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Mouse Liver Nicotinamide *N*-Methyltransferase: cDNA CLONING, EXPRESSION, AND NUCLEOTIDE SEQUENCE POLYMORPHISMS

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ABSTRACT. Nicotinamide *N*-methyltransferase (NNMT) catalyzes the N-methylation of nicotinamide and structurally related compounds. We cloned mouse liver NNMT cDNA to make it possible to test the hypothesis that large differences among strains in levels of hepatic NNMT activity might be associated with strain-dependent variation in NNMT amino acid sequence. Mouse liver NNMT cDNA was 1015 nucleotides in length with a 792 nucleotide open reading frame (ORF) that was 83% identical to the nucleotide sequence of the human liver NNMT cDNA ORF. The mouse liver cDNA encoded a 264 amino acid protein with a calculated M_r value of 29.6 kDa. NNMT cDNA ORF sequences were then determined in five inbred strains of mice with very different levels of hepatic NNMT enzymatic activity. Although multiple differences among strains in nucleotide sequence were observed, none altered encoded amino acids. cDNA sequences for C57BL/6J and C3H/HeJ mice, prototypic strains with "high" and "low" levels of hepatic NNMT activity, respectively, were then expressed in COS-1 cells. Both expression constructs yielded comparable levels of enzyme activity, and biochemical properties of the expressed enzyme, including apparent K_m values for substrates and IC_{50} values for inhibition by N^1-methylnicotinamide, were very similar to those of mouse liver NNMT. Growth and development experiments were then conducted, which demonstrated that, although at 8 weeks of age average hepatic NNMT activity in C57BL/6J mice was 5-fold higher than that in C3H/HeJ mice, activities in the two strains were comparable by 30 weeks of age—indicating strain-dependent variation in the developmental expression of NNMT in mouse liver. These observations will serve to focus future studies of strain-dependent differences in murine hepatic NNMT on the regulation of the enzyme activity during growth and development. BIOCHEM PHARMACOL **54**;10:1139–1149, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. nicotinamide *N*-methyltransferase; methyltransferase; nicotinamide; N-methylation; mouse cDNA

NNMT† (EC 2.1.1.1) is an Ado-Met-dependent cytosolic enzyme that catalyzes the N-methylation of nicotinamide and structurally related compounds [1, 2]. Although other methyltransferase enzymes are capable of catalyzing the N-methylation of heterocyclic compounds, only NNMT has been shown to utilize nicotinamide as a methyl acceptor substrate [2–4]. Human liver NNMT activity shows a 5-fold individual variation and has a bimodal frequency distribution, with approximately 25% of liver biopsy samples included in a subgroup with high levels of enzyme activity [5]. These observations raised the possibility that human liver NNMT activity, like those of several other methyltransferase enzymes in humans [6–9], might be regulated by a genetic polymorphism that could contribute to individual differences in drug and xenobiotic metabolism. Genetic polymorphisms for at least three human methyltransferase enzymes result from inherited variation in amino acid sequence [10–12].

It would be useful if there were an experimental animal model in which to study molecular genetic mechanisms involved in the regulation of NNMT, as well as possible pharmacological or toxicological consequences of variation in the enzyme activity. The inbred mouse is an ideal laboratory animal in which to perform genetic studies [13, 14], and in preliminary experiments we demonstrated large (over 10-fold) strain-dependent differences in hepatic NNMT activity among 7- to 9-week-old inbred mice [15]. In the present experiments, we set out to clone mouse liver NNMT cDNA as one step in the study of possible molecular mechanisms responsible for strain-related differences in mouse liver NNMT activity. The hypothesis tested was that variation among strains in hepatic NNMT activity, like inherited differences in levels of several human methyltransferase enzymes [10–12], may result from variation in amino acid sequences encoded by different alleles for the enzyme. Therefore, after we cloned mouse liver NNMT cDNA, ORF sequences were determined in five inbred

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† *Abbreviations:* NNMT, nicotinamide *N*-methyltransferase; Ado-Met, *S*-adenosyl-L-methionine; PCR, polymerase chain reaction; ORF, open reading frame; UTR, untranslated region; and HSS, high-speed supernatant.

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TABLE 1. Primers used to clone and amplify mouse liver NNMT cDNA

Primer designation	Primer sequence
Human liver cDNA	
hF181	5′-GACATCGGCTCTGCCCCCACTATC-3′
hR491	5′-AGTGTGCTGAGCACGCAGTCAGC-3′
Mouse liver cDNA	
mF296	5′-GAAGGAACCAGGAGCCTTTGACTG-3′
mR398	5′-ACGCCTCAACTTCTCCTCCTTCTC-3′
mF(−124)	5′-ATGGGCCGAGCTGGAGGAAGATTG-3′
mR827	5′-ACCAGCAGGCCTTTAATAGGGATC-3′
Vector	
T3	5′-AATTAACCCTCACTAAAGGG-3′
T7	5′-GTAATACGACTCACTATAGGGC-3′
p91023(B)	5′-CTTGAGATCTGGCCATAC-3′

Abbreviations used include: h, human; m, mouse; F, forward; and R, reverse. Numbers indicate nucleotide positions, with the "A" of the ATG translation initiation codon for either the human or mouse cDNA designated number 1. Positive numbers are located 3′, and negative numbers are located 5′, to the position of the A in the initial cDNA codon.

strains of mice with very different levels of hepatic NNMT activity. Although multiple strain-dependent differences in nucleotide sequence were observed, none altered encoded amino acids. However, an unexpected observation was the expression of two NNMT mRNA species in some strains, possibly as a result of alternative RNA splicing. Since no differences among strains in NNMT amino acid sequence had been observed, we next turned to studies of the effects of growth and development on hepatic NNMT activity in the mouse. The enzyme activity increased with increasing age, and there was a striking strain-dependent difference in the rate of this increase—variation that accounted for the large differences in enzyme activity among strains at 7–9 weeks of age. These observations will serve to focus future studies on mechanisms responsible for strain-dependent differences in the developmental expression of NNMT in the mouse liver.

