Molecular cloning and functional characterization of the cDNA encoding the murine thiopurine *S*-methyltransferase (TPMT)

Michael Y. Fessing[a,b], Vladimir M. Belkov[a,b], Eugene Y. Krynetski[a,b], William E. Evans[a,b,*]

[a] *Department of Pharmaceutical Sciences, St. Jude Children's Research Hospital, 332 North Lauderdale St., Memphis, TN 38105, USA*
[b] *University of Tennessee, Memphis, TN 38101, USA*

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Abstract Thiopurine *S*-methyltransferase (TPMT) is a cytosolic enzyme that catalyzes *S*-methylation of aromatic and heterocyclic sulfhydryl compounds, including anticancer and immunosuppressive thiopurines. Here we report the isolation and functional characterization of the murine TPMT cDNA. The screening of expressed sequence tags database led to isolation of a murine cDNA clone containing an uninterrupted ORF encoding the protein with an amino acid sequence that is 82% similar and 78% identical to the human TPMT. The expression product of the murine cDNA in rabbit reticulocyte and wheat germ lysate coupled transcription-translation systems showed TPMT enzymatic activity. We conclude that the isolated cDNA clone represents the murine TPMT cDNA.

Key words: Thiopurine *S*-methyltransferase; cDNA; Biological methylation; Pharmacogenetics

1. Introduction

Thiopurine *S*-methyltransferase (TPMT, EC 2.1.1.67) is a cytoplasmic transmethylase originally found in kidney and liver of rodents [1]. Using *S*-adenosyl-methionine as a methyl donor, this enzyme catalyzes methylation of sulfur atoms in aromatic and heterocyclic compounds. TPMT was shown to be present in erythrocytes, kidney and liver, and TPMT mRNA was detected in most human tissues, e.g. heart, blood cells, placenta, pancreas, intestine [2,3]. Human TPMT has a molecular mass of 28 kDa and comprises 245 amino acids. Interest in this enzyme is enhanced by the crucial role TPMT plays in metabolism of the important antileukemic and immunosuppressive drugs mercaptopurine, thioguanine and azathioprine [4]. TPMT activity shows co-dominant genetic polymorphism, with about 10% of the population inheriting a lower activity of this enzyme. Recent studies have elucidated the molecular basis for inherited TPMT deficiency [2,5–7] and provided genotyping assays to detect individuals with impaired activity of TPMT [8]. Isolation of the human TPMT chromosomal gene [7,9] and characterization of its promoter region have made it possible to characterize the regulation of TPMT (Fessing et al., unpublished). Despite significant progress in molecular genetics of TPMT, many biochemical questions remain unanswered including its endogenous substrate and biological functions. Comparison of the human TPMT amino acid sequence revealed little homology with other methyltransferases, and no SAM-binding motifs were found in its primary structure [10–12].

To date, the amino acid sequence deduced from the cDNA was determined only for human TPMT [2,11]. Here we report the first isolation and characterization of the murine TPMT cDNA. Isolation of the murine TPMT provides an important new tool for gaining further insight into the biochemical functions and regulation of this enzyme.

2. Materials and methods

A computer search for homologous sequences was performed at the National Center for Biotechnology Information, using the BLAST network service. The University of Wisconsin Genetic Computer Group software package was used to analyze sequence information (Genetic Computer Group, 1991).

cDNA clones MGI:314202 (GenBank accession number AA109870) isolated from C57/BL6 cDNA library and MGI:610617 (GenBank accession number AA637718) isolated from the C2C12 cell line cDNA library were obtained from Genome Systems (St. Louis, MO) and propagated according to the manufacturer's instructions. Plasmid DNA was isolated using the Qiagen Plasmid Midi Kit (Valencia, CA). cDNA inserts were bidirectionally sequenced with an Applied Biosystems DNA sequencer Model 373A and the ABI PRISM dye Terminator Cycle Core Sequencing Kit, with AmpliTaq DNA Polymerase, FS (Perkin Elmer, Foster City, CA), in the Center for Biotechnology at SJCRH.

