Rabbit Lung Indolethylamine N-Methyltransferase

cDNA AND GENE CLONING AND CHARACTERIZATION*

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Indolethylamine N-methyltransferase (INMT) catalyzes the N-methylation of tryptamine and structurally related compounds. This reaction has been studied because of its possible role in the in vivo synthesis of psychoactive compounds or neurotoxins and has been characterized biochemically in preparations of rabbit lung. Therefore, we set out to purify rabbit lung INMT, to clone and express its cDNA, and to clone and structurally characterize its gene as steps toward understanding the function and regulation of this enzyme. Rabbit lung INMT was purified and partial amino acid sequence was obtained. A polymerase chain reaction-based approach was then used to clone a rabbit lung INMT cDNA with a 792-base pair open reading frame that encoded a 263-amino acid protein with a predicted molecular mass of 29 kDa. When the cDNA was expressed in COS-1 cells, the encoded protein catalyzed the methylation of tryptamine and structurally related compounds, and was inhibited by two products of the reaction, S-adenosyl-L-homocysteine (AdoHcy) and N,N-dimethyltryptamine, as well as antimigraine drugs that are structurally related to N,N-dimethyltryptamine. Northern blot analysis demonstrated the presence of 2.0-kilobase mRNA species in rabbit lung, liver and, at lower levels, in brain. The cDNA was then used to clone the rabbit INMT gene. That gene had three exons and was structurally similar to the genes for nicotinamide N-methyltransferase and phenylethanolamine N-methyltransferase in several species. Cloning and expression of a rabbit lung INMT cDNA and cloning of the rabbit INMT gene represent important steps toward determination of the function and regulation of this mammalian methyltransferase enzyme.

Methylation is an important reaction in the metabolism of many drugs, other xenobiotics, and endogenous molecules (1). The methylation of tryptamine and structurally related compounds by a cytosolic S-adenosyl-L-methionine (AdoMet)-dependent methyltransferase (MT) was first described in the rabbit lung over 30 years ago (2). That reaction, with tryptamine as a substrate, is shown in Fig. 1. Interest in this reaction originally focused on the possible role of N-methylation in the metabolism of biogenic amines to form psychoactive agents such as N,N-dimethyltryptamine and N,N-dimethyl-5-hydroxytryptamine (2). Subsequently, there was also interest in the possibility that the N-methylation of compounds such as the β-carbolines and the isoquinolines might generate neurotoxins (3–7). As a result, a series of enzymes capable of methylyating tryptamine have been characterized biochemically (2, 8–12), and at least two tryptamine N-MT isoforms have been described in both rabbit lung (9) and rabbit liver (10, 12). Although these activities have been referred to by a variety of names including rabbit lung MT (2), nonspecific MT (2), aromatic alkylamine N-MT (13), indolamine N-MT (9, 14), arylamine N-MT (10), and amine N-MT (12), we will refer to the enzyme that we have studied as indolethylamine N-MT (INMT) (11). No cDNA for any of these activities or enzymes has been cloned and expressed to make it possible to associate the activity being studied with a single protein. Therefore, it has remained controversial whether tryptamine methylation is catalyzed by a single gene product with a broad substrate specificity or by a series of related enzymes.

We set out to purify rabbit lung INMT, clone its cDNA, and clone and structurally characterize its gene to make it possible to study the function of this enzyme and to search for orthologues in other species, including humans. Since tryptamine had been used as a “prototypic” methyl acceptor substrate for most of the original biochemical studies of this activity, tryptamine was used as a substrate in our experiments. We studied the rabbit lung because of its high INMT specific activity and because of the extensive previous biochemical characterization of INMT activity in that tissue. The experimental strategy involved the purification of rabbit lung INMT to obtain partial amino acid sequence, followed by the application of a PCR-based approach to clone the cDNA. Use of that strategy resulted in the cloning of a rabbit lung INMT cDNA, followed by expression and biochemical characterization of the protein encoded by the cDNA. The cDNA was then used to clone the rabbit INMT gene. These results represent important steps toward understanding the function and regulation of INMT and will make it possible to seek orthologues in other species as well as related enzymes in the rabbit.