MATERIALS AND METHODS

Materials

[^{14}C-*methyl*]Ado-Met (58 μCi/μmol) was purchased from DuPont NEN (Boston, MA). [α-^{32}P]dCTP (>3000 Ci/mmol) was obtained from ICN, Amersham Corp. (Arlington Heights, IL). Bio-safe II liquid scintillation counting fluid was purchased from the Research Products International Corp. (Mount Prospect, IL). Low molecular weight protein markers, SDS–PAGE reagents, and Bio-Rad protein assay dye reagent were obtained from Bio-Rad Laboratories (Richmond, CA). Nicotinamide was purchased from the Aldrich Chemical Co., Inc. (Milwaukee, WI). 1-Heptane sulfonic acid was obtained from the Fisher Scientific Co. (Fair Lawn, NJ). Toluene and isoamyl alcohol were purchased from the J. T. Baker Chemical Co. (Phillipsburg, NJ). N^1-Methylnicotinamide chloride was obtained from the Sigma Chemical Co. (St. Louis, MO). *Eco*RI was purchased from Gibco BRL (Gaithersburg, MD).

Cloning of Mouse Liver NNMT cDNAs

Mouse liver NNMT cDNA was cloned by use of the PCR with a strategy based on the assumption that there might be sequence homology between the known human cDNA sequence and that of the mouse [16]. In an initial experiment, a 311 bp NNMT cDNA nucleotide sequence was amplified with template DNA from a C57BL/6J × CBA crossbred mouse liver cDNA library in the vector λ ZAP (Stratagene, La Jolla, CA) using primers hF181 and hR491. These primers had been designed on the basis of the human liver NNMT cDNA sequence [16]. The sequences of hF181 and hR491, as well as those of all other primers described subsequently, are listed in Table 1. This PCR amplification was performed in a model 2400 Perkin–Elmer Cetus DNA thermal cycler (Emeryville, CA) in a 50-μL reaction volume that contained 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 deoxyribonucleoside triphosphates; and 1 unit of *Thermus aquaticus* (*Taq*) DNA polymerase. Reaction conditions included 2 min at 94°, followed by 35 cycles of 30 sec at 94°, 1 min at 57°, and 1 min at 72°, with a final 7-min extension at 72°. Unless otherwise stated, these same conditions were used to perform all PCR amplifications described subsequently. Portions of the mouse liver NNMT cDNA that were not amplified with primers hF181 and hR491 were amplified by performing the PCR with T3 or T7 vector primers paired with mouse liver NNMT-specific primers mF296 or mR398 with the same cDNA library as template (Table 1). The entire ORF of the mouse NNMT cDNA was then amplified with primers mF(−124) and mR827, and was subcloned into the vector pCR2.1 (Invitrogen, San Diego, CA) prior to DNA sequencing.

NNMT cDNA ORF sequences were then determined for five inbred mouse strains with very different levels of hepatic NNMT activity [15]. "Low" NNMT activity strains included C3H/HeJ (C3H) and AKR/J (AKR); DBA/2J (D2) was an "intermediate" activity strain; and "high" activity strains included C57BL/6J (B6) and C57BR/cdJ

(C57BR). All mice were obtained from the Jackson Laboratory, Bar Harbor, ME. These experiments were reviewed and approved by the Mayo Clinic Animal Care and Use Committee. Tissue from male animals 7–8 weeks of age was used for the cDNA ORF amplification studies. Growth and development experiments utilized 3-, 8-, 14-, 20-, and 30-week-old male C3H and B6 mice. The mice were killed by cervical dislocation, followed by decapitation. Hepatic tissue was removed, placed in ice-cold 0.9% saline, weighed, and homogenized in 5 mM potassium phosphate buffer, pH 7.5, with a Polytron homogenizer (Brinkmann Instruments, Inc., Westbury, NY), and a 100,000 *g* HSS was prepared as described previously [15]. The hepatic HSS preparations were used immediately to perform enzyme assays. Total RNA was extracted from liver samples by use of the RNeasy™ total RNA isolation kit (QIAgen, Chatsworth, CA), followed by first-strand cDNA synthesis performed with a first-strand cDNA synthesis kit (P-L Biochemicals, Pharmacia Biotech, Inc., Piscataway, NJ). These cDNA samples were then used as template for the PCR amplification of ORF sequences with primers mF(−124) and mR827, primers that annealed to cDNA 5′- and 3′-UTR sequences (Table 1). To maximize amplification fidelity, these reactions were performed with *Pyrococcus furiosus* (*Pfu*) DNA polymerase [17] (Stratagene). These amplification products were also subcloned into pCR2.1, and at least five separate, independent clones were sequenced for each strain studied.

COS-1 *Cell Expression*

C3H and B6 NNMT cDNA ORF sequences were used to transfect COS-1 cells to perform transient expression experiments. Specifically, first-strand cDNA generated from RNA isolated from C3H and B6 liver samples was used as template for the PCR with primers mF(−124) and mR827 and with *Pfu* as the DNA polymerase. Amplification products from these reactions were subcloned into pCR2.1. Inserts were removed from these clones by *Eco*RI restriction digestion and were subcloned into the eukaryotic expression vector p91023(B) [18, 19]. Nucleotide sequences of the inserts were confirmed by automated DNA sequencing, and their orientations within the expression vector were determined by performing PCR amplifications with primers p91023(B) and mR398 (Table 1). These expression constructs were used to transfect COS-1 cells with the DEAE-dextran method [20, 21] as described by Honchel *et al.* [22]. All cells were also cotransfected with the β-galactosidase construct pSV-β-galactosidase (Promega, Madison, WI) to make it possible to correct for transfection efficiency.

