDS-PAGE, and individual proteins were excised in gel slices and were dried by lyophilization. Dried gel slices were treated for 16 hr at 20° with cyanogen bromide in 0.5 ml of aqueous 70% formic acid, with approximately 40 μg of cyanogen bromide/μg of protein. Cyanogen bromide and formic acid were then removed by centrifugal lyophilization, and peptide fragments generated during the procedure were separated by tricine-SDS-PAGE (24). The peptide fragments were then transferred to PVDF membranes before amino acid sequencing.

Amino acid sequencing. Amino acid sequencing was performed by the Mayo Research Resource Protein Core with peptide fragments electroblotted onto PVDF membranes. This procedure was performed either with a Proton 2090E integrated microsequencing system (Proton Instruments Inc., Tarzana, CA) or with an ABI 470A/120A microsequencer (Applied Biosystems Inc., Foster City, CA) using on-line reverse phase high performance liquid chromatography performed with narrow-bore C_{18} columns. Data were collected with an Everex 286/12 computer and were analyzed using Proton Instruments protein sequence analysis software.

RNA preparation and PCR. Total RNA was isolated from a frozen sample of human liver by extraction with guanidine HCl, followed by centrifugation through CsCl (25). First-strand cDNA for use as a template for PCR was synthesized with an oligo(dT) primer and murine reverse transcriptase. Oligonucleotide primers for PCR were designed on the basis of amino acid sequence information obtained after limited proteolysis of TPMT. The amino acid sequence IQEFFT was used to design a sense primer containing all possible codons as well as an *Xba*I restriction site [5′-GTCTCTAGAAT(A/C/T)CA(A/G)GA(A/G)TT(T/C)TT(T/C)AC-3′]. The amino acid sequence EEPIT was used to design an antisense primer containing all possible codons and an *Eco*RI restriction site [5′-GTCGAATTCGT(A/G/T)AT(A/G/C/T)GG(C/T)TC(C/T)TC-3′]. The PCR reaction was performed in a 100-μl reaction volume (containing 10 mM Tris, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, 50 μM concentrations of each of the four deoxynucleoside triphosphates, 4 μM concentrations of each primer, and 1 unit of *Thermus aquaticus* polymerase) in a Perkin Elmer Cetus DNA thermal cycler (Emeryville, CA). The amplification conditions used were 30 cycles of 1 min at 94°, 1 min at 46°, and 1 min at 72°, followed by a final 10-min incubation at 72°. The PCR reaction mixture was then applied to an 8% polyacrylamide gel, and PCR products were visualized by ethidium bromide staining. A 71-nucleotide PCR product was excised from the gel, and DNA was isolated with the MerMaid kit (BIO 101, Inc., La Jolla, CA). Because the recovery of DNA from the polyacrylamide gel after the initial cycle of amplification was low, the reaction was repeated using the 71-nucleotide species as template. The product of the second cycle of amplification was applied to a 2.5% agarose gel, the 71-nucleotide PCR product was excised from the gel, and DNA was isolated using the MerMaid kit. The ends of the PCR product were filled in by use of the Klenow fragment of DNA polymerase, the product was ligated into the *Sma*I site of pBluescript (Stratagene, La Jolla, CA), and the plasmid was used to transform *Escherichia coli* DH5α made competent by the method of Hanahan (26). The insert present in positive clones was sequenced by the dideoxy method of Sanger *et al.* (27), with the ^{35}S-sequencing protocol of the Sequenase kit version 2.0 (United States Biochemical Corp., Cleveland, OH).

Screening of cDNA libraries. A nondegenerate sense 53-nucleotide probe designed on the basis of the sequence of the 71-nucleotide PCR product was used to screen both human liver and T84 human colon carcinoma cell cDNA libraries constructed in the Uni-Zap XR

vector (Stratagene). The oligonucleotide probe was end-labeled with [γ-^{32}P]dATP (25). Approximately 1 × 10^6 plaque-forming units were screened from each library. Plaques were transferred to nitrocellulose filters, and hybridization was performed at 46° for 40 hr as described by Sambrook *et al.* (25). Five clones from the T84 cell library remained positive through secondary screening. Tertiary screening of these clones was performed both with the nondegenerate 53-nucleotide probe and with a nondegenerate 17-nucleotide probe, [5′-ACAGAGCA-GAATCTTTC-3′], the sequence of which was based on the non-primer-dependent region of the 71-nucleotide PCR product. One clone from the T84 cell library remained positive when screened with both probes. This clone was excised from Uni-Zap XR *in vivo* and was sequenced with the Sequenase kit version 2.0. Both strands of this clone were sequenced completely.

***In vitro* transcription and translation.** The coding region of the T84 cell cDNA clone was amplified by PCR from nucleotide −5 to nucleotide +739, with the addition of *Eco*RI restriction sites at each end. The primers used were 5′-GAATTCAAACCATGGATGGTA-CAAGAACT-3′ at the 5′ end and the antisense primer 5′-GAATTCT-CATTTACTTTTCTGTAAGTAGAT-3′ at the 3′ end of the coding region. The single nucleotide underlined in the 5′-end primer was changed from thymidine to cytidine to enhance translation efficiency (28). The PCR conditions used were the same as those already described except that 100 ng of the cDNA clone served as template, 100 ng of each primer were used, and amplification conditions involved 35 cycles of 1 min at 94°, 2 min at 50°, and 3 min at 72°, followed by a final 10-min incubation at 72°. The PCR mixture was then applied to a 1% agarose gel, and the PCR products were visualized by ethidium bromide staining. A PCR product of 756 nucleotides was excised from the gel, and DNA was isolated with the GeneClean kit (BIO 101). The ends of the PCR product were filled in by use of the Klenow fragment of DNA polymerase. The product was then ligated into the *Sma*I site of pBluescript, and this construct was used to transform *E. coli* DH5α made competent by the method of Hanahan (26). The insert present in positive clones was sequenced with the Sequenase kit version 2.0 to determine its orientation. The clone was then linearized with *Sst*I, and T7 RNA transcripts were synthesized using the mCAP capping kit (Stratagene). These RNA samples were translated *in vitro* with a rabbit reticulocyte lysate system (29). Translated proteins were analyzed by SDS-PAGE together with a standard that consisted of human kidney TPMT peak I that had been photoaffinity labeled with [*methyl*-^{3}H] Ado-Met (19).