EXPERIMENTAL PROCEDURES

Materials—[1-14C-CH₃]AdoMet (60 μCi/µmol) and [9H-CH₃]AdoMet (56.1 Ci/µmol) were obtained from NEN Life Science Products Inc. (Boston, MA). [α-32P]dCTP (3000 Ci/µmol) was purchased from Amer-
sham Life Science (Arlington Heights, IL). Ultrapure agarose, restriction enzymes, Superscript II reverse transcriptase, terminal transferase, Dulbecco’s modified Eagle’s medium, and fetal calf serum were obtained from Life Technologies, Inc. (Gaithersburg, MD). DEAE-Sepharose CL-6B, Sephacryl S-200, and Sephadex G-25 were purchased from Pharmacia LKB Biotechnology, Inc. (Piscataway, NJ). TA cloning kits (pCR2.1 and pCR3.1) were obtained from Invitrogen (San Diego, CA). A protein low molecular weight marker kit and Wizard Miniprep and Maxiprep DNA purification systems were purchased from Promega (Madison, WI). Bovine serum albumin, AdoHcy HCl, AdoMet HCl, amitriptyline HCl, chloroquine diphosphate, desipramine HCl, dimethyl sulfoxide, N,N-dimethyltryptamine, N-methyltryptamine, tryptamine HCl, (−)-epinephrine (−)-bitartrate salt, epinine HCl, ethanolamine HCl, harmaline HCl, harmalol HCl, harmane-1,2,3,4-tetrahydrocarboxylic acid, histamine 2HCl, imipramine HCl, melatonin, nortriptyline HCl, β-phenylethylamine HCl, quinacrine 2HCl, (6)-salsolinol HCl, and tryptamine HCl were obtained from Sigma. 2,3-Dichloro-a-methylbenzylamine was purchased from Research Biochemicals International (Natick, MA). 3,4-Dimethoxy-5-hydroxybenzoic acid, serotonin creatine sulfate, and tropolone were obtained from Regis Chemical Co. (Morton Grove, IL). Ethylamine HCl, harmol HCl, methyamine, α-methyltryptamine, N′-methyl Nicotinamide, nicotinamide, (±)-norepinephrine HCl, (±)-octopamine, and 1,2,3,4-tetrahydrosoquinoline HCl were purchased from Aldrich Chemical Co. Dopamine HCl and tyramine HCl were obtained from Calbiochem (San Diego, CA). SKF 525A was a gift from Dr. Russell Van Dyke (Mayo Clinic). Naratriptan, sumatriptan, and zolmitriptan were gifts from Glaxo Wellcome (Research Triangle Park, NC). The HCl salt of phenylethanolamine was prepared by refluxing with HCl, followed by recrystallization.

**Tissue Acquisition and Preparation**—Frozen rabbit lung tissue was purchased from Pel-Freez Biologicals (Rogers, AR) and was homogenized in 50 mM Tris-HCl, pH 7.3 (1:2, w:v), with an Osterizer blender, followed by homogenization with a Polytron homogenizer (Brinkman Instruments, Inc., Westbury, NY). A 100,000 g “high-speed supernatant” was then prepared as described previously (15).

