Cloning and Characterization of a New Member of the Nudix Hydrolases from Human and Mouse* 

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Hanjing Yang‡‡, Malgorzata M. Slupska‡‡, Ying-Fei Wei§§, Jennifer H. Tai‡, Wendy M. Luther‡, Yu-Rong Xia**, Diana M. Shih**, Ju-Huei Chiang‡, Claudia Baikalov‡‡, Isabella T. Phan‡, Alexis Conrad‡, and Jeffrey H. Miller‡§§

From the §§Department of Microbiology and Molecular Genetics and the Molecular Biology Institute, University of California, Los Angeles, California 90095, the **Division of Cardiology, Department of Medicine, 47-123 CHS, School of Medicine, University of California, Los Angeles, California 90095, and ¶¶Human Genome Sciences, Rockville, Maryland 20850

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Proteins containing the Nudix box “GXEXX,REUXE-XGU” (where U is usually Leu, Val, or Ile) are Nudix hydrolases, which catalyze the hydrolysis of a variety of nucleoside diphosphate derivatives. Here we report cloning and characterization of a human cDNA encoding a novel nudix hydrolase NUDT5 for the hydrolysis of ADP-sugars. The deduced amino acid sequence of NUDT5 contains 219 amino acids, including a conserved Nudix box sequence. The recombinant NUDT5 was expressed in Escherichia coli and purified to near homogeneity. At the optimal pH of 7, the purified recombinant NUDT5 catalyzed hydrolysis of two major substrates ADP-ribose and ADP-mannose with Km values of 32 and 83 μM, respectively; the Vmax for ADP-mannose was about 1.5 times that with ADP-ribose. The murine NUDT5 homolog was also cloned and characterized. mNudT5 has 81% amino acid identity to NUDT5 with catalytic activities similar to NUDT5 under the optimal pH of 9. Both NUDT5 and mNudT5 transcripts were ubiquitously expressed in tissues analyzed with preferential abundance in liver. The genomic structures of both NUDT5 and mNudT5 were determined and located on human chromosome 10 and mouse chromosome 2, respectively. The role of NUDT5 in maintaining levels of free ADP-ribose in cells is discussed.
Free ADP-ribose is converted to AMP and ribose 5-phosphate by ADP-ribose pyrophosphatase. The activities of ADP-ribose pyrophosphatase are detected in microorganisms as well as in higher eukaryotes. So far, three genes encoding ADP-ribose pyrophosphatase activities have been identified and characterized. ORF186 from *E. coli* was characterized as a Nudix hydrolase with broad substrate specificity (7). It catalyzes the conversion of ADP-ribose to AMP and ribose 5-phosphate. It can also catalyze the hydrolysis of Ap₆A and NAD with similar efficiencies. Recently, the product of the Mu1149 gene, another Nudix hydrolase from thermophilic Archaeon *Methanococcus jannaschii*, was characterized as a highly specific ADP-ribose pyrophosphatase (15). A bifunctional enzyme shl0787 from *Synechocystis* sp. was shown to have both ADP-ribose pyrophosphatase and NMN adenylyltransferase activity (16). Four other Nudix hydrolases, YSA1 of *S. cerevisiae* (ScYSA1), ORF209 of *E. coli* (EcORF209), YQKG from *Bacillus subtilis* (BsYQKG), and Hi0398 from *Hemeophilus influenzae* (1, 7). The functions of ADP-ribose pyrophosphatases were proposed to be the “housecleaning enzyme” that removes the highly reactive free ADP-ribose molecule as well as reutilize it after hydrolysis to form AMP and ribose 5-phosphate (1).

Although several biochemically distinct ADP-ribose pyrophosphatase activities have been identified from human erythrocytes, rat liver, and Artemia cysts (27–30), no gene has been cloned and characterized. In this work, we report cloning and characterization of a novel Nudix hydrolase NUDT5 from human and its murine homolog mNUDT5. The major substrates for NUDT5 and mNUDT5 are ADP-sugars with preference to ADP-ribose. The RNA transcripts of NUDT5 and mNUDT5 are expressed in all tissues analyzed with preferential expression in liver. Furthermore, the genomic structure and chromosome localization for both NUDT5 and mNUDT5 are also described.

**EXPERIMENTAL PROCEDURES**

**Identification and Cloning of NUDT5 and mNUDT5 cDNA—**A cDNA fragment containing the NUDT5 coding region was identified in the Human Genome Sciences human cDNA sequence data base during searches for fragments encoding protein sequence homologous to *E. coli* MutT using the program BLAST (31). A λ phage library containing mouse liver cDNA (Stratagene, La Jolla, CA) was screened using a probe containing the NUDT5 cDNA coding region obtained after PCR amplification. The hybridization and washing condition were according to the manufacturer protocols. Recombinant plagues containing mNUDT5 were isolated and DNA samples were prepared using protocols as described previously (32). The sequencing reactions were carried out using α-[32P]dATP and a SequiTherm Cycle Sequencing Kit (Epicycle Technologies, Madison, WI). The oligonucleotides used for sequencing were synthesized on a Beckman oligo1000 DNA synthesizer (Beckman Instruments). All oligonucleotides were deprotected in ammonium hydroxide and used without further purification.