Expression in COS-1 cells. The cDNA clone isolated from the T84 cell cDNA library was digested with *Sst*I to excise the coding region, the 5′-untranslated region, and a portion of the 3′-untranslated region. This insert was ligated into pBluescribe to create a second *Eco*RI site at the 3′ end. The pBluescribe clone was then digested with *Eco*RI, and the insert was cloned into the *Eco*RI site of the expression vector P91023(B) (30, 31). Positive P91023(B) clones were isolated by colony screening performed with the *Sst*I fragment of the TPMT clone that had been radioactively labeled with random primers (25), and the orientation of the insert was determined by partial restriction mapping and partial sequencing. The expression constructs were designated A3 and A6, whereas constructs that contained the coding region in the antisense orientation were designated A1 and A2. COS-1 cells were then plated in 100-mm dishes at a density of 1.5–1.8 × 10^6 cells/dish, in DMEM with 10% fetal calf serum, and were allowed to grow overnight. Three dishes of cells were used for each transfection. Purified plasmid (0.5 μg) was mixed with DEAE-dextran and DMEM, and this mixture was added to the cell culture dishes (32, 33). Control cells were treated with no DNA, with expression vector alone, or with constructs that contained the cDNA clone in an antisense orientation. After 1 hr the DNA-DEAE-dextran solution was replaced for 2 min with DMEM containing 10% dimethylsulfoxide, followed by incubation for 2 hr with 0.1 mM chloroquine in DMEM (34). Cells were then grown for 40–42 hr in DMEM with 10% fetal calf serum. The cells from each transfection were harvested and pooled, cell pellets were washed with 5 ml of phosphate-buffered saline, and the cell pellets were homogenized for 30 sec in 2 ml of 5 mM potassium phosphate buffer, pH 7.5. Homogenates were centrifuged at 15,000 × *g* for 15 min at 4°, supernatants from that step were centrifuged at 100,000 × *g* for 1 hr at 4°, and 0.25-μl aliquots of 100,000 × *g* supernatant preparations were then assayed for TPMT enzymatic activity.

Data analysis. The University of Wisconsin Genetics Computer Group software package was used to analyze sequence information and to make comparisons between the sequence of the TPMT cDNA clone and the sequences of other cloned proteins. The GenBank Genetics Sequence Data Bank and the EMBL Nucleotide Sequence Database were used to search for nucleotide homologies, whereas the Swiss-Prot Protein Sequence Database was used to search for protein structural homologies. IC_{50} values were calculated with the GraphPAD InPlot program (GraphPAD, San Diego, CA).

Materials. [*Methyl*-^{3}H]Ado-Met (84.3 mCi/μmol) and [*methyl*-^{14}C] Ado-Met (58.0 μCi/μmol) were purchased from DuPont-NEN (Boston, MA). [γ-^{32}P]dATP (>7000 Ci/mmol) was obtained from ICN Biomedicals, Inc. (Costa Mesa, CA). [α-^{35}S]dATP (>1000 Ci/mmol) and [α-^{32}P]dCTP (>3000 Ci/mmol) were purchased from Amersham Corp. (Arlington Heights, IL). T_4 polynucleotide kinase, DNA ligase, murine reverse transcriptase, DMEM, Ham's F-12 medium, and fetal calf serum were obtained from GIBCO BRL (Gaithersburg, MD). Restriction enzymes were purchased from GIBCO BRL and Boehringer Mannheim Corp. (Indianapolis, IN). DEAE-Sepharose CL-6B, Sephadex G-100 Superfine, and DEAE-dextran were obtained from Pharmacia LKB Biotechnology, Inc. (Piscataway, NJ). Hydroxylapatite and tricine were purchased from Calbiochem (San Diego, CA). Low molecular weight protein markers, SDS-PAGE reagents, and Bio-Rad Protein Assay Dye Reagent were obtained from Bio-Rad Laboratories (Richmond, CA). Allopurinol, bovine serum albumin, dithiothreitol, *S*-adenosyl-L-homocysteine HCl, Ado-Met HCl, 6-MP, 6-MMP, cyanogen bromide, *S. aureus* V8 protease, papain, and salmon testis DNA were purchased from Sigma Chemical Co. (St. Louis, MO). DMHBA was obtained from ICN Pharmaceuticals, Inc. (Plainview, NY). Amodiaquine HCl was donated by Warner Lambert Co. (Ann Arbor, MI), SKF-525A was a gift from Dr. R. Van Dyke, Henry Ford Medical Center (Detroit, MI), and 2,3-dichloro-α-methylbenzylamine was obtained from Research Biochemical, Inc. (Natick, MA). RESOLUTION autoradiogram enhancer was purchased from E.M. Corporation (Chestnut Hill, MA). A low-range molecular weight marker kit was obtained from Diversified Biotech (Newton Centre, MA), and Immobilon-PVDF membranes were purchased from Millipore Corporation (Bedford, MA).

Results

Protein purification, limited proteolysis, and partial amino acid sequence. Amino acid sequence information was obtained with TPMT purified from human renal tissue, a tissue with a high specific activity for the enzyme (2). Human kidney TPMT was partially purified by sequential ion exchange, gel filtration, and hydroxylapatite chromatography as described in Materials and Methods. After these three chromatographic steps, the enzyme was purified approximately 500-fold, compared with a 100,000 × *g* renal supernatant preparation (19). Photoaffinity labeling of TPMT with [*methyl*-^{3}H]Ado-Met confirmed that its approximate molecular mass was 35 kDa, as estimated by SDS-PAGE (19) (Figs. 1 and 2, *UNCLEAVED*). The amino terminus of the enzyme was blocked to Edman degradation. Therefore, limited proteolysis was performed to generate peptide fragments that could be used in amino acid sequencing. Enzymatic cleavage with *S. aureus* V8 protease or with papain yielded a series of peptide fragments (Fig. 1, *CLEAVED*). The fragments indicated by arrows in Fig. 1 were subjected to amino acid sequencing. Cyanogen bromide cleavage yielded two peptides that did not have blocked amino termini