Preparation of plasmids with human *TPMT*1* and *TPMT*3A* cDNA and transcription-translation experiments in the rabbit reticulocyte lysate system (RRL) and wheat germ extract system (WGL) (Promega, Madison, WI) were performed essentially as described earlier [13].

Enzymatic activity of translation products was determined after incubation of 1 μg of the plasmid with the corresponding cDNA insert, and 25 μl of RRL or WGL in the 50 μl reaction mixture, for 1.5 h at 30°C. Immediately thereafter, 200 μg of 6MP in 10 μl of DMSO and 5 μl of [^{14}C-CH$_3$]*S*-adenosyl-L-methionine (59 mCi/mmol, NEN Life Science Products, Boston, MA) were added, and the reaction mixture was incubated for another 1.5 h to determine TPMT-catalyzed 6MP *S*-methylation activity. The methylation products were extracted with 1 ml of chloroform, dried in vacuum and applied on a TLC plate coated with Kieselgel 60 F_{254} (Merck, Germany). Analysis of methylation products was carried out by TLC in nPrOH-nBuOH-formic acid-water (60:20:19:1). Chromatograms were treated with En^{3}hance surface autoradiography enhancer (DuPont, Boston, MA). Radioautography was carried out at 70°C overnight.

3. Results and discussion

3.1. Isolation of the murine TPMT cDNA

Using the sequence of the open reading frame (ORF) of the human TPMT as a query, we screened the GenBank dbEST (expressed sequence tags) database [14] for homologous sequences. Two sequences (GenBank accession numbers AA109870 and AA637718) derived from the murine cDNA libraries were identified with 70–80% homology to the human TPMT ORF. When translated into polypeptide sequences, 5′-terminal sequences of both clones present in the GenBank revealed 80% homology to the N-terminal fragment of the