**Complementation of *E. coli* MutT Using the Program BLAST (31)—** A λ phage library containing mouse liver cDNA (Stratagene, La Jolla, CA) was screened using a probe containing the NUDT5 cDNA coding region obtained after PCR amplification. The hybridization and washing condition were according to the manufacturer protocols. Recombinant plagues containing mNUDT5 were isolated and DNA samples were prepared using protocols as described previously (32). The sequencing reactions were carried out using α-[32P]dATP and a SequiTherm Cycle Sequencing Kit (Epicycle Technologies, Madison, WI). The oligonucleotides used for sequencing were synthesized on a Beckman oligo1000 DNA synthesizer (Beckman Instruments). All oligonucleotides were deprotected in ammonium hydroxide and used without further purification.

**Expression in E. coli and Purification of NUDT5 and mNUDT5—**The NUDT5 gene was cloned between the SalI and XbaI sites of the bacterial expression vector pQE9 (Qiagen, Chatsworth, CA) after PCR amplification. Transformants of *E. coli* M15/pREP4 with the pQE9/NUDT5 were grown at 37 °C in 100 ml of LB medium with 100 μg/ml ampicillin and 25 μg/ml kanamycin. The plasmid pREP4 constitutively expresses the Lac repressor protein encoded by the lacI gene in order to reduce the basal level of expression (Qiagen). When the culture grew to an _A_₆₀₀₈ of 0.7, a final concentration of 1 mM isopropyl-1-thio-β-D-galactopyranoside was added to induce the expression of NUDT5 protein for 2 h. Bacterial lysates were prepared by French press (16,000 psi) buffer containing sodium phosphate, pH 8.0, 300 mM NaCl plus 0.5 mM phenylmethylsulfonyl fluoride. After clarification by centrifugation the lysate was mixed with 3 ml of Ni²⁺-NTA matrix (Qiagen) for 1 h at 4 °C with gentle shaking. Then the mixture was poured into a column, washed with buffer A containing 0.1 M imidazole, and eluted with buffer A containing 1 M imidazole. Western blot analysis was performed using a primary antibody (RGS-His Antibody, Qiagen) and a secondary alkaline phosphatase-conjugated antibody (Sigma) to monitor the recombinant NUDT5 protein during purification. The fractions containing the recombinant NUDT5 proteins were dialyzed overnight in 1 liter of buffer B (50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM dithiothreitol, and 50% glycerol) with two changes. A clear protein sample was obtained after centrifugation of the dialyzed sample, aliquots were stored at −80 °C.

The mNUDT5 gene was cloned between SpI and HindIII sites of the bacterial expression vector pQE30 (Qiagen) after PCR amplification. The expression condition and purification protocols were similar to those of NUDT5.

**Electrospray Mass Spectrometry**—A Perkin-Elmer Sciex (Thornhill, Canada) API III triple quadrupole mass spectrometer equipped with an IonSpray source was tuned and calibrated as described previously (33). Positive ion protein spectra were produced by injection of the proteins dissolved in water onto a C₅ reverse phase column (Keystone Scientific BDS Hypersil C₅, 100 × 1 mm, 5-μm particle size, 120 Å pore size, 20 μl injection) equilibrated in water/acetonitrile/trifluoroacetic acid (95/5/0.1) all by volume. The column was eluted (40 μl/min) with a linearly increasing concentration of acetonitrile (2%/min) and the column eluant was passed in series through a UV detector (215 nm) and the Ionspray source. Data was recorded with the mass spectrometer scanning from m/z 400—2000 (step size 0.3 Da, dwell time 1 ms, 5.61 s/scan, orifice voltage 90). The average of the spectra contributing to the peak in ion current was computed. Calculation of molecular mass from the series of multiply charged ions found in the protein was achieved with the MacSpec™ computer program (version 3.3, PE Sciex, Ontario, Canada). Calculation of theoretical protein average (chemical) molecular mass was achieved with the MacBioSpec™ computer program (version 1.0.1, PE Sciex) based on the MacProMass computer program of Lee and Vermuri (35).

**Enzyme Assay**—A slightly modified colorimetric procedure (15) was used to assay the hydrolysis of the substrates. A standard reaction mixture contained in (50 μl): 50 mM Tris, pH 7.0 (for NUDT5) or pH 9.0 (for mNUDT5), 5 mM MgCl₂, 1 mM dithiothreitol, 2 μM substrate, 1 unit of calf intestinal alkaline phosphatase, and 140 ng of NUDT5 or 400 ng of mNUDT5. The reaction mixture was incubated at 37 °C for 15 min, terminated by the addition 250 μl of 20 mM EDTA. The inorganic orthophosphate produced was quantified by the colorimetric assay of A. M. Lowry and R. H. Rosebrough (36).

For product identification by electrospray mass spectrometry, the cecal intestinal alkaline phosphatase was omitted from the standard assay. Spectra of reaction mixtures were obtained by flow injection analysis of samples diluted (2/100) in water/acetonitrile/triethylamine (50/50/0.1, all by volume). Aliquots of the solution (10–20 μl) were injected into a stream of the same solvent entering the IonSpray source (34) (10 μl/min) while the mass spectrometer was scanning in the negative ion mode from 200 to 1000 Da (0.3 Da step size, 5.47 s/scan, orifice voltage 60–80).