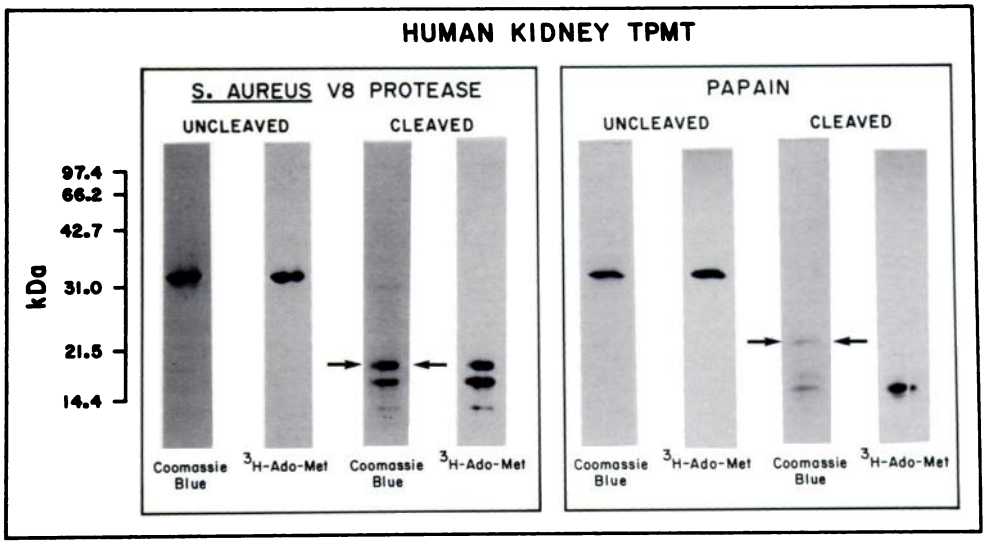

Fig. 1. Limited proteolysis of human kidney TPMT peak I by *S. aureus* V8 protease and papain. TPMT had been purified by ion exchange, gel filtration, and hydroxylapatite chromatography. *Left*, SDS-PAGE of uncleaved protein and protein cleaved by *S. aureus* V8 protease. The photograph shows gels stained with Coomassie Blue as well as autoradiographs of the same gels that show photoaffinity labeling of proteins with the methyl donor for the TPMT reaction, [*methyl*-^{3}H]Ado-Met. *Right*, SDS-PAGE of uncleaved protein and protein cleaved by papain. The photograph shows gels stained with Coomassie Blue as well as autoradiographs of the same gels that show photoaffinity labeling of proteins with [*methyl*-^{3}H]Ado-Met. *Arrows*, peptide fragments for which amino acid sequence was obtained.

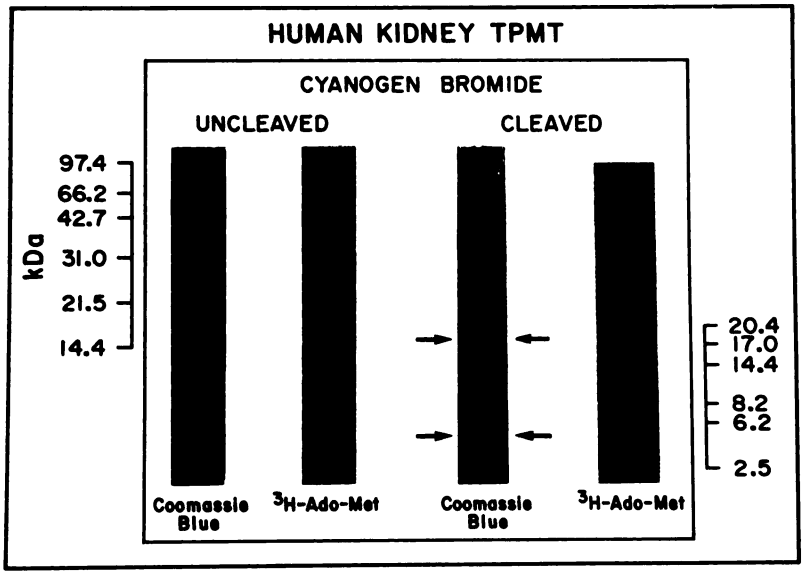

Fig. 2. Limited proteolysis of human kidney TPMT peak I by cyanogen bromide. TPMT had been purified by ion exchange and gel filtration chromatography. SDS-PAGE of uncleaved protein and protein cleaved by cyanogen bromide was performed. The photograph shows gels stained with Coomassie Blue as well as autoradiographs of the same gels that show photoaffinity labeling of proteins with [*methyl*-^{3}H]Ado-Met. *Arrows*, peptide fragments for which amino acid sequence was obtained.

and were present in adequate quantity for amino acid sequencing, one 5 kDa and the other 19 kDa in apparent molecular mass (Fig. 2). Each of these fragments yielded a sequence of 12 amino acids (Fig. 3). The 21-kDa fragment generated by papain and the 18-kDa fragment generated by *S. aureus* V8 protease contained overlapping amino acid sequences (Fig. 3). Combined information obtained from these two fragments yielded a sequence of 25 amino acids with one unresolved residue (designated X; Fig. 3). Figs. 1 and 2 also show that not all of the peptide fragments generated by limited proteolysis were photoaffinity labeled with [*methyl*-^{3}H]Ado-Met, an observation that may prove useful in future studies of the binding site of the methyl donor for the reaction. The amino acid sequence information obtained after papain and *S. aureus* V8 protease enzymatic cleavage (Fig. 3) was then used to obtain an oligonucleotide probe for use in screening cDNA libraries.

Screening of human liver cDNA library. TPMT is highly expressed in human kidney, liver, and colon (2, 14, 35). We did not have ready access to a human kidney cDNA library, but we did have human liver and T84 human colon carcinoma cDNA libraries. We chose initially to screen the human liver cDNA library. Because of the degeneracy of the genetic code for the amino acid sequences depicted in Fig. 3, a stepwise strategy was used to obtain a specific oligonucleotide probe. The least degenerate portions of the papain-*S. aureus* V8 protease amino acid sequence were used to design PCR primers (Fig. 4). Restriction sites were added to the PCR primers to assist in ligation of amplification products into vector. PCR performed with human liver cDNA resulted in the amplification of three PCR products. One of the amplification products was of approximately the anticipated size, i.e., 71 nucleotides. DNA sequencing revealed that the PCR product did consist of 71

