

A human synaptic vesicle monoamine transporter cDNA predicts posttranslational modifications, reveals chromosome 10 gene localization and identifies *TaqI* RFLPs

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A human vesicular monoamine transporter cDNA has been identified by screening a human brainstem library using sequences from the rat brain synaptic vesicle monoamine transporter (SVMT) [(1992) Cell 70, 539–551; (1992) Proc. Natl. Acad. Sci. USA 89, 10993–10997]. The hSVMT shares 92% amino acid identity with the rat sequence, but displays one less consensus site for asparagine N-linked glycosylation and one more consensus site for phosphorylation by protein kinase C. The human SVMT gene maps to chromosome 10q25 using Southern blotting analysis of human/rodent hybrid cell lines and fluorescent *in situ* hybridization approaches. The cDNA, and a subclone, recognize *TaqI* polymorphisms that may prove useful to assess this gene's involvement in neuropsychiatric disorders involving monoaminergic brain systems.

Monoamine transporter; Chromosomal mapping; Polymorphism; N-linked glycosylation

1. INTRODUCTION

The vesicular monoamine transporter acts to accumulate cytosolic monoamines into synaptic vesicles, using the proton gradient maintained across the synaptic vesicular membrane. Its proper function is a key to the correct activity of the monoaminergic systems that have been implicated in a number of human neuropsychiatric disorders [3–5]. The transporter is a site of action of important drugs, including reserpine and tetra-*benazine* [6–8]. Amphetamine and its derivatives induce release of vesicular monoamines by mechanisms that may include their uptake via the SVMT [7,9,10]. In addition, it has been documented to sequester monoaminergic neurotoxins, such as that producing one of the best current experimental models of Parkinson's disease, MPP⁺ [1,11,12].

Recent elucidation of cDNAs encoding the rat synaptic vesicular monoamine transporter [1,2] provides hybridization probes useful for isolation of its human cognate. We now describe the human cDNA, the unique features that it predicts for its encoded mRNA and protein, the location to which its gene maps, RFLPs identified by this cDNA, and estimates of the population frequencies of these genetic polymorphisms. These results provide the groundwork for examination of this

interesting gene's roles in the normal function of human aminergic systems, and in human disorders of these systems.

2. MATERIALS AND METHODS

2.1. Screening human and rat brain cDNA libraries

Partial cDNA hybridization probes of 1.0 and 0.8 kb corresponding to TM domains 1–8 and 4–12, respectively, were generated by PCR (polymerase chain reaction) amplification of a size-selected rat ventral midbrain cDNA library [13], using 20-nucleotide primers corresponding to previously described rat brain cDNA sequences from these transmembrane domains [1]. Each probe was purified by agarose gel electrophoresis and ³²P-labeled by random priming (Prime It Kit, Boehringer Mannheim). Human brainstem (Stratagene) and rat ventral midbrain [13] cDNA libraries were plated and blotted onto duplicate nitrocellulose (Schleicher and Schuell) filters which were prehybridized for 4 h at 42°C in 6 × SSPE containing 29% deionized formamide, 2.5 × Denhardt's solution, 0.1% SDS and 0.4 mg/ml herring sperm DNA. 1 × 10⁶ plaques were screened in each case using 2 × 10⁶ cpm/μl of the 4–12 (rat library) or 1–8 (human library) partial cDNA probes in the prehybridization solution at 37°C overnight. Nonspecific hybridization was eliminated with three 0.5 h washes with 0.4 × SSPE, 0.5% SDS at 54°C. Clones detected by autoradiography as reproducibly positive for hybridization were isolated as Bluescript II SK plasmids via *in vivo* excision mediated by helper phage R408 (Stratagene), and both strands were sequenced on an Applied Biosystems automated sequencer. Sequences obtained were analyzed using the GCG software package [14,15], with focus on the human cDNA clone phSVMT1 that appeared to represent the human homolog of previously reported rat SVMT sequences.

