Methods in Enzymology
Volume 296
(B)

Methods in Enzymology

Volume 296

NEUROTRANSMITTER TRANSPORTERS

METHODS IN ENZYMOLOGY

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Expression Systems and Molecular Genetic Approaches

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[28] Homologies and Family Relationships among Na^+/Cl^- Neurotransmitter Transporters

By HOLGER LILL and NATHAN NELSON

Introduction

Synaptic transmission involves the release of a neurotransmitter into the synaptic cleft, interaction with the postsynaptic receptor, and subsequent removal of the transmitter from the cleft. The majority of transmitters are removed from the cleft by rapid sodium-dependent uptake systems in the plasma membrane of the presynaptic cells and their surroundings. These reuptake systems are catalyzed by transporters specific for the various neurotransmitters in the brain. A defining moment in the advancement of our knowledge in neurotransmitter transport came in 1990 when the first cDNAs encoding neurotransmitter transporters were cloned and sequenced.[1–8] A family of transporters with a common structure of presumably 12 transmembrane helices has been defined. Sequence analysis revealed that this family of transporters contains four subfamilies of monoamine, γ-aminobutyric acid (GABA), amino acid, and "orphan" (NTT4) transporters.[9]

GABA is a major inhibitory neurotransmitter in the mammalian brain and is widely distributed throughout the nervous system.[10–12] Molecular cloning studies have resulted in the isolation and characterization of

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[2] H. Nelson, S. Mandiyan, and N. Nelson, *FEBS Lett.* **269,** 181 (1990).
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cDNAs encoding four different GABA transporters, GAT1, GAT2, GAT3, and GAT4,[13] as well as creatine,[14] betaine,[15] and taurine[16,17] transporters.

The subfamily of monoamine transporters contains the transporters of noradrenaline, serotonin, and dopamine.[9,18] Cloning of a cDNA encoding a noradrenaline transporter revealed that all the fundamental properties of noradrenaline uptake in the brain are encoded by this single cDNA species.[3] Dopamine transporter appears to be the most important site for the behavioral effects of amphetamines and cocaine.[19,20] The identity between the GABA and noradrenaline transporters was used for designing degenerative oligonucleotide probes corresponding to the regions of greatest identity. The attempt resulted in the isolation of a cDNA clone encoding the serotonin transporter in rat brain.[8] Simultaneously, using expression cloning, the serotonin transporter from rat basophilic leukemia cells was also cloned.[6] Expression of both cDNAs in nonneuronal cells generated sodium-dependent serotonin uptake ability which was sensitive to antidepressants.

The subfamily of amino acid transporters contains the transporters of glycine and proline. The amino acid glycine is a classical inhibitory neurotransmitter localized in the spinal cord, brain stem, and retina.[21] cDNAs encoding the amino acid transporters of glycine (GLYT1 and GLYT2) and proline were cloned and sequenced.[22-26] GLYT2 differs from GLYT1 in

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molecular structure, tissue specificity, and pharmacological properties. The cDNA of GLYT2 encodes for 799 amino acid residues with an extended amino-terminal peptide of 200 amino acids containing potential phosphorylation sites for protein kinase C, cAMP-dependent kinase, and calmodulin-dependent kinase.[26] Although a specific proline receptor has not yet been identified, it was argued that this amino acid also functions as a neurotransmitter.[25] It was shown that radiolabeled L-proline is released from brain slices and synaptosomes in a Ca^{2+}-dependent manner following K^+-induced depolarization.[27] These and other experimental findings supported a synaptic role for L-proline in specific excitatory pathways in the brain.

The subfamily of "orphan" (NTT4) transporters consists of four gene products that appear to encode transporters which differ structurally from the three other subfamilies.[28,29] They display large second and fourth extracellular domains with sites for N-linked glycosylation in both large domains. Their function is not known and attempts to identify their substrates thus far have been unsuccessful.[30]

Genomic cloning of genes encoding neurotransmitter transporters and search for sequences without known function in the GenBank, revealed that insects and *Caenorhabditis elegans* contain genes encoding potential neurotransmitter transporters.[31] Therefore, it was assumed that this family of transporters evolved from a common ancestor about 1 billion years ago.[9] We have identified and cloned a bacterial gene with relatively high homology to neurotransmitter transporters.[32] This gene is present in a potential tryptophan operon of *Symbiobacterium thermophilum* that contains the gene encoding the enzyme tryptophanase. The entire genomic sequences of *Haemophilus influenzae* and *Methanococcus jannaschii* have

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[31] Q.-R. Liu, S. Mandiyan, H. Nelson, and N. Nelson, *Proc. Natl. Acad. Sci. USA* **89,** 6639 (1992).