	PAPAIN 21 kDa	V8 18 kDa	CNBr 5 kDa	CNBr 19 kDa
1	V		F	D
2	E		S	G
3	I		L	T
4	S		L	R
5	E		D/G	T
6	L		K	S
7	G		K	L
8	I		F	D
9	Q		S/Q	I
10	E		Y	E
11	F-------F		L	E
12	F-------F		L	Y
13	T-------T			
14	E-------E			
15	Q-------Q			
16	N-------N			
17	L-------L			
18		S		
19		Y		
20		"X"		
21		E		
22		E		
23		P		
24		I		
25		T		

Fig. 3. Amino acid sequences of peptide fragments isolated after limited proteolysis of human kidney TPMT performed with papain, *S. aureus* V8 protease (*V8*), or cyanogen bromide (*CNBr*). Amino acid sequences obtained after limited proteolysis with papain and *S. aureus* V8 protease were overlapping. Residue 20 in the papain-*S. aureus* V8 protease amino acid sequence was unresolved (*X*). Residues 5 and 9 in the 5-kDa cyanogen bromide fragment could have been either of the amino acids indicated.

nucleotides and that it encoded the amino acid sequence generated by papain-*S. aureus* V8 protease treatment of TPMT (Fig. 4). The previously unresolved residue (X in Fig. 3) was found to be serine. A nondegenerate 53-nucleotide probe based on the entire sequence of the PCR product, minus the restriction sites, was then synthesized (Fig. 4). This oligonucleotide probe was used, unsuccessfully, in an attempt to screen a human liver cDNA library. Therefore, we next turned our attention to the T84 human colon carcinoma cell cDNA library. However, it was first necessary to determine whether these cells expressed TPMT.

TPMT in T84 human colon carcinoma cells. T84 human colon carcinoma cells were grown in culture, harvested, and homogenized, and 100,000 × *g* supernatant preparations were used to determine whether the cells expressed TPMT. Cytosol from T84 cells did contain TPMT enzymatic activity. The optimal pH for assay of TPMT activity in these preparations was determined in the presence of 150 mM potassium phosphate buffer at seven different buffer pH values from 5.5 to 8.5. Optimal activity for T84 cell TPMT, like that for human kidney and liver TPMT (2, 14), was present at pH 6.5. Apparent K_m values of T84 cell TPMT for the two substrates for the TPMT reaction, 6-MP and Ado-Met, were then determined. When enzyme activity was measured in the presence of 25 μM Ado-Met and seven concentrations of 6-MP that varied from 0.11 to 6.8 mM (Fig. 5A), an apparent K_m value of 0.71 mM for 6-MP was calculated. This value was similar to those that have been reported for human liver and kidney TPMT (2, 14). When an apparent K_m value for Ado-Met was calculated on the basis of data obtained in the presence of 3.4 mM 6-MP and six concentrations of Ado-Met that varied from 0.78 to 25 μM (Fig. 5B), a value of 5.1 μM was calculated. This K_m value was similar to but slightly higher than those reported for human kidney and liver TPMT (2, 14). Finally, human kidney and liver TPMT activities are inhibited by DMHBA (14, 36). IC_{50} values for inhibition of T84 cell and human kidney TPMT by DMHBA were calculated on the basis of data obtained in the presence of seven concentrations of DMHBA that varied from 0.01 μM to 10 mM (Fig. 5C). The IC_{50} values calculated from these data were 6.6 and 7.8 μM for T84 cell and human kidney TPMT, respectively. Therefore, T84 cells expressed TPMT activity, and the biochemical properties of this enzyme were very similar to those of TPMT in other human tissues. These results encouraged us to proceed with the screening of a T84 cell cDNA library.

Screening of T84 human colon carcinoma cell cDNA

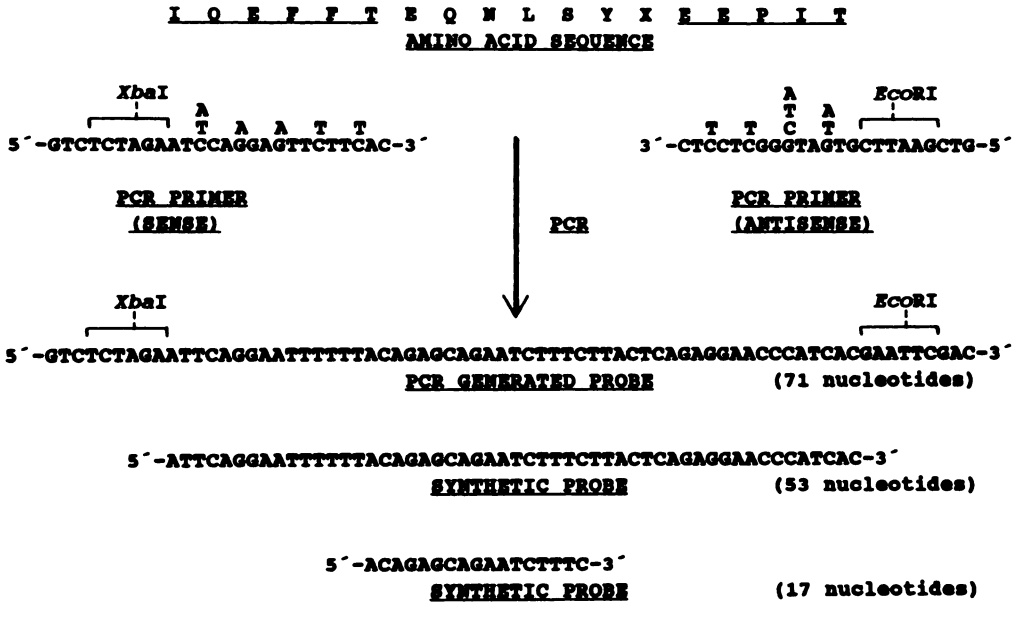

Fig. 4. Schematic representation of the PCR-based strategy used to generate oligonucleotide probes for screening cDNA libraries. Sequentially, from *top* to *bottom*, the figure depicts use of the portions of the amino acid sequence determined after papain-*S. aureus* V8 protease treatment with the least degenerate genetic code to design degenerate PCR primers that included nine additional nucleotides at the 5′ and 3′ ends that contained *Xba*I and *Eco*RI restriction sites, respectively. Use of PCR led to the amplification of a 71-nucleotide PCR product that encoded the amino acids located between the two primers in the papain-*S. aureus* V8 protease sequence. These results were used to synthesize a nondegenerate 53-nucleotide probe, as well as a nondegenerate 17-nucleotide probe that contained the sequence located between the two PCR primers. Both the 53- and 17-nucleotide probes were used to screen cDNA libraries (see text for details).