**Northern Blot Analysis**—Mouse multiple tissue Northern (MTN™) blot containing 2 μg of poly(A)⁺ RNA from various adult tissues (CLONE-TECH) was hybridized with a 32P-Random primed 300-bp mNUDT5 cDNA fragment containing exon 1–4 (Prime-II II kit, Stratagene). The hybridization and washing conditions were according to the manufacturer protocol “Multiple Tissue Expression Array User Manual.” The blot was hybridized at 65 °C overnight and washed in solution 1 (2 × SSC and 1% SDS) 5 times at 65 °C for 20 min and twice in solution 2 (0.1 × SSC and 0.5% SDS) at 55 °C for 20 min. The blot was stripped by incubation for 10 min in 0.5% SDS at 90–100 °C and reprobed with β-actin cDNA control probe.

The Human Multiple Tissue Expression (MTE™) Array (CLONE-TECH, Palo Alto, CA) hybridization was carried out according to the manufacturer protocol “Multiple Tissue Expression Array User Manual” with a 32P-Random-primed 700-bp NUDT5 cDNA PCR probe containing the coding region (Prime-II II kit, Stratagene). The blot was washed under the same condition as for the MTN blot (see above).
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Determination of the Exon-Intron Organization—A λ phage library containing mouse genomic DNA (strain 129 Svj, Stratagene) was screened using a NUDT5 cDNA probe containing the coding region. The recombinant phage was isolated and the DNA sample was prepared using protocols as described previously (32). The DNA was partially sequenced with primers designed on the basis of mNUDT5 cDNA sequence to obtain the intron-exon organization. The 3′ end of mNUDT5 gene containing exons 7–9 was obtained from two overlapping PCR products from mouse genomic DNA (strain 129 Svj). The PCR products were cloned in pcR2.1-TOPO vector using TOPO TA cloning kit (Invitrogen, Carlsbad, CA) and partially sequenced. The sizes of introns were determined from other sequencing of the genomic clone (introns 1, 3–5, and 7) or from PCR analysis (introns 2, 6, and 8).

To determine the exon-intron organization of the NUDT5 gene, 8 sets of PCR primers were designed to construct a contig of genomic DNA fragments covering the NUDT5 locus. Human genomic DNA (CLONTECH) was used for PCR. PCR products were analyzed on agarose gel, cloned into the pcR2.1-TOPO vector using TOPO TA cloning kit (Invitrogen), and partially sequenced to obtain the exon-intron organization. The sequences of primers are available upon request.

Chromosomal Mapping of NUDT5 and mNUDT5—Radiation hybrid mapping technique was used to map both mNUDT5 and NUDT5.

The 100 cell lines of the T31 radiation hybrid panel, which carries fragments of the mouse genome on a hamster background (37), were used as templates for PCR amplification with mNUDT5 primers 5′- CAAAGAAACCTGGACCATGA-3′ and 5′-TACCTACCTGACCTTTCA-CAC-3′ derived from the sequence expanding the region of exon 4-intron 4 of the mNUDT5 genomic DNA. Reactions were performed with 112.5 ng of hybrid clone DNA, 0.4 μM each primer, 250 nM each dNTP, 0.625 units of TaKaRa E Taq polymerase (TaKaRa Shuzo Co., Japan) and the supplied buffer, in a total volume of 25 μL. After an initial denaturation at 94 °C for 3 min, 30 cycles of denaturation at 94 °C for 30 s, annealing at 62 °C for 1 min, and extension at 72 °C for 1.5 min were performed, followed by a final extension at 72 °C for 7 min. The PCR products were analyzed by gel electrophoresis in 1.2% agarose gel, stained with ethidium bromide, and scored for the presence or absence of the 230-bp product. In preliminary experiments, a single product of expected size 190 bp was detected using human genomic DNA as template. Two independent reactions were performed with mNUDT5 primers for the entire GB4 panel. All data were submitted to the Jackson Laboratory Mouse Radiation Hybrid Data base for mapping analysis.

The 93 cell lines of the Gene Bridge 4 (GB4) radiation hybrid panel, which carries fragments of the human genome on a hamster background (38), were used as templates for PCR amplification with NUDT5 primers 5′-TCGATAATATAAGGCACAGAA-3′ and 5′-CTCGACTAGAACTAAGAAGCTCT-3′ derived from the 3′- untranslated region of the NUDT5 cDNA. PCR conditions were the same as described above for the mNUDT5 gene. In preliminary experiments, a single DNA band of expected size 190 bp was detected using human genomic DNA as template while no product was detected using hamster genomic DNA as template. Two independent reactions were performed with NUDT5 primers for the entire GB4 panel. All data were submitted to the Whitehead Institute/MIT Center for Genome Research’s Human Radiation Hybrid Data base for mapping analysis.

Phylogenetic Analysis—Distance analysis was performed using neighbor joining in the PAUP program (39). A multiple sequence alignment of the “MutT” domain was retrieved from the Pfam website (40). Representative sequences were selected from the Pfam alignment and five additional sequences were manually added (NUDT5, mNUDT5, Tm, Pa, and Ap, see Fig. 10, legend).