NNMT Enzymatic Assay

The radiochemical enzymatic assay for NNMT was based on the conversion of nicotinamide to radioactively labeled N^1-methylnicotinamide with [^{14}C-methyl]Ado-Met as the methyl donor, followed by organic solvent extraction of the reaction product in the presence of the ion-pairing reagent 1-heptane sulfonic acid [5]. Blank samples did not contain nicotinamide. All results were corrected for quenching, counting efficiency, and partitioning of the reaction product into the organic phase. One unit of activity represented the formation of 1 nmol of N^1-methylnicotinamide per hour of incubation at 37°. As a control for studies of the mouse enzyme, NNMT activity was also measured in a pooled sample of human liver HSS with each assay. A human liver preparation was used for this purpose because, unlike the murine enzyme, human liver NNMT is stable when frozen [5, 15].

Protein Assay

Protein concentrations were measured by the method of Bradford [23] with bovine serum albumin as a standard.

Western Blot Analysis

Western blot analysis was performed with proteins that had been separated by SDS–PAGE. The proteins were transferred to nitrocellulose membranes after electrophoresis, and the membranes were probed with 1000-fold diluted rabbit polyclonal antiserum against a 12 amino acid peptide located at positions 3–14, near the amino terminus of both mouse and human liver NNMT. The amino acid sequence selected (SGFTSKBTYLSH) was not homologous to known sequences present in other methyltransferase enzymes. This 12 amino acid peptide, with cysteine added to the C-terminus so that it could be conjugated to keyhole limpet hemocyanin, was synthesized by the Mayo Research Resource Protein Core Laboratory. The synthetic NNMT peptide, conjugated to keyhole limpet hemocyanin, was then used by Cocalico Biologicals Inc. (Reamstown, PA) to generate polyclonal antibodies by the immunization of a New Zealand white rabbit. Serum from the second test bleed, obtained 8 weeks after the initial injection, was used to perform Western blots with the ECL Western Blotting Analysis System (Amersham Life Sciences, Arlington Heights, IL) as described elsewhere [24]. Recombinant human NNMT [16] was used as a positive control for these experiments.

Northern Blot Analysis

A commercial BALB/c mouse Multiple Tissue Northern Blot (MTN) membrane (Clontech, Palo Alto, CA) was probed with [α-^{32}P]dCTP radioactively labeled random primed full-length B6 NNMT cDNA. Random priming was performed with the Oligolabelling Kit (Pharmacia Biotech, Inc.).

Data Analysis

The University of Wisconsin Genetics Computer Group (GCG) software package, (Version 8.0) [25], was used to

analyze nucleotide and protein sequence information. The IC_{50} values were calculated with the GraphPAD Inplot curve-fitting program (GraphPAD Inplot Software, San Diego, CA). Apparent K_m values were calculated by the method of Wilkinson [26] with a computer program written by Cleland [27]. Statistical comparisons of mean values for the growth and development studies were performed by the use of Student's *t*-test and ANOVA. These calculations were performed with SAS software (SAS Institute Inc., Cary, NC).

RESULTS

Mouse Liver NNMT cDNA Cloning

Mouse liver NNMT cDNA was cloned by the use of a PCR-based strategy (see Materials and Methods for details) with a B6 × CBA λ ZAP mouse liver cDNA library as template. The full-length cDNA isolated by this approach was identified subsequently as the larger of two cDNA species present in B6 liver preparations. It consisted of 1015 nucleotides with a 792 nucleotide ORF that was 83% identical to the nucleotide sequence of the human liver NNMT cDNA ORF (Fig. 1). The mouse liver cDNA encoded a 264 amino acid protein with a calculated M_r value of 29.6 kDa. The sequence environment surrounding the ATG translation initiation codon closely approximated the optimal sequence for translation initiation in higher eukaryotes described by Kozak [28]. The 3′-UTR of the cDNA ended with a poly(A) tract, and an ATTAAA polyadenylation signal [29] was located 34 nucleotides 5′-upstream from the poly(A) tract (Fig. 1). The polyadenylation signal for human liver NNMT cDNA is also ATTAAA [16].

NNMT cDNA ORF sequence was then determined for five different inbred strains of mice shown previously to have over 10-fold differences in levels of hepatic NNMT activity at 7–9 weeks of age. Specifically, two C57 strains, B6 and C57BR, had high activity; D2 had intermediate activity; and C3H and AKR mice had low NNMT enzymatic activity [15]. Although there were multiple differences in NNMT cDNA ORF nucleotide sequences (Table 2), none altered encoded amino acids. At least five independent clones were sequenced for each strain to assure that the sequences listed in the table were correct. cDNA ORF sequences for the two C57 strains were identical, while D2 and C3H mice had five shared differences in nucleotide sequence when compared with that of the B6 cDNA ORF (Table 2). The AKR strain ORF had four nucleotide differences when compared with those of the other strains, although differences with those strains varied (Table 2). GenBank accession numbers for these mouse liver NNMT cDNA sequences are: U86105 (C57BL/6J and C57BR/cdJ), U86106 (AKR/J), U86107 (C3H/HeJ), and U86108 (DBA/2J). An unanticipated observation was that both of the C57 strains studied, B6 and C57BR, expressed two separate NNMT cDNA ORF sequences (Fig. 2), one of which contained a 131 bp deletion (underlined sequence in Fig. 1), a deletion that would result in a frameshift if the sequence were translated. The GenBank accession number for this truncated cDNA is U89377. The 131 bp deletion began with "GT" and ended with "AG" (Fig. 1)—signal sequences for intron splicing [30]. Three of the nucleotide differences between C57 and the other strains studied were located within the 131 bp deletion (Table 2). If the cDNA with the 131 bp deletion were translated, it would encode a 157 amino acid protein with a calculated M_r value of 18 kDa, and the 21 C-terminal amino acids in that protein would differ from those encoded by the full-length NNMT cDNA. It should be emphasized that the cDNA with the 131 bp deletion was amplified during five separate independent PCR amplifications in which B6 or C57BR hepatic cDNA was used as template. However, since these amplifications were not performed under conditions that allowed quantification, the relative proportions of the two species amplified—that with and that without the 131 bp deletion—are not known. Phylogenetic analyses of the nucleotide sequences of the full-length NNMT cDNAs in these five strains were performed using both distance matrix and maximum parsimony techniques [31, 32]. Both "trees" fit the known genealogic relationships among these inbred mice (data not shown) [13, 14].

