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Expression cloning of a reserpine-sensitive vesicular monoamine transporter

(vaccinia virus/transfection/biogenic amine uptake/digitonin permeabilization/photoaffinity labeling)

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ABSTRACT **A cDNA for a rat vesicular monoamine transporter, designated MAT, was isolated by expression cloning in a mammalian cell line (CV-1). The cDNA sequence predicts a protein of 515 amino acids with 12 putative membrane-spanning domains. The characteristics of [^{3}H]serotonin accumulation by CV-1 cells expressing the cDNA clone suggested sequestration by an intracellular compartment. In cells permeabilized with digitonin, uptake was ATP dependent with an apparent K_m of 1.3 μM. Uptake was abolished by the proton-translocating ionophore carbonylcyanide *p*-trifluoromethoxyphenylhydrazone and with tri-(*n*-butyl)tin, an inhibitor of the vacuolar H$^+$-ATPase. The rank order of potency to inhibit uptake was reserpine > tetrabenazine > serotonin > dopamine > norepinephrine > epinephrine. Direct comparison of [^{3}H]monoamine uptake indicated that serotonin was the preferred substrate. Photolabeling of membranes prepared from CV-1 cells expressing MAT with 7-azido-8-[^{125}I]iodoketanserin revealed a predominant tetrabenazine-sensitive photolabeled glycoprotein with an apparent molecular mass of ≈75 kDa. The mRNA that encodes MAT was present specifically in monoamine-containing cells of the locus coeruleus, substantia nigra, and raphe nucleus of rat brain, each of which expresses a unique plasma membrane reuptake transporter. The MAT cDNA clone defines a vesicular monoamine transporter representing a distinct class of neurotransmitter transport molecules.**

Vesicular monoamine transporters (MATs) facilitate the ATP-dependent accumulation of biogenic amine neurotransmitters into secretory organelles of neurons, enterochromaffin cells, platelets, and mast cells. Monoamine transport occurs in exchange for intravesicular protons (substrate/H$^+$ antiporter) and is requisite for vesicular amine storage prior to secretion via exocytosis (1–4). The biogenic amine Na$^+$-dependent transporters (reuptake transporters) located at the plasma membrane are responsible for transport of the released monoamines back into the cytoplasm, where they may be repackaged by the vesicular transporter into storage organelles or degraded by monoamine oxidases (5, 6).

Several distinguishing features of the MAT are as follows: (*i*) broad selectivity for 5-hydroxytryptamine (serotonin) (5HT), dopamine (DA), and norepinephrine (NE) uptake, (*ii*) specific inhibition of transport by reserpine (RES) and tetrabenazine (TBZ), and (*iii*) transmembrane H$^+$-electrochemical dependence of monoamine accumulation (1–4). Furthermore, photoaffinity labeling of storage vesicles from brain, adrenal medulla, and platelets from different species with 7-azido-8-[^{125}I]iodoketanserin ([^{125}I]AZIK) as well as protein purification studies have determined that the MAT is an integral membrane glycoprotein with an apparent mass of 65–85 kDa (7–9).

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It has been proposed that the type of neurotransmitter found in monoaminergic cells is governed by expression of the specific biosynthetic enzymes and the appropriate and specific plasma membrane reuptake transporter, with a vesicular transporter of relatively broad selectivity (10, 11). Recently, several biogenic amine reuptake transporters have been cloned (12–17, 47). Here, an expression cloning strategy was used to functionally identify a cDNA clone for a MAT.†

EXPERIMENTAL PROCEDURES

cDNA Cloning. A cDNA library of 2.3 × 10^6 recombinants (13) was subdivided and screened by coinfection/transfection of monkey kidney cells (CV-1 cells) using the vaccinia T7 RNA polymerase expression system (18) and subsequent accumulation of [^{3}H]5HT (0.4 μM). Positive subdivisions were identified by microscopy (13). The cDNA clone was sequenced (both strands) from fragments subcloned into M13 bacteriophage using the Sequenase kit (United States Biochemical). Sequence analysis was performed with a Genetics Computer Group sequence analysis software package (19).

[^{3}H]5HT Accumulation in Intact CV-1 Cells. The uptake studies were conducted as described (13, 17) with the uptake buffer at pH 6.8, 7.4, and 8.0.