RESULTS

Identification and Cloning of NUDT5 and mNUDT5 Gene—During the search for DNA repair genes in the Human Genome Sciences human cDNA sequence data base, a clone was identified, which encodes a protein of 28.5 and 21.8% amino acid identity to E. coli MutT and hMTH (41), respectively. The nucleotide sequence of the full-length cDNA (1125 bp accession) is shown in Fig. 1. It contains an open reading frame (ORF) of 660 nucleotides from the first translation initiation codon ATG to the termination codon TAA (nucleotides 120–779). An in-frame stop codon is located 39 base pairs upstream of the initiation codon. The complete ORF encodes a protein of 219 amino acids with a predicted molecular mass of 24357 Da and pl 4.8 (42). We confirmed the nucleotide sequence of 5′-nontranslated region with sequences obtained from the BLAST search (31). Of the six sequences from the GenBank that contained the 5′-nontranslated region (accession numbers: AA306176, AA306565, AA490510, W07480, W16891, and W92824), all of them contained the in-frame stop codon before the initiation translation codon ATG. The Nudix signature sequences of this ORF protein, together with E. coli MutT, hMTH, and other characterized Nudix hydrolases with unique substrate specificity are shown in Table I. The ORF protein contains the conserved amino acid residues in the Nudix signature sequence, which recently has been designated as NUDT5 on the website of the Human Gene Nomenclature Committee. One feature in NUDT5 Nudix sequence is Tyr-119, which is not a bulk amino acid like Ile, Leu, or Val as in the majority of other Nudix signature sequences.

A cDNA clone of a murine NUDT5 homolog (mNUDT5) from mouse liver cDNA library was identified using NUDT5 cDNA sequence as a probe. It contains a 934-bp cDNA sequence with an open reading frame of 657-bp encoding a predicted protein product of 218 amino acids. The mNUDT5 has 81% amino acid identity to the NUDT5 (Fig. 2) with a predicted molecular mass of 23986 Da and pl of 5.3 (42).

Expression of Both NUDT5 and mNUDT5 in E. coli and Purification of the Recombinant Proteins—The initial attempt to characterize NUDT5 was to test whether the predicted protein product had the MutT activity. Complementation of E. coli mutT mutant phenotype was carried out using E. coli mutT strain expressing the cloned NUDT5 gene in the plasmid pKK388-1. The experiments were done simultaneously with both E. coli mutT and hMTH genes cloned in the same vector as positive controls. While E. coli mutT and hMTH totally complemented the E. coli mutT mutator phenotype, no complementation was observed with NUDT5 (data not shown). The dGTP hydrolase activity was also examined using the crude extract from E. coli mutT strain containing pKK388-1/NUDT5. No activity was detected (data not shown).

To further characterize the NUDT5 protein, we decided to purify the protein and to test its activity on potential substrates catalyzed not by MutT, but by other Nudix hydrolases. The hexahistidine-tagged recombinant proteins for both NUDT5 and mNUDT5 were expressed in E. coli and purified to near homogeneity (Fig. 3). Both hexahistidine-tagged proteins have unusual tight binding to Ni2+-NTA resin as they eluted from the Ni2+-NTA column with 1 M imidazole at pH 8.0. The apparent molecular mass on the SDS-PAGE of both purified recombinant proteins were around 40 kDa, about 15 kDa bigger than the predicted molecular mass of both histidine-tagged proteins. Also the purified NUDT5 recombinant protein apparently has a doublet on the SDS-PAGE (Fig. 3).

Mass spectrometry was used as an alternative and more accurate method to investigate the apparent increased molecular mass and heterogeneity of both purified protein samples. LC/electrospray MS of the purified hexahistidine-tagged mNUDT5 protein gave superimposable peaks of UV absorption and ion current eluting at around 28 min (approximately 20% acetonitrile). The mass spectrum yielded a molecular mass of 25429.4 Da (data not shown), which is close to the calculated mass of the protein (25425.9 Da). The same analysis on the NUDT5 revealed superimposable peaks of UV adsorption and ion current eluting in the same region of the chromatogram. The mass spectrum revealed microheterogeneity within the sample with three predominant and roughly equally abundant components with molecular masses of 26057.3, 26048.5, and 26017.2 Da. The difference in molecular masses between the lighter two components of 31.3 Da could be due to the inclusion...
of two oxygen atoms, possibly through oxidation of any two of the four Met and 10 His residues. The explanation for the difference of 8.5 Da between the heavier two forms is not obvious. This mass difference is incongruous with known protein covalent modifications. The observed molecular masses are to be compared with a calculated molecular mass of 25635.0 Da of the NUDT5, which is 382.2 Da lighter than the smallest of the observed forms. The explanation for this mass difference is obscure at the moment.

**Substrate Specificity of the Recombinant Proteins—**

The first set of Nudix substrates tested were ADP-sugars including ADP-ribose and ADP-mannose. The reaction products were subjected to mass spectrometry analysis and the results from ADP-mannose hydrolysis by NUDT5 protein were shown in Fig. 4. The reaction products contain two new species of roughly equal amounts with the molecular mass correlated to AMP (346.1 Da) and mannose 5-phosphate (259.1 Da) suggesting that NUDT5 acts as a pyrophosphatase, which hydrolyzes ADP-mannose to generate products of AMP and mannose 5-phosphate. Similar results with AMP and ribose 5-phosphate peaks were obtained when the substrate ADP-ribose was used (data not shown).