2.2. Chromosomal localization of the human SVMT gene

The phSVMT1 cDNA clone was digested with restriction endonuclease *EcoRI* to liberate the 1.8 kb insert that was gel-purified, ³²P-

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1  GAATTCCCCG CCGGACTGAC GGAGCCCACT GCGGTGCGGG CGTTGGCGCG
51  GGCACGGAGG ACCCGGGCAG GCATCGCAAAG CGACCCCGAG CGGAGCCCCG
101 GAGCATGGC CCTGAGCGAG CTGGCGCTGG TCCGCTGGCT GCAGGAGAGC
151 CGCCGCTCGC GGAAGCTCAT CCTGTTTCATC GTGTTCTTGG CGCTGCTGCT
201 GGACAACATG CTGCTCACTG TCGTGGTCCC CATCATCCCA AGTTATCTGT
251 ACAGCATTAA GCATGAGAAG AATGCTACAG AAATCCAGAC GGCCAGGCCA
301 GTGCACACTG CCTCCATCTC AGACAGCTTC CAGAGCATCT TCTCCTATTA
351 TGATAACTCG ACTATGGTCA CCGGGAATGC TACCAGAGAC CTGACACTTC
401 ATCAGACCGC CACACAGCAC ATGGTGACCA ACGCGTCCGC TGTTCCCTTC
451 GACTGTCCCA GTGAAGACAA AGACCTCCTG AATGAAACG TGCAAGTTGG
501 TCTGTTGTTT GCCTCGAAAG CCACCGTCCA GCTCATCACC AACCCTTTCA
551 TAGGACTACT GACCAACAGA ATTGGCTATC CAATTCCCAT ATTTGCGGGA
601 TTCTGCATCA TGTTTGTCTC AACAATTATG TTTGCCTTCT CCAGCAGCTA
651 TGCCTTCCTG CTGATTGCCA GGTCGCTGCA GGGCATCGGC TCGTCCTGCT
701 CCTCTGTGGC TGGGATGGGC ATGCTTGCCA GTGTCTACAC AGATGATGAA
751 GAGAGAGGCA ACGTCATGGG AATCGCCTTG GGAGGCCTGG CCATGGGGGT
801 CTTAGTGGGC CCCCCCTTCG GGAGTGTGCT CTATGAGTTT GTGGGGAAGA
851 CGGCTCCGTT CCTGGTGCTG GCCGCCCTGG TACTCTTGGA TGGAGCTATT
901 CAGCTCTTTG TGCTCCAGCC GTCCCGGGTG CAGCCAGAGA GTCAGAAGGG
951 GACACCCTTA ACCACGCTGC TGAAGGACCC GTACATCCTC ATTGCTGCAG
1001 GCTCCATCTC CTTTGCAAAC ATGGGCATCG CCATGCTGGA GCCAGCCCTG
1051 CCCATCTGGA TGATGGAGAC CATGTGTTCC CGCAAGTGGC AGCTGGGCGT
1101 TGCCtTCTTG CCAGCTAGTA TCTCTTATCT CATTGGAACC AATATTTTTG
1151 GGATACTTGC ACACACAATG GGGAGGTGGC TTTGTGCTCT TCTGGGAATG
1201 ATAATTGTTG GAGTCAGCAT TTTATGTATT CCATTTCCAA AAAACATTTA
1251 TGGACTCATA GCTCCGAAC TTTGGAGTTGG TTTTGCAAAT GGAATGGTGG
1301 ATTCGTCAAT GATGCCTATC ATGGGCTACC TCGTAGACCT GCGGCACGTG
1351 TCCGTCTATG GGAGTGTGTA CGCCATTGCG GATGTGGCAT TTTGTATGGG
1401 GTATGCTATA GGTCTTCTG CTGGTGGTGC TATTGCAAAG GCAATTGGAT
1451 TTCCATGGCT CATGACAATT ATTGGGATAA TTGATATTCT TTTTGCCCTT
1501 CTCTGCTTTT TTCTTCGAAG TCCACCTGCC AAAGAAGAAA AAATGGCTAT
1551 TCTCATGGAT CACAACTGCC CTATTAAAAC AAAAATGTAC ACTCAGAATA
1601 ATATCCAGTC ATATCCGATA GGTGAAGATG AAGAATCTGA AAGTGACTGA
1651 GATGAGATCC TCAAAAATCA TCAAAAGTGT TAATTGTATA AAACAGTGTT
1701 TCCAGTGACA CAACTCATCC AGAACTGTCT TAGTCATACC ATCCATCCCT
1751 GGTGAAAGAG TAAAACCAA GGTATTATT TCAAAAAGG AATTC

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Fig. 1. Sequence of the human SVMT cDNA. Initiation and termination codons of the open reading frame are indicated (boxed).

labeled, and employed as a probe in Southern blot analysis of the NIGMS rodent somatic cell hybrid panel #2 (Coriell Institute for Medicine, Camden, NJ). Each human chromosome was represented in a separate gel lane as a *Hind*III digest.