[^{3}H]Monoamine Uptake in Permeabilized CV-1 Cells. CV-1 cells expressing the MAT cDNA were rinsed with intracellular buffer containing 110 mM potassium tartrate, 5 mM glucose, 0.2% bovine serum albumin, 200 μM $CaCl_2$, 1 mM ascorbic acid, 10 μM pargyline, and 20 mM Pipes (pH 6.8). The cells were then permeabilized for 10 min at 37°C in uptake buffer with 10 μM digitonin (Calbiochem). The medium was replaced with fresh buffer without digitonin containing 5 mM MgATP and [^{3}H]5HT, [^{3}H]DA, or [^{3}H]NE (New England Nuclear). Uptake was terminated by a 1-ml wash on ice. Experiments were also performed in collagen-coated (50 μg/ml) dishes without Ca^{2+} ions in uptake buffer.

Photolabeling with [^{125}I]AZIK. Bovine chromaffin granules (20) and total membranes (minus nuclei) from CV-1 cells expressing either MAT or 5HT reuptake transporter (5HTT) cDNA clones and from rat basophilic leukemia cells (RBL 2H3) (10^8 cells) were lysed in 10 mM Tris·HCl buffer (pH 7.5) with peptidase inhibitors and photolabeled with [^{125}I]AZIK (Amersham) as described (8). Photolabeled membrane samples were digested with *N*-glycopeptidase (from *Flavobacterium meningosepticum*; Boehringer) as described (8). Sam-

Abbreviations: DA, dopamine; DAT, DA reuptake transporter; FCCP, carbonylcyanide *p*-trifluoromethoxyphenylhydrazone; 5HT, 5-hydroxytryptamine; 5HTT, 5HT reuptake transporter; [^{125}I]AZIK, 7-azido-8-[^{125}I]iodoketanserin; ISHH, *in situ* hybridization histochemistry; MAT, vesicular monoamine transporter; NE, norepinephrine; NET, NE reuptake transporter; RES, reserpine; TBT, tri-(*n*-butyl)tin; TBZ, tetrabenazine.

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†The sequence reported in this paper has been deposited in the GenBank data base (accession no. L00603).