Most of the characterized Nudix hydrolases require an alkaline pH and the presence of divalent ions to become fully active. The optimal pH was determined for both NUDT5 and mNudT5 with substrates ADP-ribose and ADP-mannose (Fig. 5). While mNudT5 has its optimal activity to both ADP-sugars at the expected alkaline pH around 9, the NUDT5 has an optimal

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**Nudix signature sequences of NUDT5 with other Nudix hydrolases**

| Accession | Organism | Enzyme | Substrate | Nudix box | Ref. |
|-----------|----------|--------|-----------|-----------|------|
| AF218818  | *H. sapiens* | NUDT5 | ADP-ribose, ADP-mannose | GLIDGDPTEEAALRELEEEETGY | This work |
| P08337    | *E. coli* | MutT | dGTP, 8-oxo-dGTP | GRIEMGETEPQAVRELREEEGVI | 46 |
| P36639    | *H. sapiens* | MTH | dGTP, 8-oxo-dGTP | GRVCEGETIEGERELREGSI | 4 |
| D64443    | *M. jannaschii* | ADPBase | ADP-ribose | GFVEGETVIEAVRESREESGL | 15 |
| P39851    | *E. coli* | ORF209 | ADP-ribose | GNEIEGDEVVAVRESREESGL | 1 |
| P45799    | *E. coli* | ORF186 | Ap,A, ADP-ribose, NADH | GLIDGSRVEEANRELREEEGVF | 7 |
| P32664    | *E. coli* | ORF257 | NADH | GRVCEGETIEGERELREGSI | 13 |
| P32056    | *E. coli* | ORF1.9 | GDP-mannose | GRVCEGETIEGERELREGSI | 14 |
| P24236    | *E. coli* | ORF17 | dATP | GSEVEGETPAAREREELVEETI | 6 |
| P50553    | *H. sapiens* | ApAase | ApA | GHVEGETPEDDLTEAREREGAI | 9 |
| Q09790    | *S. pombe* | ApS1 | ApA,ApA,PP-insP,insP,PP | GGEWDEVSQQQALREGENGGL | 18 |

**Table I**

The conserved amino acid residues in Nudix box are in bold.
neutral pH around 7. The requirement for divalent ions was also studied. Both NUDT5 and mNudT5 require the presence of Mg$^{2+}$ to achieve optimal activity (1 mM Mg$^{2+}$ with NUDT5 and 5 mM Mg$^{2+}$ with mNudT5, data not shown). Similar catalytic activities were observed when Mg$^{2+}$ concentration was increased to 20 mM for both NUDT5 and mNudT5 (data not shown). Mn$^{2+}$ and Zn$^{2+}$ could partially substitute for Mg$^{2+}$ (data not shown).

Other potential substrates for both NUDT5 and mNudT5 were tested and the results were shown in Fig. 6. Under the optimal pH 7 for NUDT5 with the presence of 5 mM Mg$^{2+}$, NUDT5 prefers substrates containing ADP linked to sugar moieties, such as ADP-mannose, ADP-ribose, and ADP-glucose. The rate of hydrolysis decreased about 5–7-fold as the nucleoside in ADP-sugar changes from adenosine to guanosine or uridine. NUDT5 also displayed minor activities on NADH, as well as on Ap$_2$A. A similar pattern of substrate specificity was observed with mNudT5 under its optimal alkaline pH (Fig. 6).

Both NUDT5 and mNudT5 have no activities on ribo- and deoxyribonucleoside triphosphates (data not shown), which are the substrates for MutT (43) and another Nudix hydrolase, Orf17 (6).

The catalytic properties were studied on substrates ADP-ribose and ADP-mannose for both NUDT5 and mNudT5 (Table II). NUDT5 has $K_m$ of 32 $\mu$M for ADP-ribose and higher $K_m$ (83 $\mu$M) for ADP-mannose. However, the $V_{max}$ of NUDT5 for ADP-mannose is about 1.5 times that with ADP-ribose. Therefore, the overall catalytic efficiency ($V_{max}/K_m$) of NUDT5 is higher for ADP-mannose than for ADP-ribose. mNudT5 has slightly different catalytic properties. For ADP-ribose mNudT5 has a $K_m$ similar to NUDT5, about 38 $\mu$M. However, the $K_m$ for ADP-mannose for mNudT5 is 154 $\mu$M, about four times as much as the $K_m$ for ADP-ribose. The $V_{max}$ of mNudT5 for ADP-mannose is only about 1.2 times that with ADP-ribose. Therefore, the resulting overall catalytic efficiency ($V_{max}/K_m$) of mNudT5 is higher for ADP-ribose than ADP-mannose. According to the standard practice of naming the enzyme after the substrate with the lowest $K_m$, we suggest the name ADP-ribose pyrophosphatase for NUDT5 protein.

Tissue-specific Expression of NUDT5 mRNA—Northern blot of mouse poly(A)$^+$ RNA isolated from various tissues was carried out using mNudT5 probe containing exons 1–4. It revealed a major transcript of 1.35 kb in all eight adult mouse tissues analyzed, with the most abundant expression in liver (Fig. 7). The expression of NUDT5 mRNA was analyzed using the Human Multiple Tissue Expression Array (Fig. 8). Abundant expression of NUDT5 mRNA was observed in liver, pituitary
gland, placenta, and also HeLa cells.

**Genomic Structure and Chromosomal Localization**—The exon-intron boundaries of the *NUDT5* gene were determined by analyzing 8 PCR-amplified clones covering the entire gene. The *NUDT5* gene spans approximately 18.5 kb and is divided into 9 exons (Table III, part a).