COS-1 Cell Expression

Even though the strain-dependent differences in NNMT cDNA nucleotide sequence listed in Table 2 did not change encoded amino acids, they could potentially alter translation efficiency or mRNA stability. Therefore, cDNA sequences for a prototypic high activity strain, B6 (both the full-length cDNA and the sequence with the 131 bp deletion), as well as that of a prototypic low activity strain, the C3H, were transiently expressed in COS-1 cells. As a control, transfection was also performed with vector that lacked insert. NNMT activity was then measured in preparations from transfected COS-1 cells under optimal conditions for the assay of mouse liver NNMT activity [15]. After transfection with C3H and the full-length B6 cDNAs, the COS-1 cell preparations showed greatly enhanced ability to catalyze the methylation of nicotinamide (Table 3). Furthermore, average activities in preparations from cells transfected with full-length cDNAs from the two strains did not differ significantly (15.4 ± 3.5 and 17.9 ± 2.2 units/β-galactosidase units, mean ± SD for three independent transfections with B6 and C3H cDNAs, respectively, $P = 0.38$). There was very little detectable NNMT activity in COS-1 cell preparations transfected with vector alone or in preparations from cells transfected with B6 cDNA that contained the 131 bp deletion (Table 3).

Biochemical Properties of Recombinant NNMT

The biochemical properties of recombinant mouse liver NNMT were determined in preparations obtained from COS-1 cells transfected with the B6 NNMT cDNA expres-

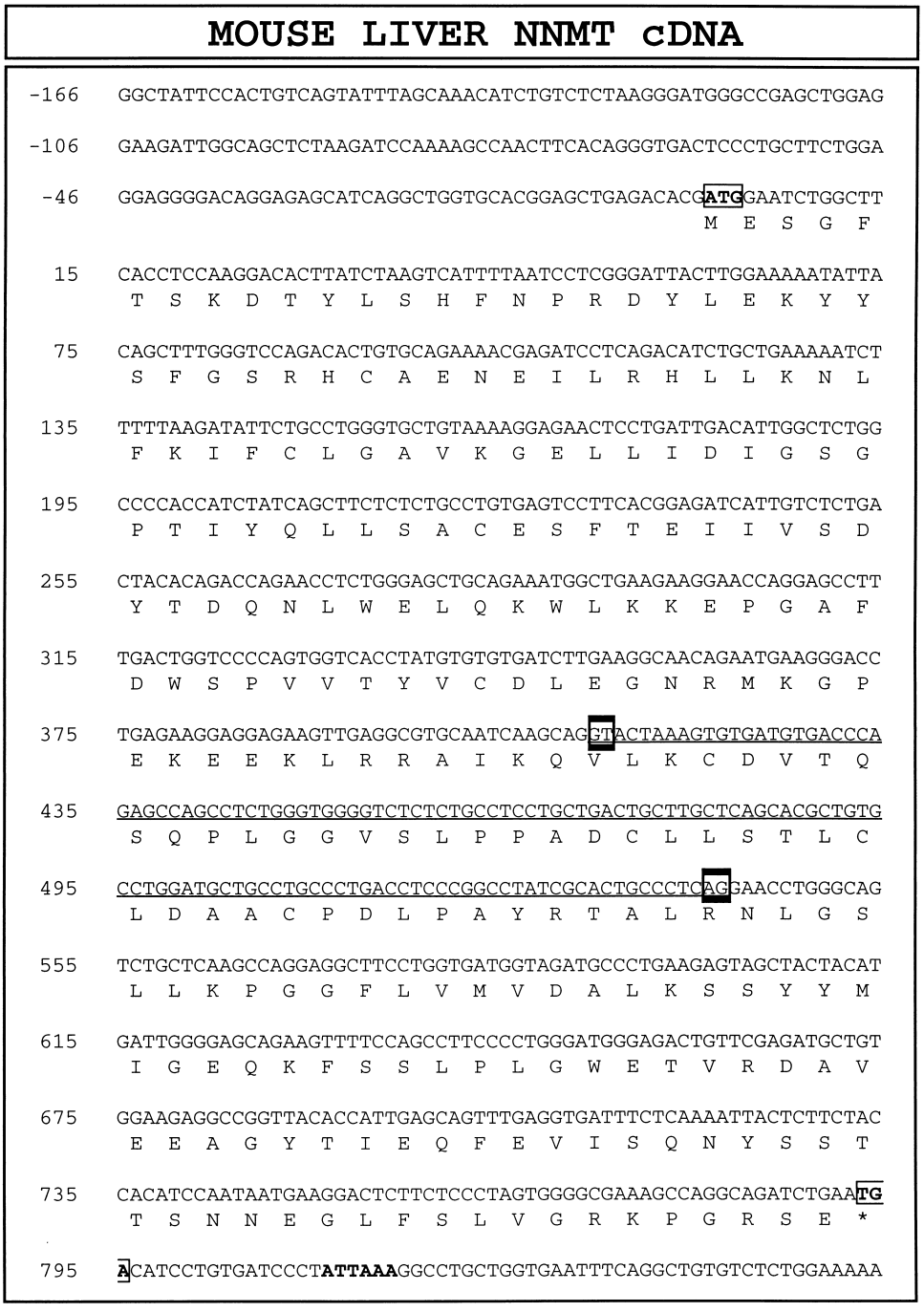

FIG. 1. Mouse liver NNMT cDNA nucleotide and deduced amino acid sequences. Nucleotides are numbered in a 5′- to 3′-direction, with the "A" in the translation initiation codon designated as +1. The amino acid sequence of the protein encoded by the full-length cDNA is listed in single-letter code beneath the nucleotide sequence. The underlined sequence is the 131 bp deletion present in one of the two cDNAs cloned from the C57 strains studied. Translation initiation and termination codons for the full-length cDNA are boxed. The "bold boxes" indicate GT and AG sequences found at potential splice junctions for the underlined 131 bp deletion. A polyadenylation signal sequence, ATTAAA, is shown in bold type.