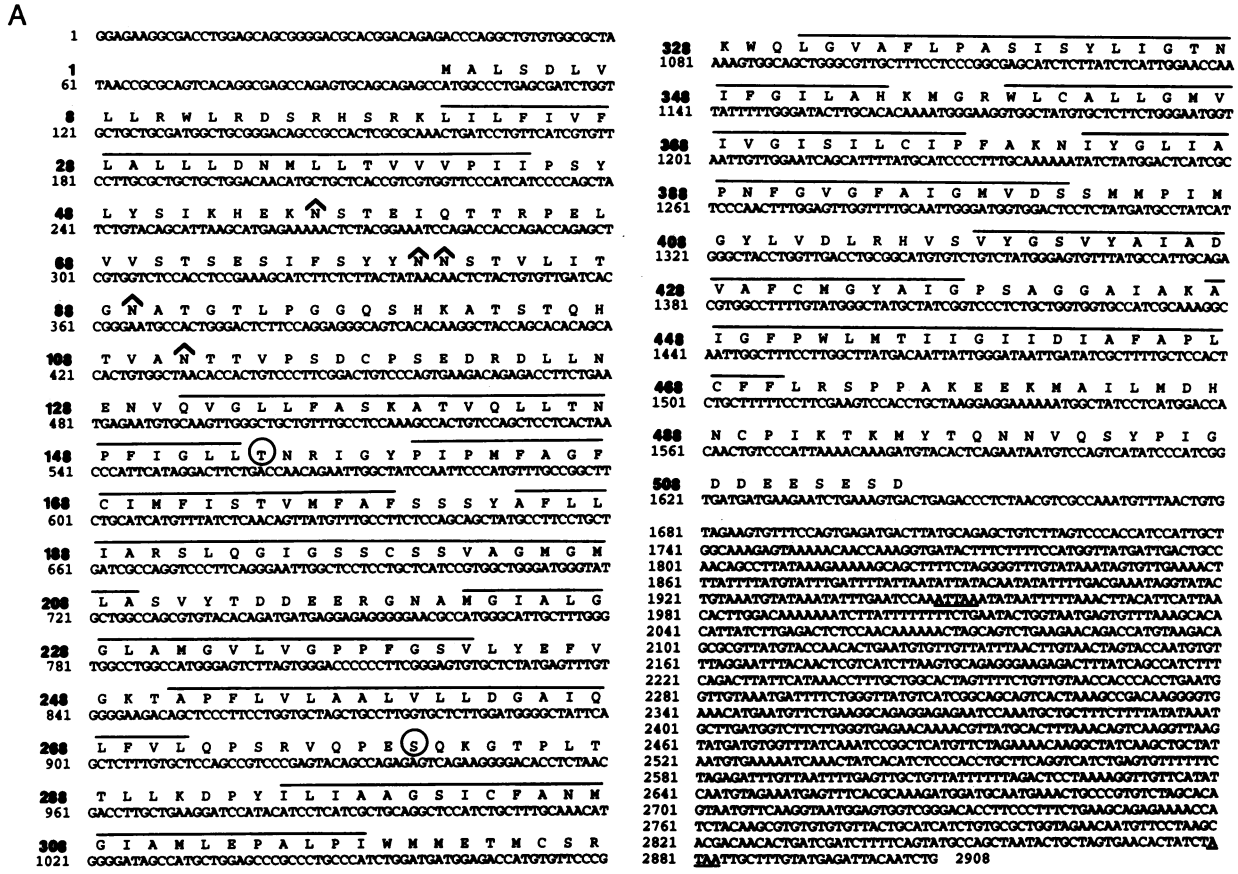

FIG. 1. Nucleotide and predicted amino acid sequences of the cDNA encoding MAT. (*A*) Predicted transmembrane domains are overlined above the amino acids. Potential N-linked glycosylation sites (^) and sites for phosphorylation by protein kinase C (o) are indicated. Polyadenylylation signals are underlined. (*B*) Model for proposed structure of MAT in intracellular organelle.

ples were analyzed by SDS/PAGE (9% acrylamide; bisacrylamide/acrylamide, 0.8:30) (21).

Northern Analysis and *in Situ* Hybridization Histochemistry (ISHH). Poly(A)$^+$ RNA from RBL 2H3 cells and rat tissues was purified as described (22). RNA blots were hybridized with MAT cDNA labeled by random priming (Boehringer Mannheim) with final wash conditions of 0.2× standard saline citrate/0.1% SDS at 55°C. Antisense oligodeoxynucleotide probes for MAT (amino acids 57–72 and 270–285), 5HTT (amino acids 40–56; ref. 13), DA reuptake transporter (DAT) (amino acids 42–57; refs. 14 and 16), and NE reuptake transporter (NET) (amino acids 221–236; ref. 15) were labeled and ISHH was performed as described (23).

RESULTS

Sequence Analysis of the MAT cDNA Clone. The nucleic acid sequence of the 2.9-kilobase (kb) MAT cDNA clone revealed an open reading frame of 1545 base pairs (Fig. 1*A*), predicting a protein of 515 amino acids with a molecular mass of ≈56.7 kDa. Hydrophobicity analysis by the Kyte–Doolittle algorithm (24) predicted 12 putative transmembrane domains (I–XII). The orientation of the protein within the