[32] H. Nelson, S. Mandiyan, S. Horinouchi, and N. Nelson, in preparation (1998).

been determined.[33,34] Each genome contains a single gene homologous to the mammalian family of neurotransmitter transporters. These findings indicate that the family of neurotransmitter transporters is rooted to the onset of life and is present not only in eukaryotes, but also in *Eubacteria* and Archaea.

Evolutionary trees were constructed for the genes that were available at the time of the particular studies. Moreover, an attempt was made to calculate evolutionary trees for individual exons in the various transporters of mammalian origin.[9] Here we describe the construction of evolutionary trees that include the various mammalian transporters together with the recently discovered bacterial transporters.

Construction of Evolutionary Trees of Neurotransmitter Transporters

All sequences used in the evolutionary calculations are available on the Internet (GenBank at http://www2.ncbi.nlm.nih.gov/genbank, EMBL

[33] R. D. Fleischmann, M. D. Adams, O. White, R. A. Clayton, E. F. Kirkness, A. R. Kerlavage, C. J. Bult, J.-F. Tomb, B. A. Dougherty, J. M. Merrick, K. McKenney, G. Sutton, W. FitzHugh, C. A. Fields, J. D. Gocayne, J. D. Scott, R. Shirley, L.-I. Liu, A. Glodek, J. M. Kelley, J. F. Weidman, C. A. Phillips, T. Spriggs, E. Hedblom, M. D. Cotton, T. R. Utterback, M. C. Hanna, D. T. Nguyen, D. M. Saudek, R. C. Brandon, L. D. Fine, J. L. Fritchman, J. L. Fuhrmann, N. S. M. Geoghagen, C. L. Gnehm, L. A. McDonald, K. V. Small, C. M. Fraser, H. O. Smith, and J. C. Venter, *Science* **269,** 496 (1995).

[34] C. J. Bult, O. White, G. J. Olsen, L. Zhou, R. D. Fleischmann, G. G. Sutton, J. A. Blake, L. M. FitzGerald, R. A. Clayton, J. D. Gocayne, A. R. Kerlavage, B. A. Dougherty, J.-F. Tomb, M. D. Adams, C. I. Reich, R. Overbeek, E. F. Kirkness, J. F. Weidman, J. L. Fuhrmann, D. Nguyen, T. R. Utterback, J. M. Kelley, J. D. Peterson, P. W. Sadow, M. C. Hanna, M. D. Cotton, K. M. Roberts, M. A. Hurst, B. P. Kaine, M. Borodovsky, H.-P. Klenk, C. M. Fraser, H. O. Smith, C. R. Woese, and J. C. Venter, *Science* **273,** 1058 (1996).

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[39] K. E. Smith, S. G. Fried, M. M. Durkin, E. L. Gustafson, L. A. Borden, T. A. Branchek, and R. L. Weinshank. *FEBS Lett.* **357,** 86 (1995).

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TABLE I
SEQUENCES USED FOR EVOLUTIONARY CALCULATIONS

Abbreviation	Name	Substrate	Accession	Refs.
BETAT-D	Dogncbta	Betaine	M80403	15
CRETR-R	Cretr	Creatine	X67252	14, 35
DOPAT-B	Bovdopatr	Dopamine	M80234	7
DOPAT-H	Humdoptra	Dopamine	M95167	36
DOPAT-R	Ratdoper	Dopamine	M80570	4
GABAT1-H	Hsgat1mr	γ-Aminobutyrate	X54673	2
GABATI-M	Gat1	γ-Aminobutyrate	M92378	31
GABAT2-M	Gat2	γ-Aminobutyrate	M97632	42
GABAT3-M	Gat3	γ-Aminobutyrate	L04663	13
GABAT4-M	Gat4	γ-Aminobutyrate	L04662	13
GABAT4-R	Rat3gat	γ-Aminobutyrate	M95763	37
GLYTI-M	Glyt1	Glycine	X67056	22
GLYT2-R	Glyt2	Glycine	L21672	26
HAEM1		?	U32703	33
METHAN1		?		34
NORAT-B	Ntt1	Noradrenaline	U09198	41
NORAT-H	Humnortr	Noradrenaline	M65105	3
NTT4	Ntt4r	?	S52051	29
NTT73	Ntt73	?	L22022	28
NTTB21	rB21a	?	S76742	39
PROT-R	Protr	Proline	M88111	25
ROSI-T	ROSIT	?	U12973	40
SEROT-R	Rsertran	Serotonin	X63253	8
STRYP1	Sat1	?	—	32
TAUT-D	Dognacltau	Taurine	M95495	16
TAUT-M	Taurt	Taurine	L03292	17
TAUT-R	Ratttransp	Taurine	M96601	38