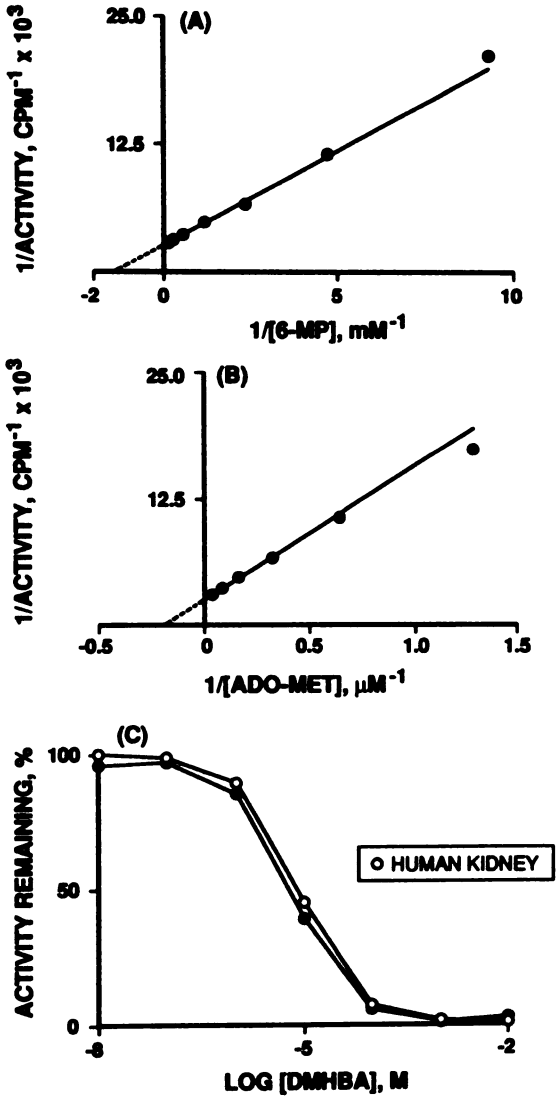

Fig. 5. T84 human colon carcinoma cell TPMT enzymatic activity. A, Double-inverse plot of the relationship between TPMT activity and 6-MP concentration. B, Double-inverse plot of the relationship between TPMT activity and Ado-Met concentration. C, DMHBA inhibition of T84 cell and human kidney TPMT enzymatic activities. Each *point* represents the average of three determinations.

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CGGCAACCAGCTGTAAGCGAGGCACGGAAGACATATGCTTGTGAGACAAAGGTGTCTCTG   -6
    1
AAACTATGGATGGTACAAGAACTTCACTTGACATTGAAGAGTACTCGGATACTGAGGTAC    55
    M  D  G  T  R  T  S  L  D  I  E  E  Y  S  D  T  E  V  Q
AGAAAAACCAAGTACTAACTCTGGAAGAATGGCAAGACAAGTGGGTGAACGGCAAGACTG   115
  K  N  Q  V  L  T  L  E  E  W  Q  D  K  W  V  N  G  K  T  A
CTTTTCATCAGGAACAAGGACATCAGCTATTAAAGAAGCATTTAGATACTTTCCTTAAAG   175
  F  H  Q  E  Q  G  H  Q  L  L  K  K  H  L  D  T  F  L  K  G
GCAAGAGTGGACTGAGGGTATTTTTTCCTCTTTGCGGAAAAGCGGTTGAGATGAAATGGT   235
  K  S  G  L  R  V  F  F  P  L  C  G  K  A  V  E  M  K  W  F
TTGCAGACCGGGGACACAGTGTAGTTGGTGTGGAAATCAGTGAACTTGGGATACAAGAAT   295
  A  D  R  G  H  S  V  V  G  V  E  I  S  F  L  G  I  Q  E  F
TTTTTACAGAGCAGAATCTTTCTTACTCAGAAGAACCAATCACCGAAATTCCTGGAACCA   355
    F  T  E  Q  N  L  S  Y  S  E  E  P  I  T  E  I  P  G  T  K
AAGTATTTAAGAGTTCTTCGGGGAACATTTCATTGTACTGTTGCAGTATTTTTGATCTTC   415
  V  F  K  S  S  S  G  N  I  S  L  Y  C  C  S  I  F  D  L  P
CCAGGACAAATATTGGCAAATTTGACATGATTTGGGATAGAGGAGCATTAGTTGCCATTA   475
  R  T  N  I  G  K  F  D  M  I  W  D  R  G  A  L  V  A  I  N
ATCCAGGTGATCGCAAATGCTATGCAGATACAATGTTTTCCCTCCTGGGAAAGAAGTTTC   535
    P  G  D  R  K  C  Y  A  D  T  M  F  S  I  L  G  K  K  F  Q
AGTATCTCCTGTGTGTTCTTTCTTATGATCCAACTAAACATCCAGGTCCACCATTTTATG   595
  Y  L  L  C  V  L  S  Y  D  P  T  K  H  P  G  P  P  F  Y  V
TTCCACATGCTGAAATTGAAAGGTTGTTTGGTAAAATATGCAATATACGTTGTCTTGAGA   655
  P  H  A  E  I  E  R  L  F  G  K  I  C  N  I  R  C  L  E  K
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  V  D  A  F  E  E  R  H  K  S  W  G  I  D  C  L  F  E  K  L
TATATCTACTTACAGAAAAGTAAATGAGACATAGATAAAATAAAATCACACTGACATGTT   775
  Y  L  L  T  E  K  *
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AATCAGCATGTGTTACCTGTACAATTAAAAAAATATTTCAAAATGCAATGCATATTAAAT  1075
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GACAGGGTCTCACTTTGTTGCCCACGCTGATGTTGAACTTGACGTGAGGCCTCAAGCAGT  2395
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CTTCATAGGCTTTTGGACTTGGGAATAGAAAAGCAACCCCGTCTCTACTGAAAGTGCAGA  2515
AGGATTGGCCGGGGCGTGGTGGCGCGTGCCTGTGGTCCCAGCTGCTTGGGGGGCTGAGGCG  2575
GGAGAATCACTTGGACCTGGGGGGCGGAGGTTGCAGTGAGCTGAGATCGTGCCACTGCAC  2635
GCCAGCCTGGGCAACAGAGCAAGACTCTGTCTCAAAAGAAAG(A)18
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Fig. 6. T84 human colon carcinoma cell TPMT cDNA clone nucleotide sequence. *Underlined areas in the deduced amino acid sequence*, sequences obtained by partial amino acid sequencing of human kidney TPMT (see Fig. 3). *Underlined areas in the nucleotide sequence*, sequences that were used to design PCR primers (see Fig. 4).