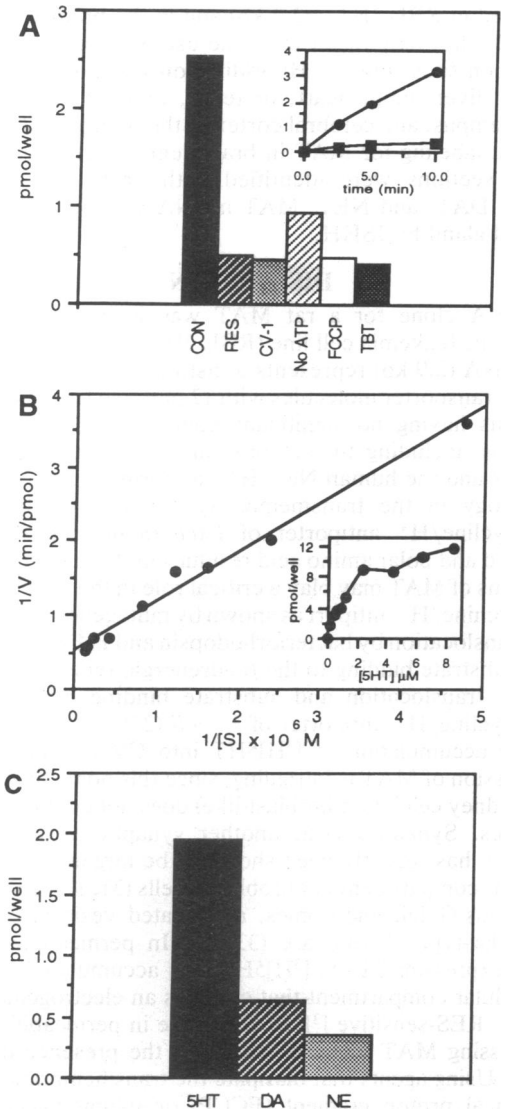

FIG. 2. Characterization of [^{3}H]5HT uptake in digitonin permeabilized CV-1 cells (≈5 × 10^4 cells) expressing MAT. (*A*) Total uptake of [^{3}H]5HT (0.4 μM) at 5 min with 5 mM ATP (CON), in the presence of 100 nM RES, in mock-transfected CV-1 cells, in the absence of ATP, or in the presence of 5 μM FCCP or 50 μM TBT. (*Inset*) Time course of RES-sensitive accumulation in the presence (●) and absence (■) of MgATP. (*B*) Lineweaver–Burk analysis of initial uptake velocity (5 min) of [^{3}H]5HT (0.2–8.4 μM). (*Inset*) Saturation isotherm of initial velocity data. (*C*) Comparison of RES-sensitive [^{3}H]monoamine uptake (0.4 μM) at 5 min. Data are representative experiments and were repeated at least twice in duplicate or quadruplicate.

vesicle membrane has been tentatively assigned (Fig. 1*B*) based on the lack of apparent signal sequence with five potential N-linked glycosylation sites facing the vesicle lumen, and NH_2 and COOH tails in the cytoplasm.

Of the 72 charged (35 negative residues and 37 positive residues) amino acids, 11 are potentially located in the transmembrane domains. The locations of transmembrane domain charged residues are aspartic acids in I, VI, XI, and XII; glutamic acid in VII; lysine in II; and arginine in IV. Transmembrane domains II–IV also contain numerous polar amino acids, while transmembrane domains VIII–XII are relatively more hydrophobic. Two potential sites for phosphorylation by protein kinase C are found on the cytoplasmic face of the molecule.

[^{3}H]5HT Accumulation by Intact CV-1 Cells Expressing MAT cDNA. The time course of RES-sensitive [^{3}H]5HT uptake in CV-1 cells expressing MAT was relatively slow with saturation occurring by 2 hr with 1 mM 5HT and was linear through 3 hr at 0.4 μM [^{3}H]5HT. Uptake of [^{3}H]5HT (0.4 μM) was maximal at pH 8.0. Using 1 mM [^{3}H]5HT, equilibration was accelerated at higher pH, but equivalent accumulation was attained by 3 hr regardless of the pH of the uptake buffer. The apparent inhibition constants (K_i) for RES and TBZ inhibition of [^{3}H]5HT uptake were 12.5 nM and 0.63 μM, respectively. Uptake was also inhibited by unlabeled 5HT, DA, and NE with apparent K_i values of 0.26, 0.38, and 1.2 mM, respectively, at pH 8.0. The effect of pH on [^{3}H]5HT accumulation was due to a change in the apparent affinity for uptake (K_m). K_m values of 0.95, 0.45, and 0.31 mM were obtained for [^{3}H]5HT uptake at pH 6.8, 7.4, and 8.0, respectively. Uptake of [^{3}H]5HT was temperature sensitive and was only reduced ≈25% with choline replacement of sodium in the buffer for 120 min at pH 8.0.

Table 1. Pharmacologic sensitivity of [^{3}H]5HT uptake in digitonin-permeabilized CV-1 cells expressing MAT

Inhibitor	Concentration, μM	[^{3}H]5HT uptake, pmol per well	% inhibition
Control	—	17.19 ± 0.87	—
TBZ	2	1.03 ± 0.11	98.5
5HT	10	1.43 ± 0.05	96
DA	10	2.84 ± 0.12	87
NE	10	4.45 ± 0.09	78
EPI	10	9.98 ± 0.20	62

CV-1 cells (4 × 10^5 cells) were plated on collagen-coated 35-mm dishes and [^{3}H]5HT uptake (0.4 μM) was measured after 5 min at 37°C. CV-1 cells alone = 0.79 ± 0.08 pmol per well. Data represent means ± SEM from three separate plates; experiments were repeated twice. EPI, epinephrine.