**INMT Enzyme Assay**—The assay used to measure INMT activity during purification of the rabbit lung enzyme was a modification of procedures described by Lyon and Jakoby (10) and Ansher and Jakoby (12). This assay utilized [14C-CH3]AdoMet as a methyl donor and tryptamine as the methyl acceptor substrate. The formation of 14C-methylated tryptamine was determined after incubation for 30 min at 37 °C in a total volume of 200 μl that contained 25 mM Tris-HCl, pH 8.5, 34 μM [14C-CH3]AdoMet (24 μCi/μmol), and 1 mM tryptamine. The reaction was terminated by the addition of 0.5 ml of 0.5 M potassium borate, pH 10. The reaction mixture was then added to 5 ml of Econofluor-2 (Packard Instrument Co., Meriden, CT), vortexed, and radioactivity was measured in a Beckman LS6000 SC liquid scintillation counter (Beckman Instruments, Inc., Fullerton, CA). Blank samples contained no tryptamine. Activity increased in a linear fashion with both enzyme concentration and time of incubation under these reaction conditions. A modification of this assay was used to characterize substrates for INMT and to perform enzyme inhibition studies. For those experiments, the reaction mixture included 250 μg/ml bovine serum albumin and the incubation time was extended to 60 min, followed by extraction of the methylated product into 5 ml of 3% isoamyl alcohol in toluene by vortexing. After centrifugation, 3.5 ml of the organic phase was added to 5 ml of BioSafe II (Research Products International Corp., Mount Prospect, IL) prior to the determination of radioactivity. The intra-assay coefficient of variation for this modified assay was less than 2%. All results were corrected for quench and for partitioning of methylated tryptamine into the organic phase (68%).
Assays for all substrate and inhibition experiments were performed in triplicate, and values reported are averages of triplicate determinations. Protein concentrations were measured with the dye binding assay of Bradford (16) with bovine serum albumin as a standard.

INMT Purification—Rabbit lung high speed supernatant (284 ml) was subjected to DEAE-Sepharose CL-6B anion exchange chromatography in 50 mM Tris-HCl, pH 7.3, that contained 1 mM EDTA and 0.02% sodium azide. The column was eluted at a flow rate of 1 ml/min with a linear KCl gradient that varied from 0 to 0.4 M in the same buffer (Fig. 2A). The column was eluted at a flow rate of 1 ml/min with a linear KCl gradient that varied from 0 to 0.4 M in the same buffer (Fig. 2A). The column was eluted at a flow rate of 1 ml/min with a linear KCl gradient that varied from 0 to 0.4 M in the same buffer (Fig. 2A). The column was eluted at a flow rate of 1 ml/min with a linear KCl gradient that varied from 0 to 0.4 M in the same buffer (Fig. 2A).

INMT Photoaffinity Labeling and Two-dimensional Gel Electrophoresis—Partially purified rabbit lung INMT was photoaffinity labeled with 33 pmol of [3H-CH3]AdoMet (2.75 Ci) as described by Van Loon et al. (17). Specifically, the reaction mixture was placed in a 96-well microtiter plate 6 cm below a horizontal 254-nm UV light source and photolysis was performed for 30 min at 4 °C. The sample was then precipitated with −20 °C high performance liquid chromatography grade acetone, centrifuged at 5900 × g for 5 min at 4 °C, and the protein pellet was resuspended in 75 μl of urea sample buffer that contained 5 μl of creatine phosphokinase carbamylation standard (Pharmacia) prior to denaturation at 95 °C, and positive numbers 3′ to that position. IUPAC codes are used to refer to the bases in primer sequences.

### Table I

Sequences of primers used to clone and study the rabbit INMT cDNA and gene.

![Table I](http://www.jbc.org/)

| Primer designation      | Primer sequence                        |
|-------------------------|----------------------------------------|
| Degenerate primers      |                                        |
| DF1                     | 5′-CCNGARCGNARATGCTTAA-3′              |
| DR1                     | 5′-GCSACTCTCSCGRTTSCG-3′               |
| Forward primers F(−15)  | 5′-GTACCCAGGGACCC-3′                    |
| F1                      | 5′-ATGGAGGCGCGCTCAGC-3′                |
| F136                    | 5′-CACAAAGACCTTCGGCCCT-3′              |
| Reverse primers R162    |                                        |
| R176                    | 5′-GAGGCCGCCAGGGCC-3′                  |
| R225                    | 5′-CAGTGTGTCCCCCTTGGAG-3′              |
| R792                    | 5′-GACGACGCCGGAGGGACTTGGA-3′           |
| R1391                   | 5′-TCAGGACCCCGGCTTCTTG-3′              |
| Vector primers 5′-Ag11  | 5′-CCCTGCTCAAACGTAAATTTCAGACAAACAGC-3′|
| 3′-Ag11                 | 5′-CTGTTCATATGGGATGTTG-3′              |
| 5′-RACE anchor primer   | 5′-CCAGACAACTCGTATGTTG-3′              |
| (dC)15 Adaptor          | 5′-GAAAATGATCGACCCCCCCCCCCCCCCC-3′     |