sion construct, since the B6 and C3H full-length expression constructs encoded identical amino acid sequences. Mouse liver cytosol preparations from B6 mice were used as controls for these experiments. Previous studies had demonstrated that the biochemical properties of NNMT that were studied did not differ in hepatic preparations obtained from B6 and C3H mice [15]. Initial experiments involved determination of apparent K_m values for the two cosubstrates for the reaction, nicotinamide and Ado-Met. When six different concentrations of nicotinamide that ranged from 0.16 to 5 mM were tested, apparent K_m values of mouse liver and recombinant mouse NNMT for nicotinamide were 0.36 and 0.43 mM, respectively (Fig. 3A). When four different Ado-Met concentrations that ranged

TABLE 2. NNMT cDNA ORF variant nucleotides among inbred mouse strains

Hepatic NNMT activity	Strain	Mouse liver NNMT cDNA sequences: ORF nucleotide position 105	165	478*	489*	522*	684	708
High	C57BL/6J	C	A	T	G	G	C	G
	C57BR/cdJ	C	A	T	G	G	C	G
Intermediate	DBA/2J	T	G	T	A	A	T	A
Low	C3H/HeJ	C	G	C	A	A	T	A
	AKR/J	T	A	C	A	A	C	G

The levels of hepatic NNMT activity (high, intermediate, or low) were determined in the course of a previous study performed with 7- to 9-week-old male mice [15]. None of these differences in nucleotide sequence resulted in alterations in encoded amino acids. An asterisk indicates a nucleotide located within the 131 bp deletion present in the smaller of the two cDNAs expressed in the two C57 strains studied.

from 1.56 to 12.5 μM were studied, apparent K_m values of mouse liver and recombinant mouse NNMT for Ado-Met were 4.8 and 6.8 μM, respectively (Fig. 3B). All of these values were very similar to those reported previously for mouse liver NNMT [15]. Mouse liver and COS-1 cell recombinant mouse liver NNMT were also used to estimate IC_{50} values for inhibition by N^1-methylnicotinamide, a potent inhibitor of NNMT [5, 15]. The IC_{50} values for inhibition were calculated on the basis of data obtained with ten different concentrations of N^1-methylnicotinamide that ranged from 0.008 to 2 mM. The IC_{50} values for the inhibition of mouse liver and recombinant mouse NNMT were 41 and 71 μM, respectively (Fig. 3C), values that were once again quite similar to those reported previously for mouse liver NNMT [15].

Western Blot Analysis of Recombinant NNMT

Recombinant mouse NNMT was also studied by Western blot analysis. Human recombinant NNMT was used as a control for these studies. These experiments used a rabbit polyclonal antibody generated against a 12 amino acid sequence located near the amino terminus of both mouse and human NNMT (see Materials and Methods for details). This antibody reacted with a protein in the recombinant human NNMT preparation that had an apparent M_r value of 29.6 kDa (Fig. 4). The mouse COS-1 cell expressed NNMT appeared to migrate with a slightly lower apparent M_r of 29.4 kDa (Fig. 4). Western blot analysis was also performed with preparations from COS-1 cells transfected with the B6 cDNA that had the 131 bp ORF deletion. Those experiments did not show a band at the 18 kDa M_r predicted for a protein encoded by the cDNA with the deletion (data not shown).

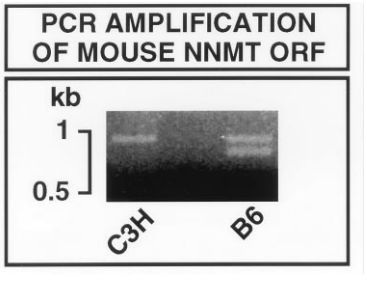

FIG. 2. PCR amplification of mouse liver NNMT cDNA ORF sequences. PCR amplification was performed with hepatic cDNA from B6 and C3H mice with primers mF(−124) and mR827 (see Table 1). B6 cDNA yielded two separate amplification products.

Northern Blot Analysis

Northern blot analysis was performed with a commercially available multiple tissue blot prepared with poly(A)$^+$ RNA from eight different BALB/c mouse tissues. A single mRNA species approximately 1 kb in length, very similar to the length of the mouse liver NNMT cDNA that we had cloned from the cDNA library (Fig. 1), was detected in lung, liver, kidney, and skeletal muscle (Fig. 5). The mRNA species in the skeletal muscle appeared to be slightly shorter than those in other tissues, and there was also a very faint transcript, approximately 4.0 kb in length, in the lung preparation (Fig. 5). No detectable NNMT mRNA was present in heart, brain, spleen, and testis preparations (Fig. 5).

Amino Acid Sequence Homology

The deduced amino acid sequence encoded by the mouse liver NNMT cDNA was compared with those of other

TABLE 3. NNMT enzymatic activity after transfection of COS-1 cells with mouse liver NNMT cDNA

	NNMT activity (units/β-galactosidase units) Experiment number 1	2	3
Control, p91023(B)	0.1	0.1	0.0
B6, deletion	0.1	0.0	0.0
B6, full-length	18.8	11.8	15.5
C3H, full-length	15.7	17.0	21.0

"Control" samples were transfected with only the vector, p91023(B). "Deletion" refers to the B6 cDNA with a 131 bp deletion, while "full-length" refers to full-length mouse NNMT cDNA sequences. Activity is expressed as units/β-galactosidase units to correct for transfection efficiency.