The slow, pH-sensitive, and low-affinity accumulation of monoamines in intact CV-1 cells expressing MAT suggested that the amines cross the plasma membrane by passive diffusion of the neutral species. Once inside the cell, the monoamines become protonated and available to MAT located on an intracellular compartment. Thus, the affinity constants for intact cells are only apparent, related to the true values for uptake at an intracellular compartment by true K_m ≈ apparent K_m × $10^{-pKa}/(H^+)$. The true K_m value for [^{3}H]5HT is ≈1.8 μM if passive diffusion of the neutral amine (pKa 9.8) across the plasma membrane (pH 7.4) must occur first.

[^{3}H]Monoamine Uptake in Digitonin-Permeabilized CV-1 Cells Expressing MAT cDNA. To access intracellular compartments, the plasma membrane was selectively permeabilized with digitonin (25, 26). Permeabilization of CV-1 cells with digitonin in intracellular medium eliminates the normal ionic gradients across the plasma membrane and abolished Na^+-dependent 5HTT function. In permeabilized cells expressing MAT, [^{3}H]5HT uptake was inhibited by 100 nM RES to levels in mock-transfected CV-1 cells (Fig. 2*A*). RES-sensitive [^{3}H]5HT uptake was reduced by >80% in the absence of ATP. In addition, [^{3}H]5HT uptake was abolished by the proton translocating ionophore carbonylcyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) (5 μM) or by inhibition of the vacuolar H^+-ATPase with tri-(*n*-butyl)tin (TBT) (50 μM). Uptake was saturable with an apparent K_m of 1.3 μM (Fig. 2*B*). The biogenic amines (10 μM) inhibit the transport of [^{3}H]5HT with 5HT > DA > NE > epinephrine, and TBZ (2 μM) abolished uptake (Table 1). [^{3}H]5HT was the preferred substrate, followed by DA > NE (Fig. 2*C*).

Photolabeling with [^{125}I]AZIK. Membranes from bovine chromaffin granules, RBL 2H3 cells, and CV-1 cells expressing MAT or 5HTT cDNA clones were photolabeled with [^{125}I]AZIK (Fig. 3). TBZ-sensitive labeling of membrane components of slightly different apparent size appeared in

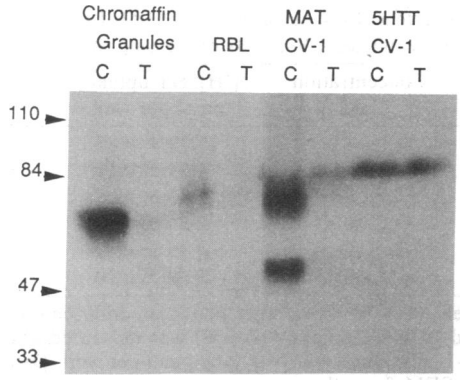

FIG. 3. [^{125}I]AZIK photolabeling of membranes from bovine chromaffin granules, RBL 2H3 cells, and CV-1 cells expressing the MAT or 5HTT cDNAs in the absence (lanes C) and presence (lanes T) of 2 μM TBZ. Membrane proteins (250 μg) were separated by SDS/PAGE and the dried gel was exposed to X-AR5 film (Kodak) for 1 hr. Molecular mass markers are in kDa.

bovine chromaffin granules (≈70 kDa) and RBL cells (≈75 kDa). In cells expressing the MAT cDNA, a membrane protein of ≈75 kDa displayed TBZ-sensitive photolabeling but was absent from 5HTT-expressing CV-1 cells. A smaller (≈50 kDa) photolabeled protein seen only in MAT transfected CV-1 cells also displayed TBZ sensitivity and may be a MAT proteolytic cleavage product. Both the ≈50-kDa membrane component and the larger ≈75-kDa photolabeled protein are glycosylated, since treatment of membranes from CV-1 cells expressing MAT with *N*-glycopeptidase caused both photolabeled polypeptides to be reduced in size by ≈20 kDa. As all potential N-glycosylation sites are in the first intraluminal loop (Fig. 1), the photolabeled site may be located within the first eight transmembrane domains of MAT. The additional photolabeled membrane protein of ≈90 kDa observed in CV-1 cells was not displaceable by TBZ, ketanserin, mianserin, chlorpheniramine, or prazosin at 2 μM and probably represents nonspecific photolabeling.