Rabbit Lung INMT cDNA Cloning—The strategy used to clone a rabbit lung INMT cDNA is depicted schematically in Fig. 3. Rabbit lung INMT amino acid sequences were determined by the Mayo Research Resource Protein Core Laboratory.
Rabbit INMT cDNA and Gene

TABLE II
INMT substrate kinetics

| Compound                  | Rabbit lung cDNA | Partially purified INMT | Recombinant INMT |
|---------------------------|------------------|-------------------------|------------------|
| N-Methyltryptamine        | ND*              | ND                      | 0.06 ± 0.006     |
| Tryptamine                | 0.27 ± 0.02      | 0.50 ± 0.17             | 0.27 ± 0.05      |
| Norharmane                | ND               | ND                      | 0.27 ± 0.02      |
| Serotonin                 | ND               | 1.38 ± 0.20             | ND               |
| AdoMet                    | 0.011 ± 0.002    | 0.015 ± 0.002           | 0.022 ± 0.005    |

* ND, not determined.

The template for the initial amplification reaction was a rabbit lung 5'-STRETCH cDNA library (CLONTECH, Palo Alto, CA), and the reaction was performed with Thermus aquaticus (Taq) DNA polymerase in a Perkin-Elmer GeneAmp PCR System 2400 thermal cycler (Foster City, CA). A 191-bp amplification product was isolated by agarose gel electrophoresis, purified with the QiAquick Gel Extraction Kit (Qiagen, Inc., Chatsworth, CA), and sequenced after cloning into pCR2.1.

RESULTS

TABLE III
INMT substrate studies

| Compound                  | Relative activity % |
|---------------------------|---------------------|
| α-Methyltryptamine        | 131                 |
| Tryptamine                | 100                 |
| Norharmane                | 58                  |
| N'-Methyltryptamine       | 44                  |
| Serotonin                 | 11                  |
| β-Phenylethylamine        | 5                   |
| Desipramine               | 4                   |
| Nortriptyline             | 3                   |
| 1,2,3,4-Tetrahydroisoquinoline | 3               |
| Phenylethanolamine        | 3                   |
| Tyramine                  | 1                   |
| (+)-Octopamine            | 1                   |
| Ethylamine                | 0.5                 |
| Histamine                 | <0.5                |
| N,N-Dimethyltryptamine    | <0.5                |
| Dopamine                  | <0.5                |
| (+)-Epinephrine           | <0.5                |
| Epinine                   | <0.5                |
| Ethanolamine              | <0.5                |
| Harmine-1,2,3,4-tetrahydroxybenzoic acid | <0.5 |
| Harmol                    | <0.5                |
| Histamine                 | <0.5                |
| Melatonin                 | <0.5                |
| Methylamine               | <0.5                |
| Nicotinamide              | <0.5                |
| (+)-Norepinephrine        | <0.5                |
| (+)-Salisolin             | <0.5                |