Higher resolution mapping of the hSVMT gene was accomplished with chromosomal in situ hybridization. phSVMT1 was nick-translated with biotin-14 dATP (BRL, Gaithersburg, MD), with 21% incorporation as determined by tritium tracer incorporation. Slides with chromosome spreads were made from normal male lymphocytes cultured with BrdU [16]. Fluorescence in situ was performed as described [17] with modifications. 20 ng/ μ l biotinylated probe in 2 \times SSCP, 50% formamide, 10% dextran sulfate, and 1 μ g/ μ l salmon sperm DNA was

denatured at 70°C for 5 min., quickly chilled on ice, placed on slides and hybridized at 37°C overnight. Slides were washed in 50% formamide/2 \times SSC at 37°C for 20 min, then twice for 5 min. Biotinylated probe was detected with FITC-avidin and biotinylated anti-avidin (Oncor, Inc.), following manufacturer's instructions.

2.3. Restriction fragment length polymorphism (RFLP) analysis using *Taq*I

DNA isolated from the leukocytes of 197 unrelated individuals subjected to experimental protocols at the Addiction Research Center, NIDA, was digested with *Taq*I and analyzed by Southern blotting as described [18]. The 1.8 kb *Eco*RI fragment of phSVMT1 was labeled

as described above, and also gel-isolated and digested with *TaqI* to yield a 1 kb fragment, 'Taq 1000', that was also used as a hybridization probe.

3. RESULTS

3.1. Identification of human and rat brain SVMT cDNAs

Human brainstem and rat ventral tegmental area (VTA) cDNA libraries were screened with the two PCR-generated probes described above. From 1×10^6 recombinant plaques in each case, 8 and 24 hybridizing cDNAs were obtained from the human and rat cDNA libraries, respectively. Preliminary sequence analyses of each clone indicated that each represented a partial SVMT cDNA. The human cDNA clone phSVMT1 was found to contain an open reading frame that encoded a 514 amino acid protein, flanked by 105 bp of 5'- and 145 bp of 3'-untranslated sequence (Fig. 1). The rat cDNA clone prSVMT1 also displays an open reading frame that encodes a protein of 515 amino acids, followed by 2.0 kb of untranslated sequence. prSVMT1 is

virtually identical to the rat brain synaptic vesicle monoamine transporters reported previously [1,2]. The open reading frame of the human hSVMT1 cDNA exhibits 88% nucleotide identity to the rat rSVMT sequence; the predicted amino acid sequences are 92% identical (Fig. 2).

3.2. Chromosomal localization of the hSVMT gene

Somatic cell hybridization analyses revealed that there was 100% concordance between the presence of chromosome 10 and the 9, 7, and 3.8 kb *HindIII* DNA fragments that hybridized with the *EcoRI* fragment of phSVMT1. There was greater than 8% discordance for each other chromosome.

Analysis of 119 metaphase cells demonstrated 25 cells (20%) that had at least one pair of hybridization signals that involved both chromatids of a single chromosome. Forty paired signals were seen; 38 (95%) were located near the terminal end of the long arm of a small C-group (chr. 8-12) chromosome. To determine the specific chromosome and band, cells were G-banded by fluorescence plus Giemsa [16] techniques, and photographs of