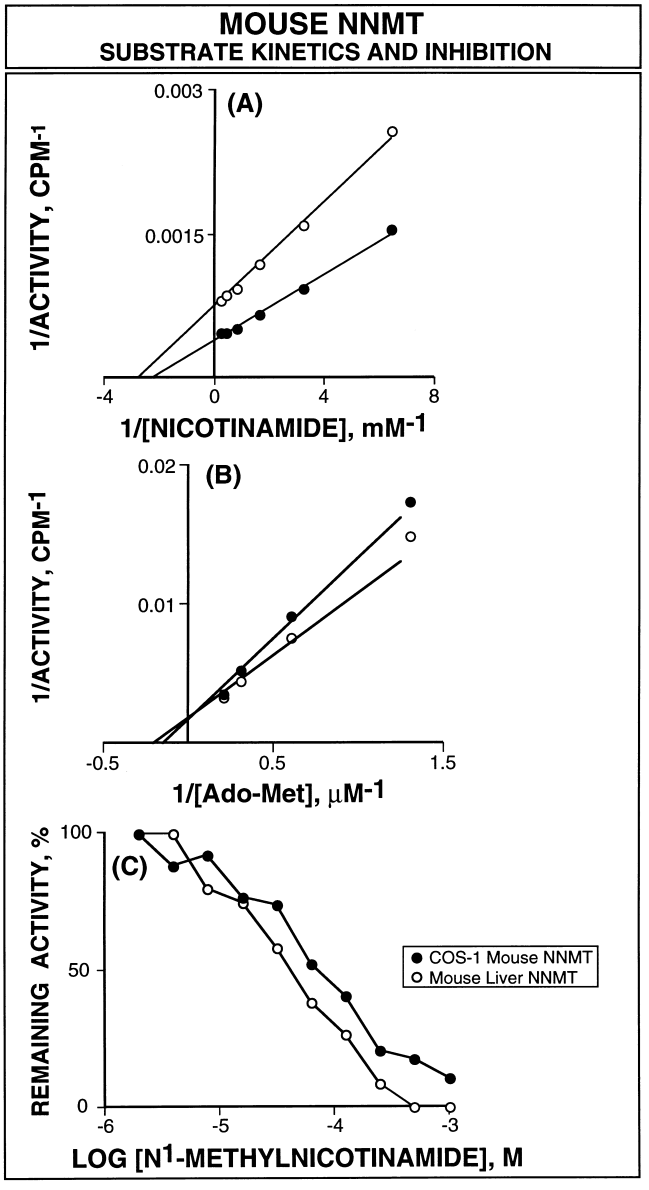

FIG. 3. Mouse liver NNMT cDNA expression in COS-1 cells. Biochemical properties of recombinant mouse liver NNMT are compared with those of NNMT in B6 mouse liver preparations. Double-inverse plots of the effect of (A) nicotinamide and (B) Ado-Met concentrations on NNMT activity in COS-1 cell and B6 hepatic preparations are shown. Panel C shows inhibition of NNMT activity in the two preparations by N^1-methylnicotinamide.

methyltransferases in the GenBank. Other than human NNMT, the mouse cDNA showed significant homology with only five other sequences. The mouse liver NNMT amino acid sequence was 85, 51, 38, 39, 38, and 38% identical to those of human liver NNMT, mouse thioether *S*-methyltransferase, and rat, mouse, human, and bovine phenylethanolamine *N*-methyltransferase, respectively [33–37] (Fig. 6B). The dendrogram shown in Fig. 6A depicts relationships among the amino acid sequences of this group of methyltransferase enzymes.

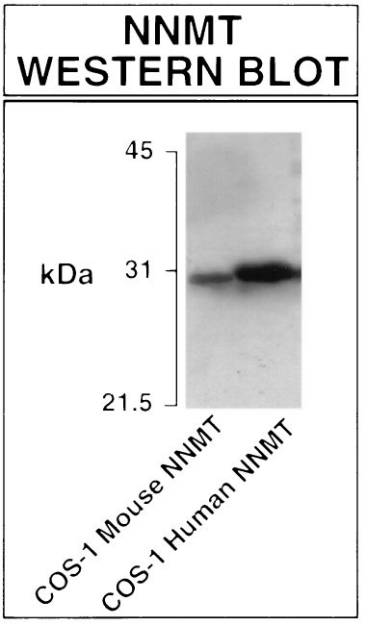

FIG. 4. Western blot analysis of recombinant NNMT. Recombinant mouse liver and recombinant human liver NNMT were probed with a rabbit polyclonal antibody to a 12 amino acid polypeptide located near the N-terminus of both proteins. See text for details.

Effect of Growth and Development

No differences in NNMT amino acid sequence that could explain strain-dependent differences in levels of this enzyme activity had been found. Therefore, we performed one additional experiment in an attempt to establish a direction for future studies of NNMT regulation in the mouse liver. Previous experiments that had demonstrated strain-dependent differences in mouse liver NNMT activity had been performed with preparations from male animals 7–9 weeks of age [15]. To determine whether the large strain-dependent differences observed in those experiments might have been influenced by the age of the animals studied, NNMT activity was measured in tissue from male B6 and C3H mice of various ages. Hepatic NNMT enzymatic activity in B6 and C3H mice did not differ significantly at 3 weeks of age, but enzyme activities in both strains increased with increasing age (Fig. 7). However, the activity increased much more rapidly in the livers of B6 mice than it did in C3H animals. Specifically, values for B6 were elevated at 8 weeks over those present at 3 weeks of age ($P < 0.001$), whereas values for C3H mice were elevated significantly only when the animals reached 20 weeks of age ($P < 0.001$). As a result, there were significant differences between hepatic NNMT activities in the two strains at 8, 14, and 20 weeks of age ($P < 0.01$), but those differences narrowed progressively, from 5-fold higher in B6 at 8 weeks to 2-fold higher at 20 weeks (Fig. 7). By 30 weeks of age, both strains had attained high levels of hepatic NNMT activity that did not differ significantly, and at that age the C3H animals had slightly higher average activity than did B6 mice (Fig. 7). Therefore, even though these two strains initially had