TABLE IV
INMT inhibition studies

| Compound or ion            | Concentration | Relative activity % | IC50 value μM |
|---------------------------|---------------|---------------------|---------------|
| AdoHcy                     | 0.1           | 2                   | 2             |
| (+)-Salisolin              | 0.1           | 3                   | 3             |
| Chloroquine                | 1.0           | 2                   | 2             |
| N,N-Dimethyltryptamine     | 1.0           | 3                   | 67            |
| Harmalol                   | 1.0           | 11                  | 11            |
| Quinacrine                 | 1.0           | 16                  | 16            |
| Naratriptan                | 1.0           | 16                  | 167           |
| Imipramine                 | 1.0           | 21                  | 166           |
| Harmaline                  | 1.0           | 22                  | 22            |
| Sumatriptan                | 1.0           | 27                  | 270           |
| Zolmitriptan               | 1.0           | 32                  | 483           |
| Amitryptaline              | 1.0           | 46                  | 46            |
| 2,3-Dichloro-o-methylenzylamine | 1.0      | 77                  | 77            |
| 3,4-Dimethoxy-5-hydroxybenzoic acid | 1.0 | 77               |
| SKF 525A                   | 1.0           | 79                  | 79            |
| Ca2+                      | 1.0           | 86                  | 86            |
| Mg2+                      | 1.0           | 91                  | 91            |
| Tropolone                  | 5.0           | 94                  | 94            |
| N'-Methylnicotinamide      | 1.0           | 101                 | 101           |

A series of potential substrates for recombinant rabbit lung INMT was tested at 1 mM concentrations under optimal conditions for the assay of tryptamine. Activity is expressed relative to that obtained with 1 mM tryptamine.

Data Analysis—The University of Wisconsin Genetics Computer Group (GCG) software package Version 8.0 (25) was used to analyze DNA and protein sequences. The TFSITES database in GCG was used to search for sequence motifs that might be involved in transcription initiation or regulation. Apparent $K_m$ values were calculated by the method of Wilkinson (26) with a computer program written by Cleland (27). The GraphPAD InPlot Program (GraphPAD Software, San Diego, CA) was used to calculate IC50 values.

RESULTS

Rabbit Lung INMT cDNA Cloning—The rabbit lung INMT cDNA cloning strategy began with purification of the rabbit lung enzyme. The purified protein was then used to obtain partial amino acid sequence, which made it possible to utilize a PCR-based approach to clone the cDNA. Specifically, rabbit
lung INMT was partially purified by sequential DEAE anion exchange and gel filtration chromatography as described under “Experimental Procedures” (Fig. 2, A and B). The enzyme was purified approximately 159-fold after these two steps. The final step in the purification was two-dimensional gel electrophoresis (Fig. 2C). INMT was identified on the gel by photoaffinity labeling with \([3H-\text{CH}_3]\) AdoMet, the methyl donor for the reaction (Fig. 2D). INMT had an apparent molecular mass value of approximately 29 kDa as determined by two-dimensional gel electrophoresis. The gel also showed a less intense radioactive signal with an identical molecular mass, but a more basic isoelectric point (Fig. 2D). However, because of very low protein concentration in that region of the gel, no amino acid sequence could be obtained. When the major protein isolated by two-dimensional gel electrophoresis was sequenced, nine sequence fragments were obtained that varied from 3 to 32 amino acids in length. Each of those sequences was later found to be present within the amino acid sequence encoded by the rabbit lung INMT cDNA, and, taken together, the sequence encoded by these fragments extended from the N terminus of the protein to amino acid 129.

The next step in cDNA cloning involved the design of two degenerate PCR primers, DF1 and DR1 (Table I), on the basis of the partial amino acid sequence of the purified protein. When

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**TABLE V**

INMT indole (A) substrates and (B) inhibitors studied at 1 mM concentrations. Activity is compared to that of 1 mM tryptamine (100%).

| A Substrates                                      | R1       | R2                          | Relative Activity % |
|--------------------------------------------------|----------|-----------------------------|---------------------|
| α-Methyltryptamine                                | H-       | CH₃                         | 131                 |
| Tryptamine                                        | H-       | CH₂NH₂                      | 100                 |
| N-Methyltryptamine                                | H-       | CH₂NH(CH₃)₂                 | 44                  |
| Serotonin                                         | HO-      | CH₂NH₂                      | 11                  |
| Melatonin                                         | H⁺CO-    | CH₂NH₂COCH₃                 | 0                   |