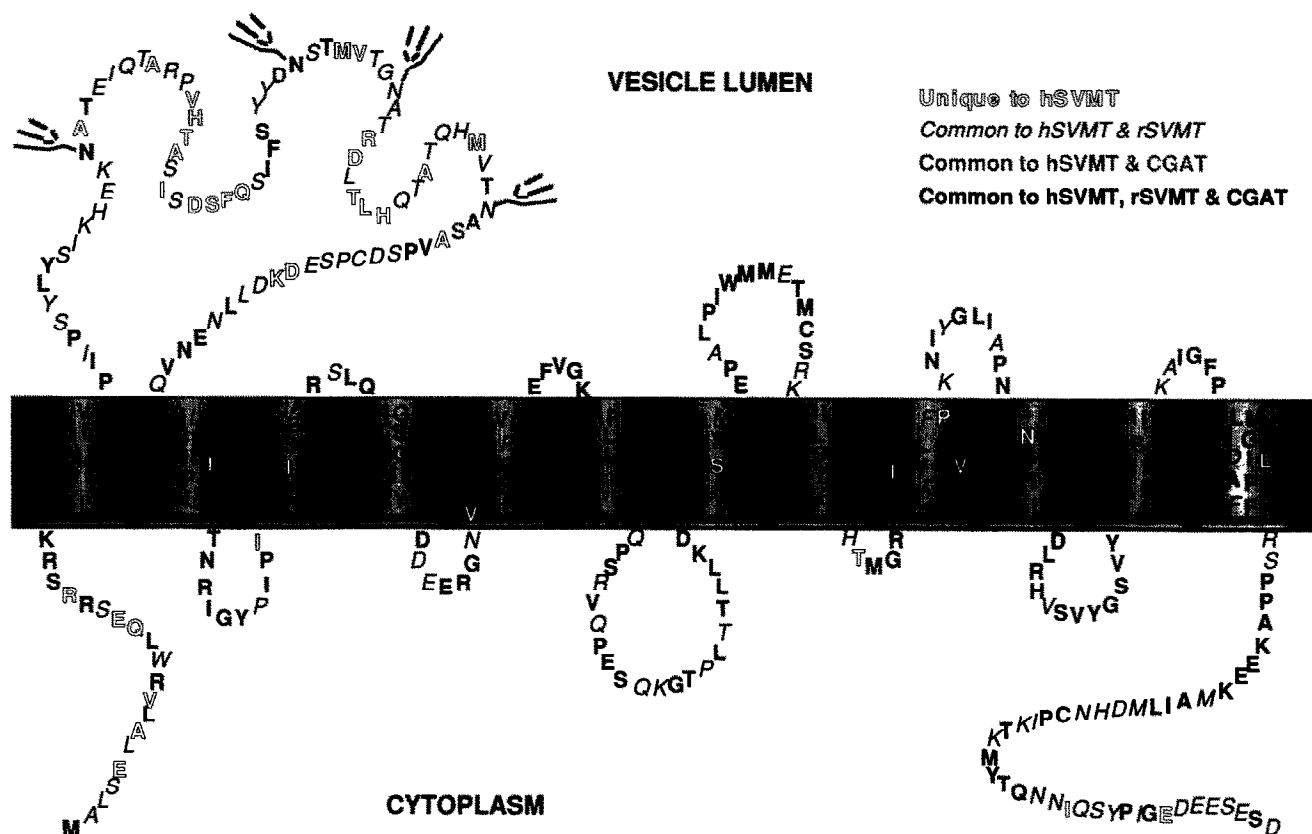


Fig. 2. Schematic representation of the hSVMT showing proposed orientation in the vesicular membrane and conservation of amino acid residues among vesicular monoamine transporters. Bold letters, residues common to human and rat SVMTs and the chromaffin granule amine transporter (CGAT; [1]); hatched letters, residues common to hSVMT and rSVMT; italic letters, residues common to hSVMT and CGAT; open letters, residues unique to hSVMT. Consensus glycosylation sites are indicated by forks.