Distribution of MAT mRNA in Rat Brain and Peripheral Tissues. MAT cDNA hybridized to mRNAs of three different sizes (Fig. 4). A 4.0-kb mRNA was identified in RBL 2H3 cells, brainstem, and stomach. Additional mRNA species

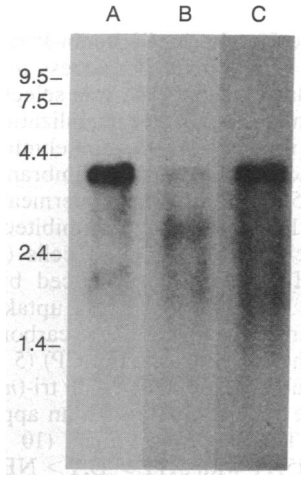

FIG. 4. Northern hybridization of MAT mRNA. Poly(A)$^+$ RNA was prepared from RBL 2H3 cells (lane A; 10 μg), rat brainstem (lane B; 10 μg), and stomach (lane C; 5 μg) specimens, size fractionated on agarose/formaldehyde gels, transferred to nylon membrane, and hybridized with ^{32}P-labeled MAT cDNA. Exposure to X-AR5 film was 5 hr for RBL 2H3 cells and 24 hr for tissues. Molecular size markers are in kb.

observed in RBL 2H3 (≈2.2 kb) and in the brainstem (−2.9 and −2.2 kb) may result from the use of different polyadenylylation sites (Fig. 1). Hybridization was not observed in kidney, liver, lung, heart, or testis, or in the cerebellum, hippocampus, and cerebral cortex of the brain. ISHH clearly showed labeling for MAT in brainstem nuclei (Fig. 5). Adjacent sections were identified with probes specific for 5HTT, DAT, and NET. MAT mRNA was not detected in adrenal gland by ISHH.

DISCUSSION

A cDNA clone for a rat MAT was isolated from a rat basophilic leukemia cell line (RBL 2H3) cDNA library. This rat cDNA (2.9 kb) represents a distinct class of neurotransmitter transporter molecules with 12 putative transmembrane domains having no significant homology to other known proteins, including the rat biogenic amine reuptake transporters and the human Na^+/H^+ antiporter. There is limited homology in the transmembrane domains of the metal-tetracycline/H^+ antiporter of *Escherichia coli* (27). The charged and polar amino acid residues in the transmembrane domains of MAT may play a critical role in the mechanism of monoamine/H^+ antiport as shown by mutagenesis studies for H^+ translocation by bacteriorhodopsin and F_0F_1-ATPase (28, 29), substrate binding to the β-adrenergic receptor (30), and in H^+ translocation and substrate binding by the metal-tetracycline/H^+ antiporter of *E. coli* (27).

The accumulation of [^{3}H]5HT into CV-1 cells following expression of MAT is intriguing, since this nonneuronal monkey kidney cell line (fibroblast-like) does not contain synaptic vesicles. Synaptophysin, another synaptic vesicle-specific protein, has recently been shown to be targeted to an intracellular compartment in fibroblastic cells (31, 32). In addition, the trans-Golgi, endosomes, and coated vesicles possess a vacuolar-type H^+-ATPase (33–36). In permeabilized CV-1 cells expressing MAT, [^{3}H]5HT was accumulated by an intracellular compartment that contains an electrogenic proton pump. RES-sensitive [^{3}H]5HT uptake in permeabilized cells expressing MAT was dependent on the presence of Mg^{2+}-ATP. Using agents that dissipate the transmembrane electrochemical proton gradient (FCCP) or inhibit vacuolar H^+-ATPase activity (TBT) (4, 37), uptake in permeabilized cells was completely inhibited. The high affinity for [^{3}H]5HT displayed by permeabilized CV-1 cells expressing MAT (K_m = 1.3 μM) is similar to the K_m for monoamine uptake in synaptic vesicles from rat brain (38–41) and RBL 2H3 cells (42). The broad specificity of transport by MAT in permeabilized CV-1 cells is also a distinguishing feature of the MAT (11, 43, 44).