| B Inhibitors                                      | R1       | R2                          | Inhibition % Activity Remaining | IC₅₀ Value, µM |
|--------------------------------------------------|----------|-----------------------------|-------------------------------|--------------|
| N,N-Dimethyltryptamine                            | H-       | CH₂CH₂N(CH₃)₂               | 3                             | 67           |
| Naratriptan                                       | CH₃NHSO₂⁻ | CH₃NH⁻                   | 16                            | 167          |
| Sumatriptan                                       | CH₂NHSO₂⁻ | CH₂CH₂N(CH₃)₂               | 27                            | 370          |
| Zolmitriptan                                      | O-CO-NH  | CH₂CH₂N(CH₃)₂               | 32                            | 483          |

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**RABBIT INMT NORTHERN BLOT ANALYSIS**

Fig. 4. Rabbit INMT Northern blot analysis. Northern blot analysis was performed with approximately 2 µg of poly[A]⁺ RNA per lane. The blots were probed with a portion of the rabbit lung INMT cDNA ORF and with human β-actin cDNA as a control. Exposure times are also listed.
the PCR was performed with these degenerate primers and a rabbit lung cDNA library as template, a 191-bp product was amplified (Fig. 3, Step 1). The sequence of this amplification product was then used to design INMT-specific primers which were paired with a series of anchor primers to perform 5'- and 3'-RACE with a rabbit lung cDNA library or with rabbit lung cDNA as template to obtain the remaining sequence of the cDNA (Fig. 3, Steps 2 and 3). The rabbit lung cDNA amplified in this fashion included a 792-bp ORF that encoded a 263-amino acid protein with a calculated molecular mass of 29 kDa. The 5'-UTR was 15 bp, and the 3'-UTR was 1094 bp in length. The length of the 5'-UTR was determined by 5'-RACE, and the length of the 3'-UTR was determined by 3'-RACE. The initial ATG in the cDNA was located within a "Kozak consensus sequence" for translation initiation (28). A polyadenylation signal (AATAAA) was located 22 bp upstream of the poly(A) tract.

Recombinant Rabbit Lung INMT Expression—The rabbit lung INMT cDNA ORF was cloned into the eukaryotic expression vector pCR3.1, and this expression construct was used to transfect COS-1 cells. The COS-1 cells expressed virtually no endogenous "INMT-like activity" under the assay conditions used. Recombinant rabbit INMT expressed in the COS-1 cells catalyzed the methylation of tryptamine, and the biochemical properties of the recombinant enzyme were very similar to those for INMT from other sources. 

![Diagram of rabbit INMT gene nucleotide and deduced amino acid sequences](image)

![Diagram of amino acid sequence relationships](image)
those of both purified rabbit lung INMT and INMT activity in rabbit lung cytosol preparations. For example, when 6 concentrations of tryptamine that varied from 0.03 to 1 mM were used to determine apparent $K_m$ values for INMT with rabbit lung high speed supernatant, purified rabbit lung INMT, and the recombinant enzyme, all of those apparent $K_m$ values were very similar (Table II). Similar results were found when 5 concentrations of AdoMet that varied from 0.8 to 25 $\mu$M were studied and apparent $K_m$ values were determined with the same three sources of enzyme (Table II). A series of compounds was then studied as possible substrates or inhibitors for the recombinant enzyme. These compounds were selected either on the basis of previous reports that they were substrates for methylation or because of their structural similarity to reported methylation substrates. The compounds tested included indolethylamines (a-methyltryptamine, N-methyltryptamine, N,N-dimethyltryptamine, 5-hydroxytryptamine, melatonin, and antimitrage drugs structurally related to N,N-dimethyltryptamine (sumatriptan, naratriptan, and zolmitriptan)), $\beta$-carbolines (norharmane, harmaine-1,2,3,4-tetrahydrocarboxylic acid, harmaline, harmol, and harmol), isoquinolines (1,2,3,4-tetrahydroisoquinoline and (6)-salsolinol), other biogenic amines (dopamine, (6)-norepinephrine, epinephrine, epinine, histamine, phenylethanolamine, $\beta$-phenylethylamine, (6)-octopamine, tyramine), and tricyclic antidepressants (desipramine and nortriptyline) among others. To determine whether these compounds were substrates for the enzyme, a single concentration that had proven optimal for the assay of tryptamine (1 mM) was tested initially (Table III), and selected compounds were subsequently studied with a series of concentrations. Obviously, the reaction conditions used to perform these assays were optimal for tryptamine methylation, but they were not necessarily optimal for the other compounds studied. Therefore, the values shown in Table III can only be compared directly with data obtained under similar assay conditions. Apparent $K_m$ values for selected compounds that were found to be INMT substrates are listed in Table II.