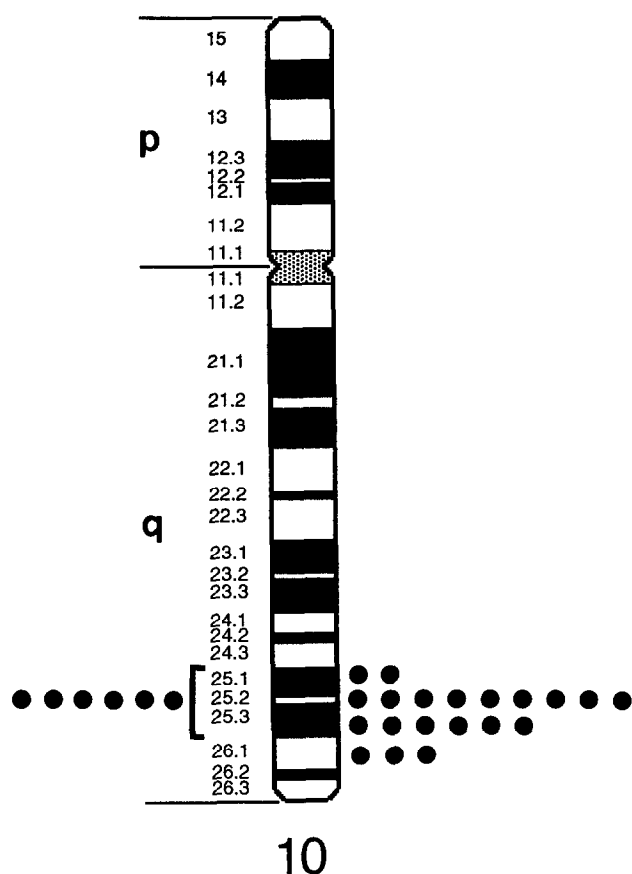


Fig. 4. Ideogram of human chromosome 10 showing localization of hSVMT cDNA to band 10q25. Each dot represents a single paired signal seen on banded chromosome 10 after hybridization. Signals plotted to the right of the ideogram were located on subbands 10q25.1–26.1, those to the left (bracket) were located on 10q25 where band could not be subdivided.

banding patterns aligned with photographs of the fluorescence in situ hybridization signals to determine sub-band location. 26 of 30 analyzable metaphases (87%) were on chromosome 10, largely on band 10q25 (Fig. 4).

3.3. Restriction fragment length polymorphism analysis

The *Taq* 1000 fragment excised from phSVMT1 revealed at least 5 different digestion patterns in *Taq*I-digested DNAs. The most striking polymorphism produced bands of approximately 7.8 or 6 kb, termed A1 and A2, respectively (Fig. 3). Fig. 3A demonstrates homozygous A1 individuals (lanes 3,5 and 6), A1/A2 heterozygotes (lanes 1 and 2), and an A2 homozygote (lane 4). Examination of 394 chromosomes revealed an A1 frequency of 0.71 and an A2 frequency of 0.29. Values in the 197 individuals surveyed showed little deviation from the Hardy-Weinberg equilibrium: 48% were A1/A1, 46% A1/A2, and 6% A2/A2. Caucasians and blacks exhibited 0.70 and 0.77 A1 frequencies, respectively, revealing no evidence of racial differences in

allele frequencies ($P=0.17$, Fisher's exact test). A second *Taq*I polymorphism also appears among the remaining lower molecular weight bands (lanes 2–4, Fig. 3B). Since some of these patterns appear to be rare (e.g. the lane 2 pattern occurs only once among the 197 individuals tested), further analyses will be required to assess their true population frequencies.

4. DISCUSSION

4.1. A human cDNA contains an open reading frame possessing high homology to the rat brain SVMT

The high homology of the sequence identified here with the rat monoamine transporter argues strongly that the cDNA identified here encodes a human vesicular monoamine transporter. The open reading frame predicted by this cDNA encodes 514 amino acids, compared to 515 amino acids for the rat sequence [1,2]. The human and rat proteins are predicted to be 92% identical, while no significant homology is observed between this human cDNA and any of the plasma membrane transporter cDNAs [13,19–22]. Each of these features enhances confidence that this cDNA encodes a human vesicular monoamine transporter. Although the possibility that other cDNAs could encode molecules with similar function remains open, the cloning of the same rat cDNA by three different laboratories and the high homology of this human cDNA does provide evidence against the existence of an abundant, closely related mRNA species that encodes a different SVMT.