The cDNA screening strategy for MAT relied on two processes of [^{3}H]5HT uptake in intact cells: diffusion of the neutral amine across the plasma membrane followed by uptake into an intracellular compartment. The rank order of potency for inhibition of [^{3}H]5HT accumulation into intact CV-1 cells expressing MAT by the biogenic amines was nevertheless identical to that in permeabilized cells. Furthermore, the potency of RES to inhibit [^{3}H]5HT uptake (12.5 nM) in intact cells was comparable to K_i values (≈10 nM) obtained with *in vitro* preparations of monoaminergic storage organelles (3, 45, 46).

The MAT cDNA clone encodes a unique vesicular biogenic monoamine transporter. High-affinity ATP-dependent uptake of monoamines, RES and TBZ inhibition of transport, transmembrane H^+-electrochemical dependence of accumulation, and [^{125}I]AZIK photolabeling are all reconstituted by expression of this single cDNA clone. The distribution of mRNA encoding MAT indicates that a common pathway may exist for vesicular biogenic amine transport in basophilic 5HT-containing cells and in noradrenergic, dopaminergic, and serotonergic neurons of the central nervous system.

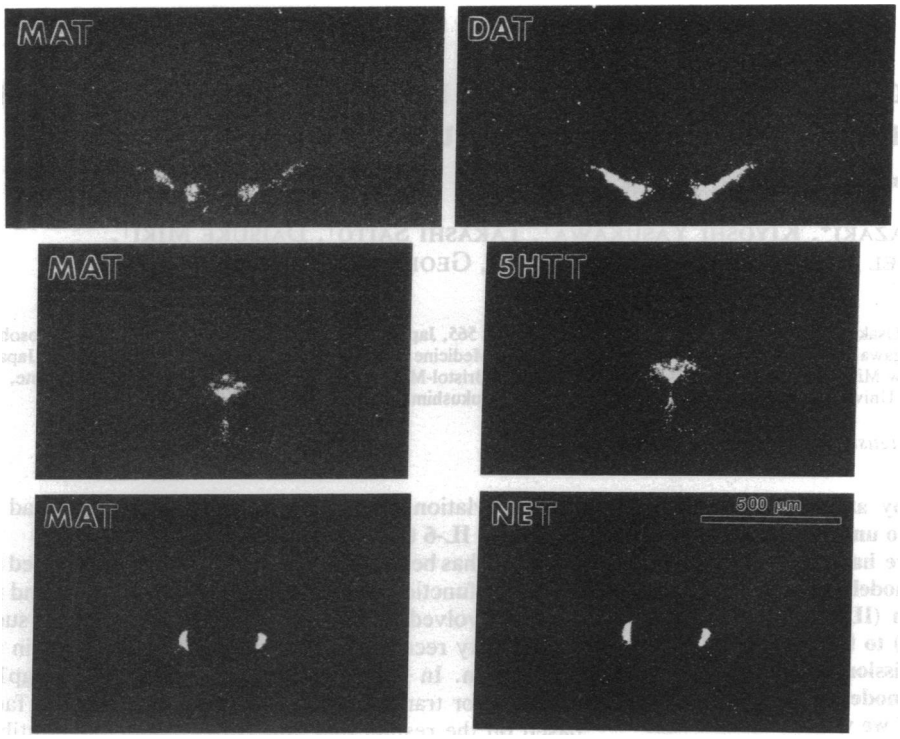

FIG. 5. *In situ* hybridization of MAT mRNA in rat brain. Adjacent sections (12 μM) were hybridized with probes specific for MAT and for DAT, NET, and 5HTT to localize the substantia nigra, locus coeruleus, and raphe nuclei, respectively. Slides were apposed to Hyperfilm β (Amersham) for 11 days.

The functional complementation of MAT within an intracellular structure of a nonneuroendocrine cell demonstrates the minimal cellular requirements for reconstitution of vesicular H^+/substrate antiport activity. This will provide a convenient *in vitro* model for detailed structural analysis of MAT as well as the transport specificity of other vesicular transporters.

Note Added in Proof. While this paper was in press, Liu *et al.* (48) described the cloning of two vesicular monoamine transporters, one of which is identical to MAT.

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