Recombinant rabbit lung INMT was also assayed in the presence of a series of possible inhibitors with 1 mM tryptamine as substrate (Table IV). These compounds were selected either because they had been shown to inhibit other MT enzymes or because of structural similarity to INMT substrates or reaction products. Of the compounds studied, other than the reaction products, AdoHcy and N,N-dimethyltryptamine, (6)-salsolinol, chloroquine, and harmalol were the most potent inhibitors under these assay conditions (Table IV). It was of interest that each of the serotonin receptor agonist antimitrage drugs studied, naratriptan, sumatriptan, and zolmitriptan, compounds related structurally to N,N-dimethyltryptamine, was an inhibitor of recombinant rabbit lung INMT. IC$_{50}$ values were also determined for selected inhibitors, especially those used in clinical practice, and are listed in Table IV. Since many of the substrates and inhibitors for rabbit INMT were indole derivatives, Table V has been included to illustrate the structural features of indole-related substrates and inhibitors that were studied in the course of these experiments.

**Rabbit INMT Northern Analysis**—Northern blot analysis performed with poly(A)$^+$ RNA from rabbit lung, liver, and brain revealed an mRNA species approximately 2.0 kb in length that was expressed in the lung, liver, and, at lower levels, in brain (Fig. 4). The length of this mRNA was compatible with the results found during the cDNA cloning studies. Of the three tissues studied, INMT was most highly expressed in lung. Longer exposure times also revealed the presence of transcripts approximately 3.6 and 5.6 kb in that tissue (Fig. 4).

**Rabbit INMT Gene Cloning**—A probe that contained the initial portion of the rabbit lung INMT cDNA ORF was used to screen a rabbit genomic DNA cosmid library. One cosmid clone...
was found which contained the entire cDNA ORF, and that clone was used to determine the rabbit INMT gene structure and sequence (Fig. 5). The rabbit INMT gene consisted of three exons and was approximately 4.5 kb in length. Both introns were approximately 1.3 kb in length (Fig. 5). The second intron was not sequenced completely because of the presence of repetitive sequence elements. All exon-intron splice junctions conformed to the “GT-AG” rule (29) (Fig. 5). No canonical TATA box sequence was present upstream of the transcription initiation site as determined by 5′-RACE, but an Sp1 motif was present approximately 50 bp upstream of that point (Fig. 5).

Comparison of Rabbit INMT with Other MT Enzymes—Cloning of the rabbit lung INMT cDNA made it possible to compare the amino acid sequence that it encoded with those of other known cytosolic “small molecule” MTs. The amino acid sequence of rabbit lung INMT was 59, 55, 55, 38, 37, 39, and 39% identical with those of mouse thioether MT (TEMT, EC 2.1.1.96); human and mouse nicotinamide N-MT (NNMT, EC 2.1.1.11); and mouse, human, rat, and bovine phenylethanolamine N-MT (PNMT, EC 2.1.1.28), respectively (Fig. 6B). The INMT amino acid sequence showed little homology to those of other MT enzymes. A dendrogram showing the relationship among these amino acid sequences is depicted in Fig. 6A. This group of proteins appears to comprise a “family” of cytosolic MT enzymes. Ingrosso et al. (30) have reported that enzymes which utilize AdoMet as a co-substrate usually contain three regions of high amino acid sequence homology, arbitrarily designated regions I, II, and III. Rabbit INMT contained areas of sequence homology to regions I, II, and III that were located between amino acids 60–67, 156–162, and 187–193, respectively (bold and underlined in Fig. 5). Finally, the relationship among INMT, NNMT, and PNMT on the basis of amino acid sequence was supported by comparisons of gene structures for these enzymes (Fig. 7). Unfortunately, the gene for the enzyme with highest homology to INMT, mouse TEMT, has not been reported, but gene structures are available for NNMT in two, and PNMT in four species. All six of these genes, like the rabbit INMT gene, consist of three exons, with a conserved central exon length (Fig. 7), observations compatible with the conclusion that all of these genes arose through a process of divergent evolution.