4.2. Structural features and post-translational modifications predicted for human and rat SVMTs

The rat SVMT protein has been tentatively assigned the 12 transmembrane (TM) domain structure characteristic of many transporter proteins, although assignment of the exact boundaries of each TM region may have some uncertainty. We have employed Chou-Fasman hydrophobicity analysis [23] and delineated many TM–cytoplasm and TM–lumen interfaces by charged residues, especially positively charged residues. We have minimized charged residues in putative TM regions and imposed a size constraint of 19–26 amino acids for membrane traversal (Fig. 2). The putative TM domains assigned in this fashion reveal remarkable conservation between human and rat brain SVMTs; only one nonconservative change (Asn-395 (human) vs. Ile-396 (rat)) in TM 10 distinguishes the two. The hSVMT displays five negatively-charged aspartic acid residues in predicted TM regions, while lysine-138 represents the lone TM region positive charge. The presence of aspartic acid residues in a TM domain is a common motif among monoaminergic receptors and neurotransmitter transporters; site-directed mutagenesis studies suggest that TM aspartic acids can be essential for ligand recognition and/or transport [24,25]. The 3rd and 4th SVMT TM domains also display serine clusters (TMs 3 and 4),

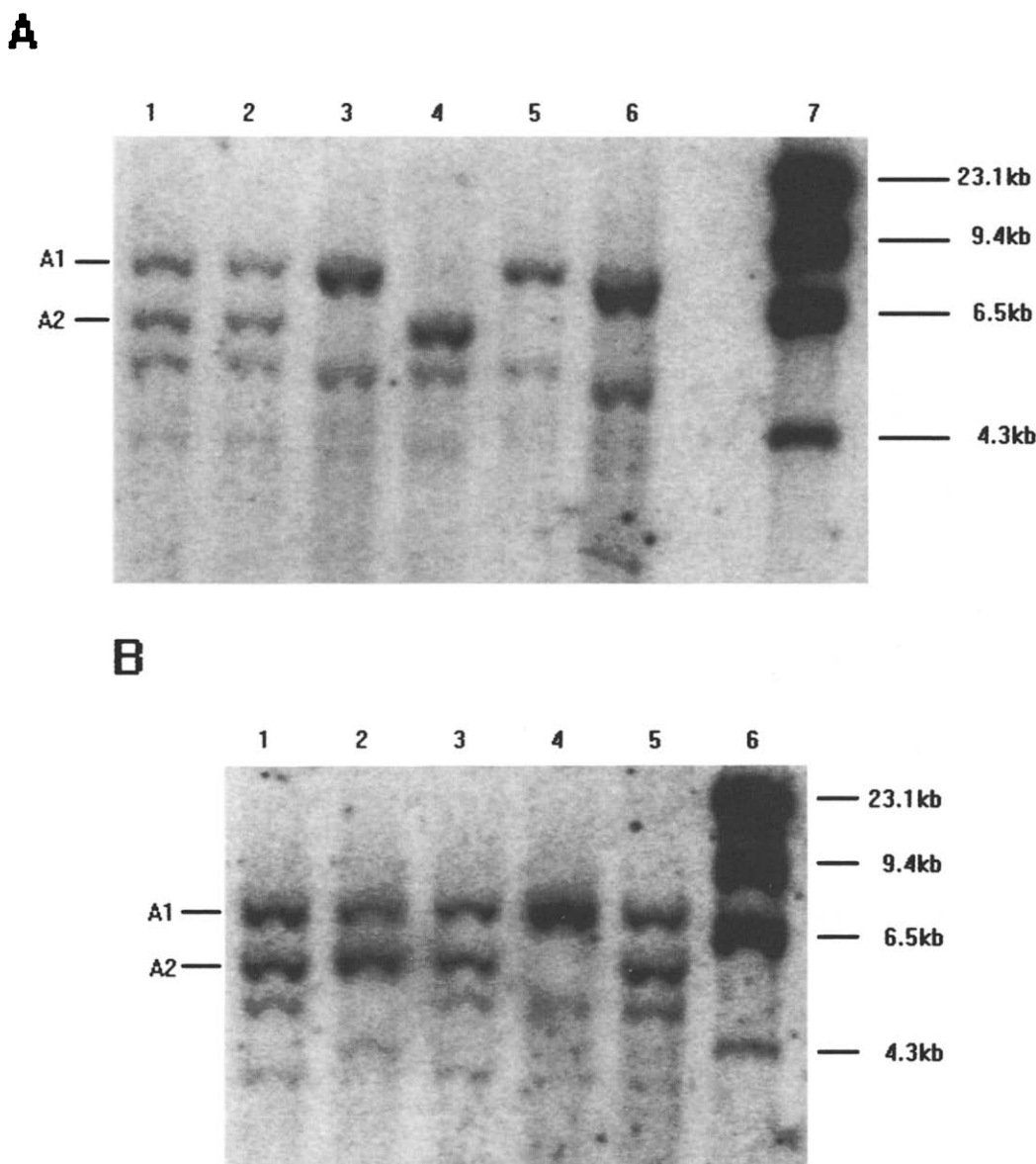


Fig. 3. *TaqI* RFLP patterns of DNAs probed with the hSVMT cDNA *Taq* 1000 fragment. The positions of A1 and A2 bands are indicated, as well as sizes of lambda DNA/*HindIII* molecular weight standards.

a feature shared by the plasma membrane receptors and transporters in which mutagenesis has suggested that they may be important for ligand recognition and/or transport [24,25].

The rat/human sequence differences in extra-membranous segments lead to differences in the two proteins' total charges. The human protein displays a net -1 charge and an isoelectric point of 5.68 while the net +2 charge of the rat protein corresponds to an isoelectric point of 6.17 [15].

The large, presumably intraluminal loop positioned between TM domains 1 and 2 contains differences between the human and rat amino acid sequences. The segment comprised of hSVMT residues 57-112 exhibits

only 59% amino acid identity and contains 4 gaps in which a residue from the human SVMT sequence has no rat counterpart, or vice versa (Fig. 2). This luminal loop region displays sites for asparagine N-linked glycosylation, based on the consensus sequence Asn-Xaa-(Ser,Thr) [26,27]. The human and rat SVMTs are predicted to contain 4 such sites at (hSVMT) residues 56, 84, 91 and 109. A fifth site is expected to be present at residue 80 in the rat sequence, occupying the same relative position as Asp-83 in the human sequence. The fact that 1 less glycosylation site is available in the human sequence may suggest that the extent of glycosylation is reduced in the human protein; however, steric hindrance from the neighboring glycosylated asparagine