library. A T84 human colon carcinoma cell cDNA library was screened with the 53-nucleotide probe shown in Fig. 4. Of the clones that were initially positive, five remained positive through secondary screening. Tertiary screening of these clones was performed with both the 53-nucleotide probe and a 17-nucleotide probe that represented the portion of the PCR product located between the two primer sequences (Fig. 4). Only one clone hybridized to both the 53- and 17-nucleotide probes. This clone was sequenced in both directions and was found to consist of 2760 nucleotides with an open reading frame of 735 nucleotides that encoded a protein of 245 amino acids (Fig. 6). All of the TPMT amino acid sequences obtained after limited proteolysis of the enzyme (Fig. 3) were encoded within the open reading frame. The T84 TPMT cDNA clone had a long 3′ untranslated region that terminated with a poly(A) tract. No consensus polyadenylation signal was present immediately upstream of the poly(A) region. The 3′ untranslated region contained two ALU-type repetitive elements. One of these elements was oriented 5′ to 3′ from nucleotides 2567 to 2695, whereas the other was oriented 3′ to 5′ from nucleotides 1101 to 1225. Finally, the predicted molecular mass of the protein encoded by the open reading frame was 28.2 kDa, less than the 35-kDa apparent molecular mass of TPMT estimated on the basis of SDS-PAGE of the photoaffinity-labeled human kidney enzyme (19) (see Figs. 1 and 2). The next step in the analysis involved expression of the cDNA to determine whether the encoded protein had an apparent molecular mass during SDS-PAGE similar to that of native human TPMT and to determine whether it could catalyze the TPMT enzymatic reaction.

Expression of TPMT activity. The coding region of the T84 TPMT cDNA clone was transcribed *in vitro* (see Materials and Methods for details), and the RNA obtained from this procedure was translated in a rabbit reticulocyte lysate system. The major translation product of this RNA comigrated during

SDS-PAGE with photoaffinity-labeled human kidney TPMT, with an apparent molecular mass of 35 kDa (Fig. 7). Therefore, the 28.2-kDa protein encoded by the T84 TPMT cDNA clone behaved during SDS-PAGE as if its molecular mass were approximately 35 kDa. The next step in evaluating the properties of the protein encoded by the T84 TPMT cDNA clone was expression in COS-1 cells.

The T84 TPMT cDNA clone was digested with *Sst*I to generate a fragment that contained the 5′ untranslated region, the coding region, and approximately 600 nucleotides of the 3′ untranslated region. The fragment generated by *Sst*I digestion was subcloned, in both orientations, into the expression vector P91023(B). COS-1 cells were transfected with constructs that contained the clone in both orientations, and TPMT enzymatic activity was measured in homogenates of these cells after transient expression. There was no increase in the low basal level of TPMT activity in COS-1 cells transfected with vector alone or with antisense constructs (Table 1). However, COS-1 cells transfected with the cDNA clone in the proper orientation had a >100-fold increase in TPMT activity (Table 1). TPMT activity expressed in the COS-1 cells transfected with the cDNA clone was inhibited by DMHBA with an IC_{50} value nearly identical to that of the TPMT present in T84 cells (Fig. 8). Finally, the response of the TPMT expressed in COS-1 cells to a panel of methyltransferase inhibitors and ions was compared with that of TPMT activity in human kidney 100,000 × *g* supernatant, partially purified human kidney peak I TPMT, and T84 cell TPMT. The results were nearly identical for all sources of enzyme (Table 2). Each activity was inhibited by the TPMT reaction products *S*-adenosyl-L-homocysteine and 6-

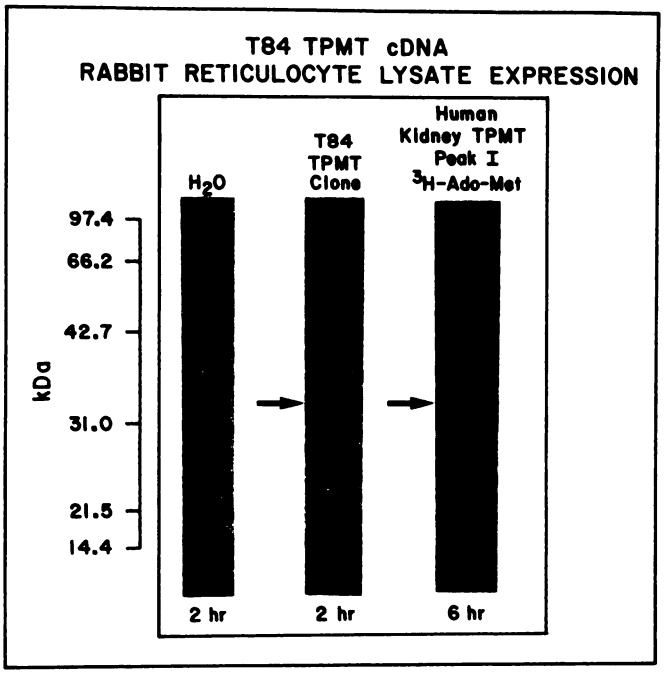

Fig. 7. T84 human colon carcinoma cell TPMT cDNA clone translation in a rabbit reticulocyte lysate system. SDS-PAGE of [^{35}S]methionine-labeled protein obtained by translation of the T84 TPMT cDNA clone in a rabbit reticulocyte lysate system is shown. The clone encoded a protein with an approximate molecular mass of 35 kDa (*arrows*). SDS-PAGE of purified human kidney TPMT photoaffinity labeled with [*methyl*-^{3}H]Ado-Met is shown for comparison. Results obtained with a control sample in which only water was added to the rabbit reticulocyte lysate system are also shown (see text for details).

TABLE 1

Expression of TPMT enzymatic activity after transfection of COS-1 cells with T84 human colon carcinoma cell TPMT cDNA in either the sense or antisense orientation

The results of two separate experiments are shown.