**FIG. 5.** pH dependence of NUDT5 and mNudT5 on hydrolysis of substrates ADP-mannose and ADP-ribose. Purified NUDT5 or mNudT5 was incubated with 2 mM ADP-mannose (A) or ADP-ribose (B) at 37 °C for 15 min in 50 mM Tris buffer (pH 4.5 to pH 10). The hydrolysis of the substrates was assayed using the colorimetric procedure described under “Experimental Procedures.” Each result represents the mean ± S.D. from three experiments.

**FIG. 6.** Substrate specificity of NUDT5 and mNudT5. Purified NUDT5 or mNudT5 was incubated with 2 mM substrate at 37 °C for 15 min in 50 mM Tris buffer (pH 7 for NUDT5 and pH 9 for mNudT5). The hydrolysis of the substrates was assayed using the colorimetric procedure described under “Experimental Procedures.” Each result represents the mean ± S.D. from three experiments.

**FIG. 7.** Expression of mNudT5 mRNA in adult mouse tissues. A Northern blot containing 2 μg of mouse poly(A)+ RNA was hybridized with a 300-bp mNudT5 cDNA probe containing exons 1–4. The membrane was stripped and reprobed with a β-actin cDNA probe. Lane 1, heart; lane 2, brain; lane 3, spleen; lane 4, lung; lane 5, liver; lane 6, skeletal muscle; lane 7, kidney; and lane 8, testis. Size markers in kb are shown on the left. mNudT5 mRNA transcripts of 1.35 kb are indicated by an arrowhead on the right.

**Table II**

**Kinetic parameters for NUDT5 and mNudT5**

The colorimetric procedure described under “Experimental Procedures” was used with concentrations of 0.01 mM to 2 mM for all substrates. *K*<sub>m</sub> and *V*<sub>max</sub> were determined from a nonlinear regression analysis (47). A unit of enzyme hydrolyzes 1 μmol of substrate/min. Each result represents the mean ± S.D. from three experiments.

| Substrate      | *V*<sub>max</sub> (units mg<sup>−1</sup>) | *K*<sub>m</sub> (μM) |
|----------------|----------------------------------------|----------------------|
| NUDT5 ADP-ribose | 9.50 ± 2.11                           | 31.61 ± 5.06         |
| NUDT5 ADP-mannose | 14.23 ± 1.19                          | 83.30 ± 11.38        |
| mNudT5 ADP-ribose | 5.67 ± 0.47                           | 37.89 ± 4.97         |
| mNudT5 ADP-mannose | 7.14 ± 0.92                           | 154.36 ± 13.88       |
For determination of \( m\text{Nud}T5 \) genomic structure one recombinant phage containing exons 1 to 6 of the \( m\text{Nud}T5 \) gene was identified and partially sequenced to determine the exon-intron boundaries. Exons 7 to 9 were determined by analyzing two overlapping PCR products covering partial intron 6 to the 3\(^\text{9}\)-untranslated region. \( m\text{Nud}T5 \) is approximately 13 kb in length and is divided into 9 exons (Table III, part b). All 5' donor and 3' acceptor splice sites conform to the consensus GT/AG rule (44). Both \( \text{NUDT5} \) and \( m\text{Nud}T5 \) have introns in the same position of their coding sequence.

Both \( \text{NUDT5} \) and \( m\text{Nud}T5 \) were mapped on their corresponding chromosomes using radiation hybrid mapping (37, 38) as described under “Experimental Procedures.” The radiation hybrid data placed \( m\text{Nud}T5 \) on mouse chromosome 2 between markers D2Mit354 and D2Mit76 (Fig. 9). The LOD score was 13.6 between D2Mit354 and \( m\text{Nud}T5 \), and 17.1 between \( m\text{Nud}T5 \) and D2Mit76. According to genetic mapping data from the 1999 Chromosome Committee Reports available from the Jackson Laboratory website, the physical map locations of WI-4124 and WI-8819 correspond to 28–29 and 29–32 cM, respectively (Fig. 9). Based on these markers, the physical map position for \( m\text{Nud}T5 \) corresponds to a locus at 29–32 cM of chromosome 10 on the genetic map (Fig. 9). Human \( \text{VIM} \) and \( \text{BMI1} \), which have been previously mapped by radiation hybrid to human chromosome 10 as well (data were obtained from Gene Map'98 provided by the International radiation hybrid mapping consortium through the NCBI website), are approximately 21 centiRad and 43 centiRad from the \( m\text{Nud}T5 \) locus (Fig. 9). Based on these markers, the physical map position for \( m\text{Nud}T5 \) corresponds to a locus at approximately 1 cM of chromosome 10 on the genetic map (Fig. 9).

Phylogenetic Analysis of ADP-ribose Hydrolases—The amino acid sequences of characterized and putative homologs of ADP-ribose pyrophosphatases and MutT were compared using multiple alignments from Pfam (40), additional manual alignments, and PAUP (39). As shown in Fig. 10, there are roughly three major branches; notably, ScYSA1, EcORF209, Hi0398, and BsYQKG are clustered near \( \text{NUDT5} \) proteins on the phylogenetic tree. EcORF186 is remotely related to \( \text{NUDT5} \) proteins. Mj1149 and slr0787 are clustered on a different branch with their putative sequence homologs from several bacteria and Archaea.