residue in the rat sequence may limit the extent of post-translational modification at the 'extra' site. Study of the human and rat proteins will help to elucidate the actual extent and sites of glycosylation.

The human and rat SVMTs also differ with respect to potential sites for phosphorylation by protein kinase C (PKC). Four sites for the consensus sequence (Ser,Thr)-Xaa-(Arg,Lys) [28,29] are found in putative intracellular segments of the human sequence (Ser-15, Ser-18, Thr-154 and Ser-281), while the Ser-15 is in the nonconsensus Ser-Arg-His context in the rat sequence and may be a poorer PKC recognition site. Both rat and human SVMTs share a fifth potential PKC site at Thr-497 (Thr-Gln-Asn), 2 protein kinase A recognition sites at Ser-18 and Ser-417 and 2 casein kinase II sites at Thr-213 and Ser-511.

4.3. RFLP patterns and chromosomal localization of the hSVMT gene

Conceivably, modifications affecting the expression or regulation of the SVMT gene could confer susceptibility to drug abuse or neuropsychiatric disorders. We therefore screened DNAs from almost 200 individuals for a *TaqI* RFLP pattern that help to assess possible gene polymorphisms that could serve as markers for associated human disorders [18]. At least two distinct polymorphisms were detected (Fig. 3), allowing the hSVMT cDNA to provide such a potential probe.

The chromosomal location of this gene to 10q25 places it in the vicinity of chromosomal breaks identified in some patients with cutis verticis gyrata with mental deficiency [30,31], although no clearly relevant disorders have been mapped to this locus to date [31].

4.4. Implications for the possible SVT gene family

Several features of the present results suggest that the size of the family of synaptic vesicular transporters may well be smaller than the size of the family of sodium-dependent plasma membrane neurotransmitter transporter gene family [32]. Each of the positively-hybridizing clones isolated from both the rat and human cDNA libraries contained a portion of the same SVMT cDNA. Other members of the vesicular transporter family have been identified on pharmacologic grounds, including a vesamicol-sensitive acetylcholine transporter [33]. Future cloning studies will identify the exact size of this possible gene family with more precision.

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