	TPMT activity	
	Experiment 1	Experiment 2
	units/mg of protein	
Control, no DNA	4	5
Control, P91023(B)	4	5
Control, A1 antisense	5	5
Control, A2 antisense	5	5
A3 sense	659	624
A6 sense	599	586

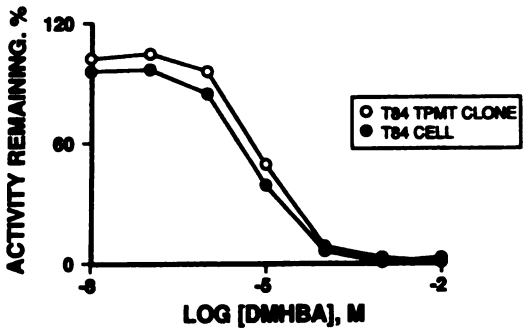

Fig. 8. T84 human colon carcinoma cell TPMT cDNA expression in COS-1 cells. DMHBA inhibition of T84 cell TPMT activity and TPMT activity expressed in COS-1 cells transfected with T84 cell TPMT cDNA is shown.

TABLE 2

Effects of methyltransferase inhibitors and ions on TPMT enzymatic activity in human kidney 100,000 × *g* supernatant, partially purified human kidney TPMT peak I, T84 human colon carcinoma cell 100,000 × *g* supernatant, and 100,000 × *g* supernatant from COS-1 cells transfected with P91023(B) that contained T84 TPMT cDNA in the sense orientation.

Compound	Concentration	Activity remaining			
		Human kidney 100,000 × *g* supernatant	Human kidney TPMT peak 1	T84 cell 100,000 × *g* supernatant	COS-1 cells transfected with T84 TPMT cDNA
	mM	%			
Ado-Hcy[a]	0.1	5	5	5	5
6-MMP	5	26	30	27	29
DCMB	1	95	95	99	95
$CaCl_2$	1	96	97	95	96
Amodiaquine	1	97	90	96	95
SKF-525A	0.5	107	98	102	101

[a] Ado-Hcy, S-adenosyl-L-homocysteine; DCMB, 2,3-dichloro-α-methylbenzylamine.

MMP. However, the enzyme was not affected by inhibitors of other methyltransferase enzymes. The other inhibitors tested, all at effective concentrations, included 2,3-dichloro-α-methylbenzylamine and SKF-525A, inhibitors of thiol methyltransferase (37, 38); amodiaquine, an inhibitor of histamine *N*-methyltransferase (39); and $CaCl_2$, an inhibitor of catechol *O*-methyltransferase (40).

T84 human colon carcinoma cell TPMT sequence, comparison with other methyltransferases. The nucleotide sequence within the open reading frame and the deduced amino acid sequence of the protein encoded by the T84 TPMT

cDNA clone were compared with sequences present in the GenBank Genetics Sequence Data Bank and the EMBL Nucleotide Sequence Database. No highly homologous sequences were found. The deduced amino acid sequence of the protein encoded by the T84 TPMT cDNA clone was then compared with those of other mammalian cytosolic Ado-Met-dependent methyltransferase enzymes (Table 3). Human TPMT displayed little homology with any of these enzymes, ranging from only 14.5% amino acid sequence identity compared with rat liver guanidinoacetate methyltransferase to 24.8% identity with the sequence of human erythrocyte protein carboxyl methyltransferase.

Ingrosso *et al.* (41) have reported that enzymes that utilize Ado-Met as a co-substrate contain three regions of sequence homology, designated I, II, and III, that might be involved in Ado-Met binding. Region I was characterized further by Wu *et al.* (42), and Gomi *et al.* (43) who reported that many, but not all methyltransferase enzymes contain characteristic "signature" sequences within both regions I and III. The protein encoded by the T84 TPMT cDNA contained no amino acid sequences with three or fewer mismatches with the hypothetical region I Ado-Met-binding sequence reported by Wu *et al.* (42) and no sequences comparable to either the region I or region III signature sequences reported by Gomi *et al.* (43).

Discussion

TPMT catalyzes an important pathway in the biotransformation of thiopurine drugs (1, 2). These drugs are used to treat transplant recipients who require immune suppression and patients with neoplastic disease such as acute lymphoblastic leukemia of childhood (10, 11). Unfortunately, the therapeutic index of thiopurines is narrow, and their use can lead to life-threatening side effects such as myelosuppression (44). The TPMT genetic polymorphism represents a striking example of the clinical importance of pharmacogenetics, in part as a result of the narrow therapeutic index of these drugs. Approximately one of every 300 Caucasian subjects lacks TPMT on a genetic basis (4) and is at risk for the occurrence of severe myelosuppression when treated with standard doses of 6-MP or azathioprine (9, 11, 45, 46). Furthermore, 11% of the population is heterozygous for this genetic polymorphism and may also require reduced doses of thiopurine drugs (8, 10). Understanding the molecular basis of the TPMT genetic polymorphism not only would expand our knowledge of mechanisms responsible for pharmacogenetic variation in drug metabolism but also would have relevance for the clinical use of thiopurine drugs.

There have been attempts to study the mechanism of the genetic regulation of TPMT in humans. Individuals who are heterozygous for the TPMT genetic polymorphism not only have decreased levels of enzymatic activity in their tissues, compared with tissue from subjects who are homozygous for high levels of activity, but also have lower levels of TPMT immunoreactive protein (12). The observation that both human liver and kidney contain two isoforms of TPMT that can be separated by ion exchange chromatography (14, 16) raised the possibility that these isoforms might provide an explanation for the biochemical mechanism of the polymorphism. However, both isoforms were present in similar relative proportions in tissues from subjects with different presumed genotypes for the polymorphism (14, 16), so the existence of the isoforms does

TABLE 3

Comparison of amino acid sequences of mammalian cytosolic Ado-Met-dependent methyltransferase enzymes

The enzymes compared include rat liver guanidinoacetate methyltransferase (rGAMT) (EC 2.1.1.2) (56), rat liver glycine methyltransferase (rGMT) (EC 2.1.1.20) (57), rat kidney histamine *N*-methyltransferase (rHNMT) (EC 2.1.1.8) (58), bovine pineal hydroxyindole *O*-methyltransferase (bHIOMT) (EC 2.1.1.4) (59, 60), mouse testis, rat brain, and human erythrocyte protein carboxyl methyltransferase (mPCMT, rPCMT, and hPCMT) (EC 2.1.1.77) (41, 61, 62), rat liver and human placental catechol *O*-methyltransferase (rCOMT and hCOMT) (EC 2.1.1.6) (63, 64), rat adrenal medulla, bovine adrenal medulla, and human pheochromocytoma phenylethanolamine *N*-methyltransferase (rPNMT, bPNMT, and hPNMT) (EC 2.1.1.28) (65–67), and T84 human colon carcinoma cell TPMT (hTPMT) (EC 2.1.1.67). The initial values represent the percentage of identity, whereas the values in parentheses represent the percentage of similarity of sequences as determined by use of the GAP program (68).