*Corresponding author. Fax: (1) (901) 525-6869.
E-mail: william.evans@stjude.org

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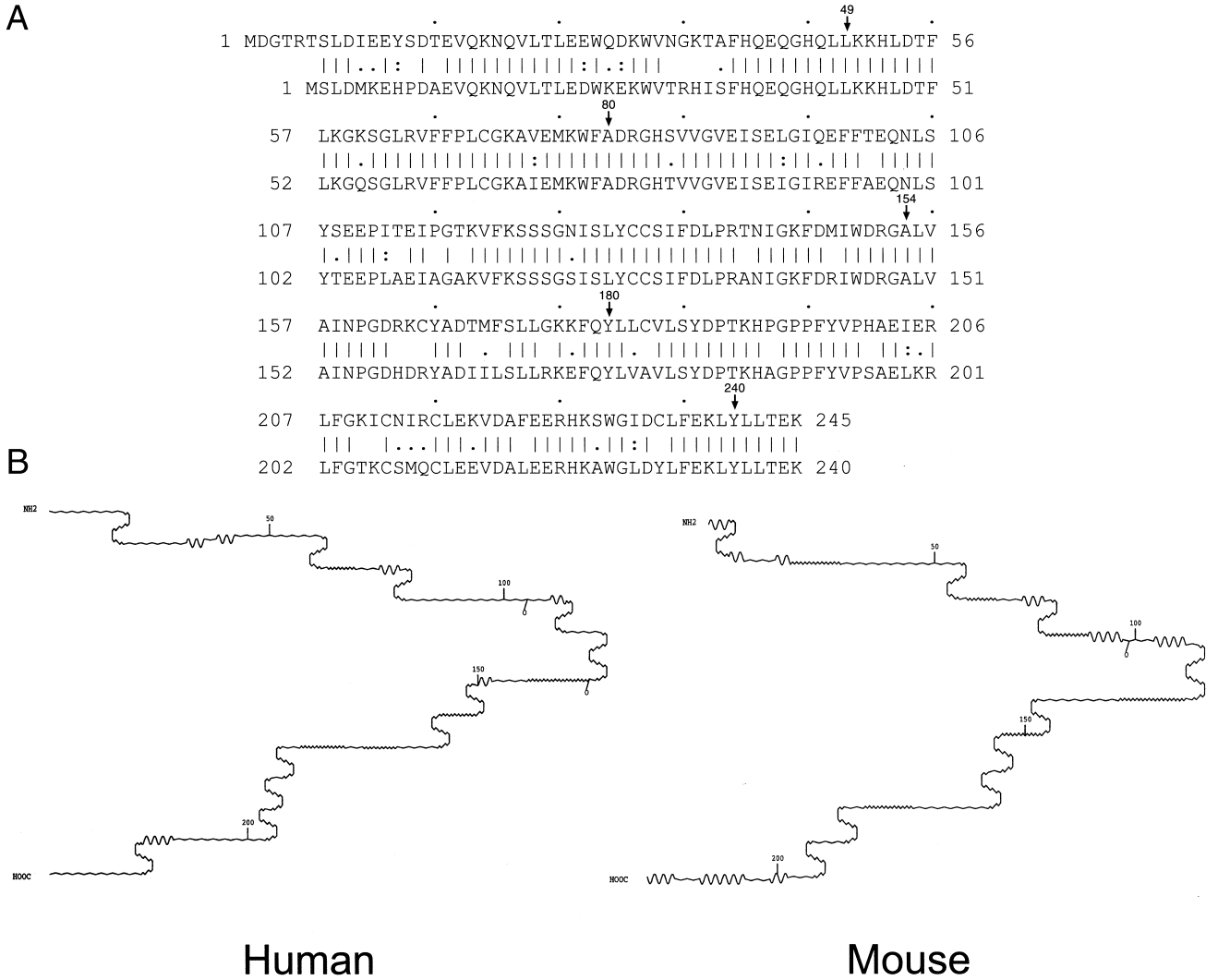

Fig. 1. A: Comparison of the amino acid sequences of the human TPMT (upper line) and the translation product of AA637718 cDNA (bottom line). Arrows indicate positions of inactivating mutations found in human TPMT (numbered according to [6,8]). B: Chou-Fasman prediction of the secondary structures of human (left) and murine (right) TPMT proteins.

human TPMT. Therefore, we further characterized these clones by bidirectional sequencing.

The nucleotide sequence of clone AA637718 contained a cDNA insert of 1068 bp with a potential ORF of 723 bp in length (the full sequence of the insert in clone AA637718 has been submitted to GenBank with accession number AF046887). This uninterrupted stretch of cDNA starts with an ATG codon and ends with a TAA codon. The 3′-end fragment of this cDNA contains a sequence identical to the polyadenylation signal ATTAAA, about 17 bp upstream from the end of the cDNA [15]. An uninterrupted ORF encoded a 240 amino acid polypeptide. The nucleotide sequence has 82% similarity to the human TPMT sequence and is more than 99% similar to the sequence of clone AA109870. The amino acid sequence of the corresponding polypeptide translated from cDNA AA637718 is 82% similar and 78% identical to that of the human TPMT (Fig. 1). Clone AA109870 derived from the C57/BL6 murine cDNA library contained an additional insert resulting in a frameshift that produced preliminary termination of the ORF, and was therefore not characterized further. Presence of this inactivating mutation indirectly supports the observation of decreased TPMT activity in the C57/BL6 strain [16], which was the source of RNA for this cDNA library. Therefore, we hypothesize that the AA109870 cDNA is from the inactive allele responsible for low TPMT activity in the C57/BL6 mouse. About 70% similarity was found between human and murine TPMT mRNA sequences in the 5′-untranslated regions (UTR), with no significant homology in the 3′-UTR of mRNA.