**DISCUSSION**

In this report, we describe the cDNA isolation and functional characterization of \( \text{NUDT5} \), a Nudix hydrolase that catalyzes the hydrolysis of ADP-sugars to AMP and sugar 5-phosphate with preference for ADP-ribose. Initiated by sequence homol-
ogy searches for potential homologs to E. coli MutT, we identified a cDNA fragment in the Human Genome Sciences human cDNA sequence database, which encodes for a protein with 28.5% amino acid identity to E. coli MutT. The analysis of the deduced 660-bp open reading frame confirms the presence of FIG. 9.

NUDT5 and mNudT5 chromosome linkage maps. Shown are physical and genetic map locations of mNudT5, NUDT5, and surrounding markers. The Radiation hybrid mapping conditions are described under "Experimental Procedures."

FIG. 10. Phylogenetic analysis. The phylogenetic tree was derived by the alignment using the programs Pfam and PAUP (39, 40). Sequences used here are as follows (species code and GenBank accession no. in parentheses): eukaryota: Homo sapiens (NUDT5, AF218818; hMTH, P36639); Mus musculus (mNudT5, AF222786); Rattus norvegicus (rMutT, P53369); S. cerevisiae (ScYSA1, Q01976); Caenorhabditis elegans (Ce, O61902); Prokaryota: E. coli (EcORF209, P36651; EcORF186, P45799; Ec1, P77788; EcMutT, P08337); H. influenzae (Hi0398, P44684; Hi1, P44932); M. tuberculosis (Tb, O33199; Tb2, O06558); B. subtilis (Bs, O06972); Proteus vulgaris (PvMutT, P29090); Thermotoga maritima (Tm, O053199; T08, O06558); Synechocystis PCC6803 (Syn1, P72646; Syn2, P55381; Syn3, P72658; Syn4, P74341; Syn_shl0787, BAAL0693); Streptococcus mutans (SmMutX, R57981); Streptococcus pneumoniae (SpMutX, P41354); Streptococcus agalactiae (Sag, O88477); Aquifex aeolicus (Aa, O67453); Thermotoga maritima (Tm, AAD9526); Archaea: Aeropyrum pernix (Ap, BA084074); Archaeoglobus fulgidus (Af, O29034); Pyrococcus abyssi (Pa, CAB49505); Pyrococcus horikoshii (Ph, O59269); M. jannaschii (Mj1149, Q58549); Methanobacterium thermoautotrophicum (Mth, O27370). The bar scale represents the number of substitutions per site. Bolded labels designate proteins with biochemically determined functions.

Table III

Intron-exon junctions of (a) NUDT5 and (b) mNudT5 genes

| Exon numbers | Exon sizes | 3’ Splice acceptors | Exon sequence | 5’ Splice donors | Intron sizes |
|--------------|------------|---------------------|---------------|-----------------|-------------|
| bp           |            |                     |               |                 |             |
| 1            | 63         | ATG-GAG             | gtataactctttttctcctcccccc | 1.3            |
| 2            | 68         | TTA-TAG             | gtaaactctttttctcctcccccc | 5.3            |
| 3            | 50         | AAC-ATG             | gtagagtcacatctctcccccc | 1.2            |
| 4            | 108        | GTG-CAG             | gtagagtcacatctctcccccc | 1.2            |
| 5            | 96         | GCG-CAG             | gtagagtcacatctctcccccc | 1.2            |
| 6            | 102        | CCG-CAG             | gtagagtcacatctctcccccc | 1.2            |
| 7            | 54         | GGG-GAG             | gtagagtcacatctctcccccc | 1.2            |
| 8            | 110        | CCC-GAG             | gtagagtcacatctctcccccc | 1.2            |

Part a

| Exon numbers | Exon sizes | 3’ Splice acceptors | Exon sequence | 5’ Splice donors | Intron sizes |
|--------------|------------|---------------------|---------------|-----------------|-------------|
| bp           |            |                     |               |                 |             |
| 1            | 60         | ATG-GAG             | gtagagtcacatctctcccccc | 1.3            |
| 2            | 68         | TTA-TAG             | gtaaactctttttctcctcccccc | 5.3            |
| 3            | 50         | AAC-ATG             | gtagagtcacatctctcccccc | 1.2            |
| 4            | 108        | GTG-CAG             | gtagagtcacatctctcccccc | 1.2            |
| 5            | 96         | GCG-CAG             | gtagagtcacatctctcccccc | 1.2            |
| 6            | 102        | CCG-CAG             | gtagagtcacatctctcccccc | 1.2            |
| 7            | 54         | GGG-GAG             | gtagagtcacatctctcccccc | 1.2            |
| 8            | 110        | CCC-GAG             | gtagagtcacatctctcccccc | 1.2            |

Part b
the Nudix signature sequence. Through detailed biochemical analysis of NUDT5 and its murine homolog, mNUDT5, we demonstrate that NUDT5 is an ADP-sugar pyrophosphatase with low $K_m$ for ADP-ribose. Unlike *E. coli* MutT, NUDT5 has no activities on ribo- and deoxyribonucleoside triphosphates. Both NUDT5 and mNUDT5 require the presence of Mg$^{2+}$. The optimal pH for NUDT5 is around neutral pH 7, while mNUDT5 requires the alkaline pH 9 to be fully active. The pH profile for NUDT5 is unusual showing two peaks of activity, which could be caused by the heterogeneity of the NUDT5 sample detected by the mass spectrometry. Both NUDT5 and mNUDT5 have similar $K_m$ (32 and 38 $\mu$M) for ADP-ribose. However, the $K_m$ for ADP-mannose was 154 $\mu$M for mNUDT5, almost twice as large as that of NUDT5 (83 $\mu$M).