	Mammalian cytosolic methyltransferase enzyme amino acid sequence comparisons												
	rGAMT	rGMT	rHNMT	bHIOMT	mPCMT	rPCMT	hPCMT	rCOMT	hCOMT	rPNMT	bPNMT	hPNMT	hTPMT
rGAMT		16.9 (44.4)	13.4 (40.2)	20.4 (44.7)	16.6 (36.6)	14.6 (42.7)	16.7 (43.4)	23.4 (43.7)	18.4 (40.8)	21.6 (46.8)	19.2 (46.6)	20.6 (48.9)	14.5 (43.0)
rGMT			18.0 (48.4)	16.9 (42.7)	23.0 (48.8)	23.1 (50.5)	21.4 (47.8)	18.3 (44.6)	20.4 (42.3)	21.0 (44.4)	17.0 (39.9)	20.2 (47.2)	18.0 (44.3)
rHNMT				17.9 (45.9)	20.2 (46.3)	14.6 (39.8)	14.3 (39.7)	17.0 (47.0)	17.1 (46.9)	16.3 (42.6)	20.9 (41.1)	17.1 (39.9)	16.0 (43.7)
bHIOMT					17.6 (47.7)	19.1 (40.9)	16.4 (43.6)	21.2 (45.7)	16.7 (45.7)	18.8 (45.3)	25.3 (47.9)	20.5 (46.8)	17.6 (43.3)
mPCMT						98.7 (99.6)*	96.0 (97.8)*	16.4 (45.6)	19.4 (48.0)	15.8 (42.3)	16.3 (45.6)	14.3 (42.1)	19.6 (46.7)
rPCMT							95.6 (98.2)*	16.7 (47.1)	19.8 (46.2)	22.0 (45.4)	17.1 (44.1)	18.7 (47.8)	19.7 (44.6)
hPCMT								16.8 (44.5)	17.1 (44.1)	20.5 (46.3)	19.7 (50.0)	19.8 (46.7)	24.8 (46.2)
rCOMT									77.7 (87.5)*	20.9 (47.0)	19.2 (44.0)	17.2 (42.9)	20.8 (51.0)
hCOMT										16.3 (38.1)	15.8 (40.2)	18.4 (38.8)	22.7 (50.7)
rPNMT											88.5 (91.5)*	85.9 (91.8)*	15.7 (43.9)
bPNMT												88.3 (92.6)*	18.5 (43.5)
hPNMT													16.7 (41.0)
hTPMT													

* Enzymes with >75% sequence identity.

not seem to be directly related to the mechanism responsible for the genetic regulation of TPMT.

One way to study the genetic regulation of TPMT would involve application of the techniques of molecular biology. Therefore, we set out to clone a cDNA for human TPMT. As a first step, TPMT was purified from human renal tissue, a tissue with high specific activity for the enzyme (2). The native protein was blocked to Edman degradation, so amino acid sequence information was obtained from peptide fragments generated by limited proteolysis. This information was used to design PCR primers. A PCR product was then amplified from human liver cDNA, a nondegenerate 53-nucleotide probe was synthesized on the basis of the sequence of the PCR product, and this probe was used, unsuccessfully, to screen a human liver cDNA library. Because TPMT has been reported to be present in the human colon (35), we next turned our attention to T84 human colon carcinoma cells. These cells were found to express TPMT activity with biochemical properties similar to those of the enzyme in human liver and kidney. Therefore, a T84 human colon carcinoma cDNA library was screened, and a 2760-nucleotide cDNA clone was isolated. The 735-nucleotide open reading frame of the clone encoded a protein of 245 amino acids, and the deduced sequence of the encoded protein contained all of the amino acid sequences obtained from peptide fragments generated by limited proteolysis of TPMT. Because the 3′ untranslated region contains repetitive elements and because no polyadenylation signal is present, the possibility exists that it might not represent the authentic 3′ untranslated region. However, translation of RNA transcribed from the open reading frame of the T84 TPMT cDNA clone in a rabbit reticulocyte lysate system yielded a protein that comigrated with purified human kidney TPMT during SDS-PAGE, and expression of the clone in COS-1 cells resulted in the expression of TPMT enzymatic activity with properties similar to those of TPMT in T84 cells.

The cloning of a cDNA for TPMT from a human tissue source will provide an important tool that can be used to study the molecular basis for the TPMT genetic polymorphism and that might be used to develop clinically useful diagnostic tests for this polymorphism. The T84 TPMT cDNA clone could also be used in an attempt to clone cDNA for TPMT from other species. Mice, like humans, exhibit a genetic polymorphism that regulates both the level of TPMT enzymatic activity and the quantity of TPMT immunoreactive protein in kidney and liver (47, 48). Although the locus *Tpmt* in the mouse is located on the midportion of chromosome 13 (49), nothing is known of the molecular biology of the enzyme in mice. Cloning of a cDNA for TPMT from a human tissue may make it possible not only to study the molecular mechanism of the genetic polymorphism at the locus *Tpmt* in the mouse but also to initiate phylogenetic studies of the enzyme.

Finally, the present results should be placed in the broader context of studies of the pharmacogenetics of other methyltransferase enzymes. Mammalian tissue contains many Ado-Met-dependent methylating enzymes and several, including catechol *O*-methyltransferase, thiol methyltransferase, and histamine *N*-methyltransferase, are regulated by inheritance in humans (50–53). When the techniques of molecular biology have been used to study other "families" of drug-metabolizing enzymes such as the cytochromes P450 or the sulfotransferases, a high degree of homology has been found among related enzymes, both within and among species (54, 55). The fact that similar homology was not present among the 12 different mammalian cytoplasmic methyltransferase enzymes that we compared with TPMT raises intriguing questions with regard to the evolutionary history of these proteins that will have to be addressed as cDNAs and genomic DNAs for more methylating enzymes are characterized in the future.

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