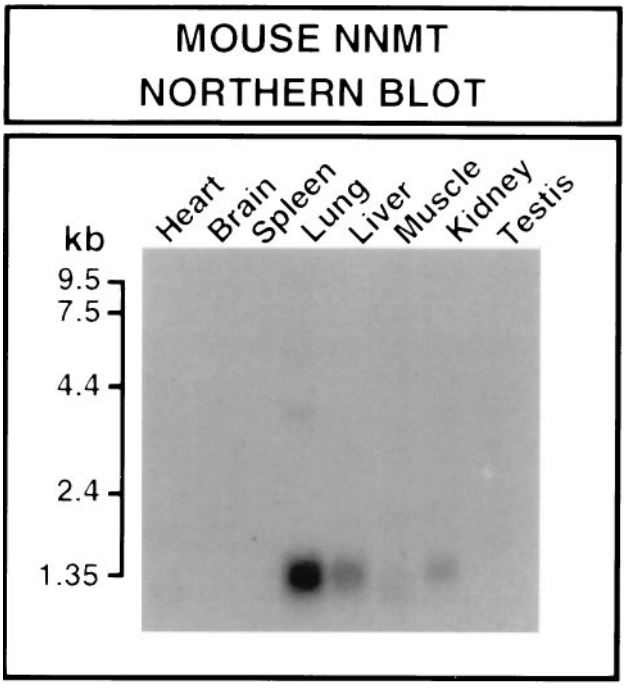

FIG. 5. Northern blot analysis of mouse NNMT. A Multiple Tissue Northern blot (2 μg of poly(A)$^+$ RNA/lane, Clontech) prepared from BALB/c mouse tissues was probed with the mouse liver NNMT cDNA ORF.

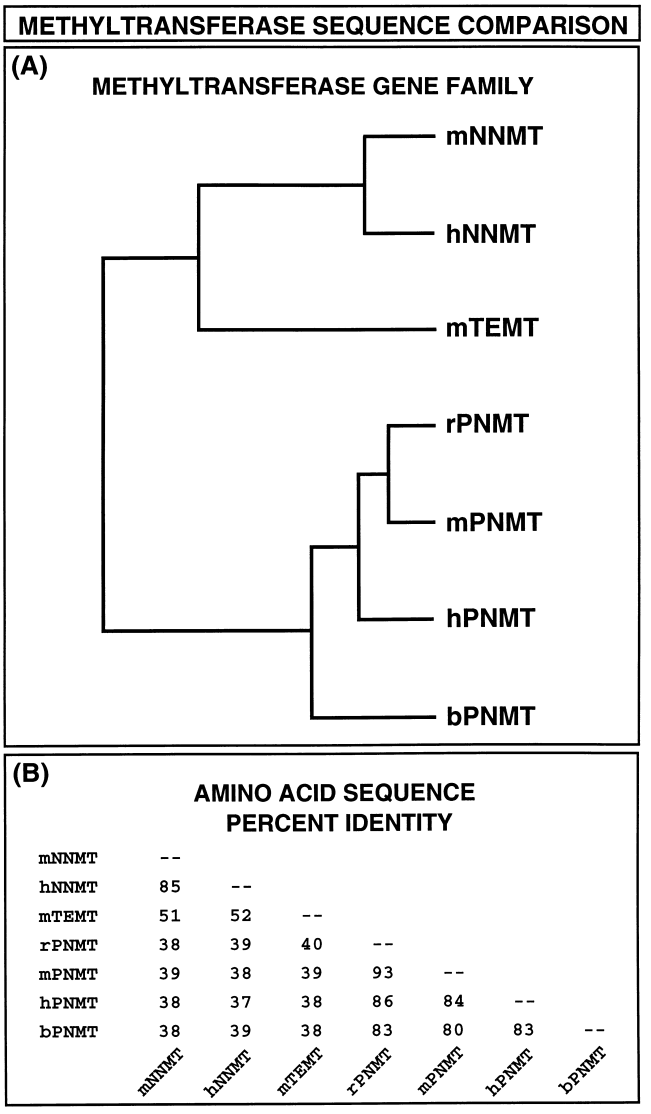

FIG. 6. Amino acid sequence comparison of selected methyltransferase enzymes. (A) The enzymes compared included NNMT, thioether methyltransferase (TEMT), and phenylethanolamine *N*-methyltransferase (PNMT). The dendrogram was generated by use of the PILEUP program [31]. Species abbreviations include "m" for mouse, "h" for human, "r" for rat, and "b" for bovine. (B) Percent amino acid sequence identities for the enzymes shown in (A) as determined by use of the GAP program [38].

similar levels of hepatic NNMT activity at 3 weeks of age, and even though both had significantly higher ($P < 0.001$), but once again similar levels at 30 weeks of age, B6 animals reached those high levels much more rapidly than did C3H mice (Fig. 7).