Amino acid sequences within the ORF of clone AA637718 and human TPMT showed striking similarity in several regions crucial for activity of human TPMT: amino acid residues at positions where inactivating mutations have been found in the human TPMT are located in the most conservative regions of the protein, with corresponding nucleotides identical in both sequences (see Fig. 1A) [6,8]. All but one nucleotide corresponding to the exon-intron boundaries in the human TPMT mRNA are conserved in the murine sequence [7,9]. The sequence of the C-terminal undecapeptide of the mouse protein (LFEKLYLLTEK) is the same as in the

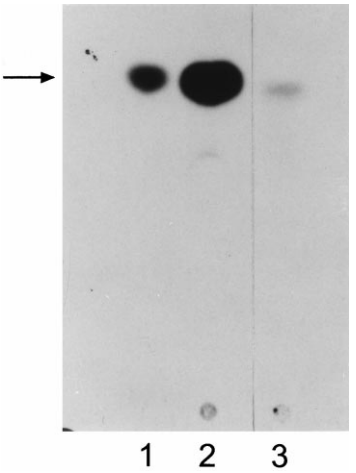

Fig. 2. Enzymatic activity of the translation product of AA637718 cDNA (lane 1), human *TPMT*1* (lane 2), and mutant human *TPMT*3A* cDNA (lane 3). After incubation of the recombinant plasmids with RRL transcription-translation system, 6-mercaptopurine and [^{14}C-CH$_3$]*S*-adenosyl-L-methionine were added to the reaction mixture as indicated in Section 2. Arrow indicates the position of authentic methylmercaptopurine.

human TPMT (Fig. 1A). When the secondary structure of the human TPMT and the putative murine TPMT polypeptide chain, as predicted by Chou-Fasman modelling [17], were compared, they showed high similarity in both the number and relative location of α-helices, β-sheets and turns (see Fig. 1B).

3.2. Functional characterization of the murine TPMT cDNA

To verify that the isolated cDNA clone encodes a functional TPMT protein, we expressed this cDNA in RRL and WGL, and documented TPMT activity in the reaction mixture. We found significant TPMT enzymatic activity in the reaction mixture after transcription-translation of the murine cDNA: incubation of 6-mercaptopurine with radioactive SAM in the presence of transcription-translation products of cDNA AA637718 resulted in the formation of ^{14}C-CH$_3$-methylated mercaptopurine. TPMT activity was substantially higher than the endogenous TPMT activity in the RRL and WGL, after expression of the cDNA encoding the inactive human *TPMT*3A* allele (Fig. 2, lane 1 versus lane 3). Human wild type cDNA was used as a positive control, and the product of its transcription-translation revealed substantially higher activity when compared to that of murine TPMT cDNA. Methylation of mercaptopurine was notably lower in WGL comparative to RRL, presumably due to lack of endogenous TPMT activity in the former. The difference in TPMT activity for the murine and human translation products could probably be explained by relatively lower activity of the murine protein, decreased efficiency of transcription of mouse versus human cDNAs (T7 versus SP6 RNA polymerases) and/or decreased efficiency of translation of the resulting murine mRNA. The latter seems quite plausible, because the murine mRNA has an approximately 100 nucleotides longer 5′-UTR compared to the human TPMT mRNA. Further experiments can now be undertaken to determine the relative activities of the human and murine TPMT enzymes.

This study demonstrated that the murine cDNA AA637718 encodes a protein with high structural similarity to the human TPMT protein sequence. When expressed, the protein encoded by this murine cDNA has thiopurine *S*-methyltransferase enzymatic activity, thus indicating that we have isolated the functional murine TPMT cDNA. Isolation of the murine cDNA permits future studies to compare the TPMT proteins from mice and humans and thereby elucidate their functional elements and mechanism(s) of enzymatic activity. This cDNA also provides an important tool for cloning the murine TPMT gene, which will facilitate future studies of the molecular biology and physiological functions of TPMT.

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