DISCUSSION

Methylation is an important pathway in the biotransformation of both exogenous and endogenous molecules (1). The N-methylation of tryptamine has been detected in a variety of species and tissues (2, 8, 9, 31–34), but it has remained unclear whether those activities were catalyzed by a single enzyme with broad substrate specificity or by members of a family of related N-MTs. Since the rabbit lung was the tissue in which INMT had been studied most intensively, we set out to purify rabbit lung INMT, to clone and express its cDNA and to clone its gene as steps toward understanding the function and regulation of this enzyme as well as those of possible orthologues in other species and paralogues in the rabbit. Rabbit lung INMT was purified, partial amino acid sequence was obtained, and a PCR-based strategy was used to clone a cDNA with a 792-bp ORF that encoded a 269-amino acid protein with a predicted molecular mass of 29 kDa. Northern blot analysis indicated that an INMT mRNA species approximately 2.0 kb in length was expressed in rabbit lung, liver and, at lower levels, in brain (Fig. 4). The protein encoded by this cDNA was able to methylate tryptamine. When substrate kinetic studies were performed with recombinant rabbit lung INMT, apparent Km values for tryptamine and AdoMet were similar to those for rabbit lung cytosol or partially purified rabbit lung enzyme. N-Methyltryptamine had the lowest apparent Km value, 0.086 mM, of the methyl acceptor substrates tested (Table III). However, the functional role of INMT in vivo remains unclear. Even though this enzyme was originally discovered as a result of interest in the possible generation of methylated metabolites of tryptamine and serotonin that might be psychoactive, our observations make that possibility less likely. First, INMT mRNA is not highly expressed in the brain, at least not in the rabbit (Fig. 3). In addition, apparent Km values of INMT for tryptamine and serotonin are relatively high (Table II), indicating that although tryptamine has served a useful purpose as a “prototypic substrate,” it is less likely to be an important endogenous substrate for the enzyme. A second line of research with regard to a possible functional role for INMT has focused on N-methylation as a step in the “bioactivation” of pro-neurotoxins such as the β-carbolines and isoquinolines (3–7). Therefore, we also tested those compounds as potential substrates for recombinant rabbit lung INMT and found that the enzyme could catalyze the N-methylation of some of these compounds (Table IV), but once again with relatively high apparent Km values (Table II). Rabbit lung INMT was inhibited by products of the reaction that it catalyzed with tryptamine as a substrate as well as antimigraine serotonin receptor agonists that are structurally related to N,N-dimethyltryptamine and tricyclic antidepressants such as imipramine (Table IV). Therefore, if an orthologue of INMT with similar biochemical properties is expressed in human tissues, the potential effects of these commonly prescribed drugs on that enzyme will have to be evaluated. Finally, rabbit lung INMT appeared to be a member of a family of cytosolic small molecule MTs, a family that included TEMT, NNMT, and PNMT. That conclusion was based on comparisons of both amino acid sequences and gene structures (Figs. 6 and 7). Whether other members of this emerging gene family remain to be discovered is unknown. In summary, the cloning and expression of a rabbit lung INMT cDNA as well as the cloning and characterization of its gene represent important steps toward understanding the function and regulation of this MT enzyme and will now make it possible to seek and study possible orthologues in other species, including humans, as well as paralogues in the rabbit.

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