Several biochemically distinct ADP-ribose pyrophosphatase activities have been identified in mammalian tissues. In rat liver, three types of enzymes that hydrolyze ADP-ribose and other related substrates have been described (30). One type strictly requires the presence of Mn$^{2+}$ rather than Mg$^{2+}$. The other two types, ADPRibase I and ADPRibase II, require the presence of Mg$^{2+}$ to be fully functional. The catalytic property of NUDT5 is closer to ADPRibase II. ADPRibase II has $K_m$ of 50 $\mu$M for ADP-ribose with substrate specificity similar as NUDT5 (30). ADPRibase I, on the other hand, is highly specific for ADP-ribose with a low $K_m$ of 0.5 $\mu$M.

NUDT5 is different from the three characterized Nudix hydrolases, ORF186 from *E. coli*, MJ1149 from *M. jannaschii*, and slr0787 from *Synechocystis* sp. Functionally, they have different substrate specificity. The slr0787 and the thermostable MJ1149 is highly specific for ADP-ribose and its closely related derivative, 2'-phospho-ADP-ribose (15, 16), while ORF186 has similar catalytic activities on three substrates, ADP-ribose, NADH, and Ap2A (7). For NUDT5, the major substrates are ADP-sugars including ADP-ribose, ADP-mannose, and ADP-glucose. NUDT5 can hydrolyze NADH and Ap2A, but with 5–10-fold lower efficiency. Structurally, NUDT5, MJ1149, and ORF186 have diverged amino acid sequences, despite having the conserved Nudix box. These enzymes are remotely related on the phylogenetic tree, which suggests that they may belong to different subfamilies of ADP-ribose pyrophosphatases. Several ORFs (1, 7), ScYSA1, EcORF209, Hl0398, and BsYQKG, are clustered with NUDT5 on the phylogenetic tree, which suggest that they might be functional homologs of NUDT5 with similar catalytic properties. Recently, a human “YSA1” homolog was mentioned to have NDP-sugar pyrophosphatase activity (5), which is similar to NUDT5 described in this paper.

The physiological function of NUDT5 is not fully understood. It has been proposed that the function of ADP-ribose pyrophosphatase is to remove the ADP-ribose, which is a potentially deleterious metabolite and to recycle it by hydrolyzing to AMP and ribose 5-phosphate (1, 15). Perhaps the final understanding of the physiological function of ADP-ribose pyrophosphatase would be to study the mutant phenotype lacking the activity of ADP-ribose pyrophosphatase. Although the potential overlapping substrate spectra between Nudix hydrolases make this task difficult, cloning and characterization of NUDT5 certainly provide the first step toward understanding the role of ADP-ribose pyrophosphatase in *vivo*. NUDT5 is preferentially expressed in liver, pituitary gland, and placenta, which may indicate the importance of NUDT5 activity in these tissues. NUDT5 maps to human chromosome 10 between markers WI-4124 and WI-8819 at the end of the short arm of chromosome 10. Our mapping is consistent with data from GenMap’99 where the ESTs similar to ScYSA1 were mapped on chromosome 10 between markers: D10S189 and D10S191. According to this server, the gene mapped in the similar region (D10S189 and D10S191) on chromosome 10 is FIP2, coding for tumor necrosis factor. In close vicinity is the gene encoding phytic acid hydrolase, which has been linked to a Refsum disease, a rare disorder of lipid metabolism. Screening through the Online Mendelian Inheritance in Man data base (OMIM) revealed that several other disorders have been mapped to the region, that at least partially overlapped with the localization of NUDT5, and have not been connected with any specific gene. They are HDR (hyperthyroidism, sensorineural deafness, and renal dysplasia), prostate adenocarcinoma 1, DiGeorge syndrome, glaucoma1, and athabaskan severe combined immunodeficiency.

It is worth noting that during the cloning of the genomic NUDT5, we have identified a pseudogene (data not shown) that had all the attributes of a processed pseudogene (45). It extended from the first base of the cDNA to the polyadenylation site, contained no introns, and was lacking the ATG translation start codon. The pseudogene was flanked by 15-bp direct repeats: GAAAAGATGAGCCAT. Two frameshifts caused by 3 deletions and 4 insertions and an in-frame stop codon interrupted the reading frame of this pseudogene. The overall amino acid sequence identity between the pseudogene and the active gene is 86.7%. The sequence of this pseudogene is consistent with a reported nucleotide sequence (accession number Z95152) on human chromosome 6p21.1–21.33.

As an increasing number of complete genome sequences become available, more and more putative Nudix hydrolases are identified. So far, every characterized genome contains at least one putative Nudix protein (15). In *E. coli*, it has 10, among which 6 are characterized with different enzymatic activity (1). It becomes a challenge to annotate these putative Nudix proteins. Phylogenetic analysis may provide a powerful tool to cluster protein homologs together according to their unique conserved motifs. One could predict the function of the putative ORF if it is clustered with a characterized Nudix protein or one could identify a putative “novel Nudix protein” if it is clustered with the unknowns. The Nudix motif has been proven to be an excellent scaffold for enzymes that hydrolyze nucleoside diphosphate derivatives or related substrates.

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