DISCUSSION

Methylation is an important pathway in the biotransformation of many drugs, xenobiotics, and endogenous compounds [39]. NNMT catalyzes the N-methylation of nicotinamide and structurally related compounds to form pyridinium ions [1]. Several *N*-methylpyridinium compounds are toxic. Included among toxic *N*-methylpyridinium compounds are the herbicide paraquat and the neurotoxin 1-methyl-4-phenylpyridinium [40–42]. Therefore, the possibility exists that selected pyridine substrates for NNMT might function as "protoxicants." Furthermore, the prototypic substrate for NNMT, nicotinamide, is a precursor of NAD. In addition to its important function as a coenzyme, NAD serves as a substrate for the formation of ADP-ribosylated proteins that participate in DNA repair [43]. Furthermore, NNMT activity has been reported to be increased in the mouse liver after the intraperitoneal implantation of Ehrlich ascites tumor, leading to the suggestion that hepatic NNMT activity might be used as a "tumor burden marker" [44]. Although the possible participation of NNMT in the regulation of DNA repair and tumor development remains hypothetical, the existence of a subgroup of human subjects with high levels of hepatic NNMT activity [5] has raised the possibility that a genetic polymorphism might contribute to the regulation of this activity in humans—just as genetic polymorphisms are involved in the regulation of other cytosolic methyltransferase enzymes in humans [6–9]. Functionally important genetic polymorphisms for several of those enzymes result from alterations in encoded amino acids [10–12].

It would be useful if there were an animal model in which to study mechanisms involved in the molecular genetic regulation of NNMT as well as possible pharmacological or toxicological consequences of that variation. The inbred mouse is an ideal experimental animal in which to perform such studies, and large variations in hepatic NNMT activity

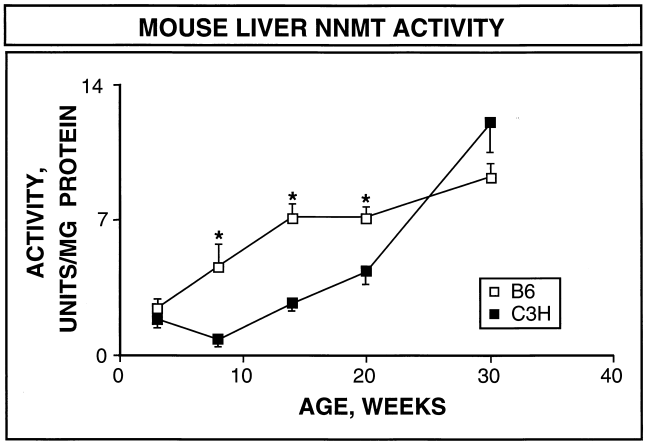

FIG. 7. Effect of growth and development on mouse liver NNMT activity. Hepatic NNMT activities in male B6 and C3H mice of various ages are shown. Values are means ± SEM for 4–8 animals. Key: (*) $P < 0.01$ when compared with animals of identical age of the other strain.

have been reported among inbred strains of mice at 7–9 weeks of age [15]. Therefore, we set out to clone NNMT cDNA from mouse liver as a first step in the study of possible molecular genetic mechanisms involved in the regulation of NNMT activity in this laboratory animal. We succeeded in cloning a mouse liver NNMT cDNA with an ORF nucleotide sequence that was 83% identical to that of human liver NNMT cDNA. Recombinant mouse NNMT had biochemical properties similar to, or identical with, those of native mouse liver NNMT. Northern blot analysis showed that NNMT was expressed primarily in mouse lung and liver, but mRNA was also detectable in kidney and skeletal muscle (Fig. 5). In adult humans, the enzyme is expressed predominantly in liver [16]. The amino acid sequence encoded by mouse liver NNMT cDNA displayed homology with those of mouse thioether *S*-methyltransferase and with phenylethanolamine *N*-methyltransferase in several species (Fig. 6). When NNMT cDNA ORF sequences were amplified from strains of mice with very different levels of hepatic NNMT activity at 7–9 weeks of age, multiple nucleotide sequence variants were found, but none altered encoded amino acids (Table 2). An unexpected observation was that both of the C57 strains studied expressed two NNMT cDNA ORF sequences, one of which contained a 131 bp deletion (Figs. 1 and 2), a deletion that could result from alternative splicing or variation in RNA processing. The underlying mechanism responsible for this observation can best be addressed after the structure of the mouse NNMT gene has been determined. However, no splice junctions are present in the human NNMT gene at locations comparable to the ends of the 131 bp deletion in the smaller mRNA cDNA expressed in C57 mice [45]. The possibility of variable RNA processing exists because the 131 bp deletion is bounded by "GT-AG" sequence motifs that could serve as signals for intron excision during RNA processing [30]. Transient expression studies conducted in COS-1 cells showed no detectable NNMT enzymatic activity after transfection with the cDNA that contained the deletion. However, Western blot analysis performed with an antibody that would have been expected to detect the approximately 18 kDa protein encoded by that cDNA failed to detect the presence of such a protein in preparations from transfected cells. Therefore, it remains unclear whether such a protein was translated, or, if translated, whether it was unstable.

Since we did not detect strain-dependent alterations in NNMT amino acid sequence that could explain differences in levels of enzyme activity, we performed an additional experiment in which we studied the effects of growth and development on mouse liver NNMT enzymatic activity in prototypic high and low activity strains, B6 and C3H, respectively. Although hepatic NNMT activity in B6 mice increased more rapidly during development than did that in the C3H strain, both strains reached approximately equal levels of activity during adulthood (Fig. 7). Those observations indicated that mechanisms responsible for strain-dependent variation in the developmental regulation of mouse liver NNMT should be addressed during future experiments. We have also shown previously the existence of gender-dependent variation in the expression of NNMT activity in the mouse liver [15]. Mechanisms ranging from variation in transcription regulation to variation in NNMT protein stability during development could explain strain-dependent variations in developmental regulation of hepatic NNMT activity. Included among future steps in this series of experiments will be cloning of the mouse NNMT gene—both to make it possible to study the regulation of its transcription and to investigate mechanisms involved in the expression of two different cDNA species in C57 mice. The results of the present experiments represent an additional step in an ongoing effort designed to expand our understanding of mechanisms involved in the regulation of the expression of cytosolic methyltransferase enzymes in mammals.

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