at http://www.ebi.ac.uk, SwissProt at http://expasy.hcuge.ch). In Table I, we compiled the names, synonyms, substrates, and accession numbers of all the sequences employed, if available. Prior to the evolutionary calculations, the protein sequences have been aligned by means of the programs PILEUP and LINEUP of the GCG package.[43] Three regions, showing a relatively high degree of similarity between all sequences and only few gaps in the alignment have been chosen for further analysis (Fig. 1). The respective DNA sequences were aligned and then run with evolutionary tree building software of the PHYLIP package developed by Felsenstein[44] on a DEC 3000 workstation under OpenVMS ALPHA 1.5. The DNAML program

[43] J. Devereux, P. Haeberli, and O. Smithies, *Nucleic Acid Res.* **12,** 387 (1984).
[44] J. Felsenstein, "PHYLIP 3.2 Manual." University of California Herbarium, Berkeley, 1989.

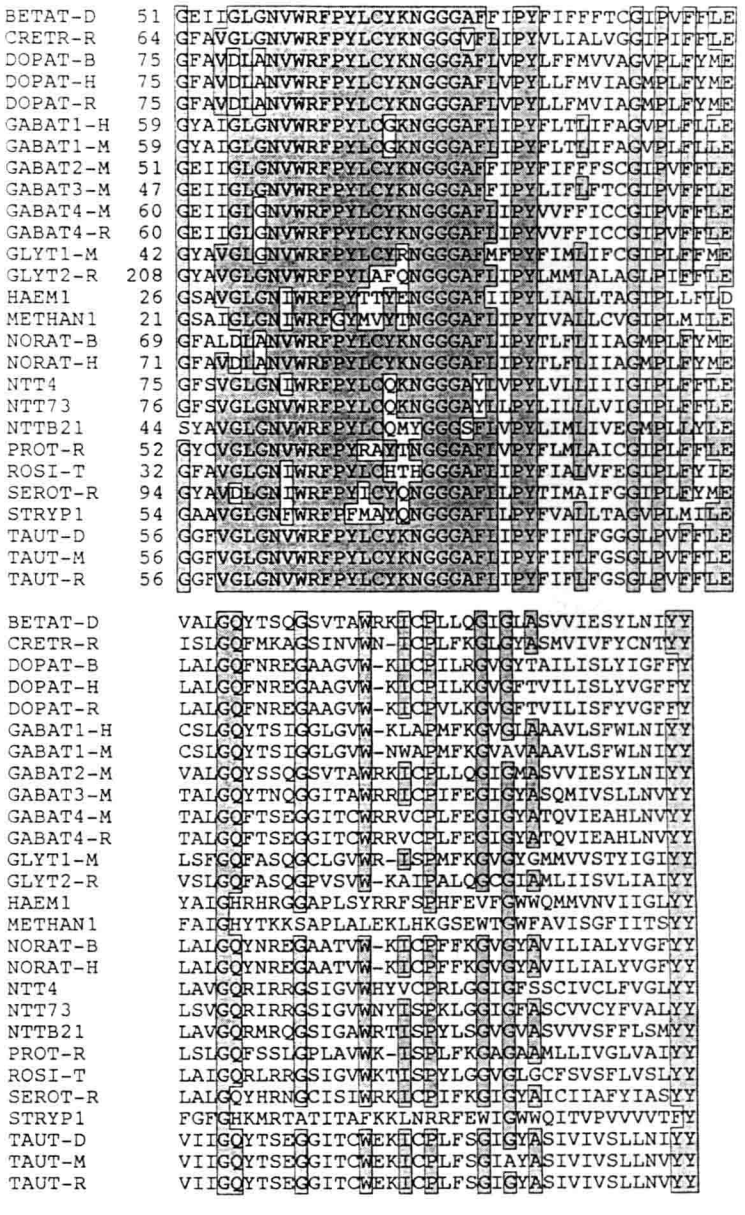

FIG. 1. Amino acid sequence alignment of the three regions in the various neurotransmitter transporters used for calculating the evolutionary trees. The abbreviations of the various transporters are listed in Table I. The source of some transporters is indicated by the last letter: D, dog; H, human; B, bovine, R, rat; M, mouse. HAEM1 is a gene of the *Haemophilus influenzae* genome,[33] MRTHAN1 is a gene of the *Methanococcus jannaschii* genome,[34] and STRYP1 is a gene of the *Symbiobacterium thermophilum* genome.[32]

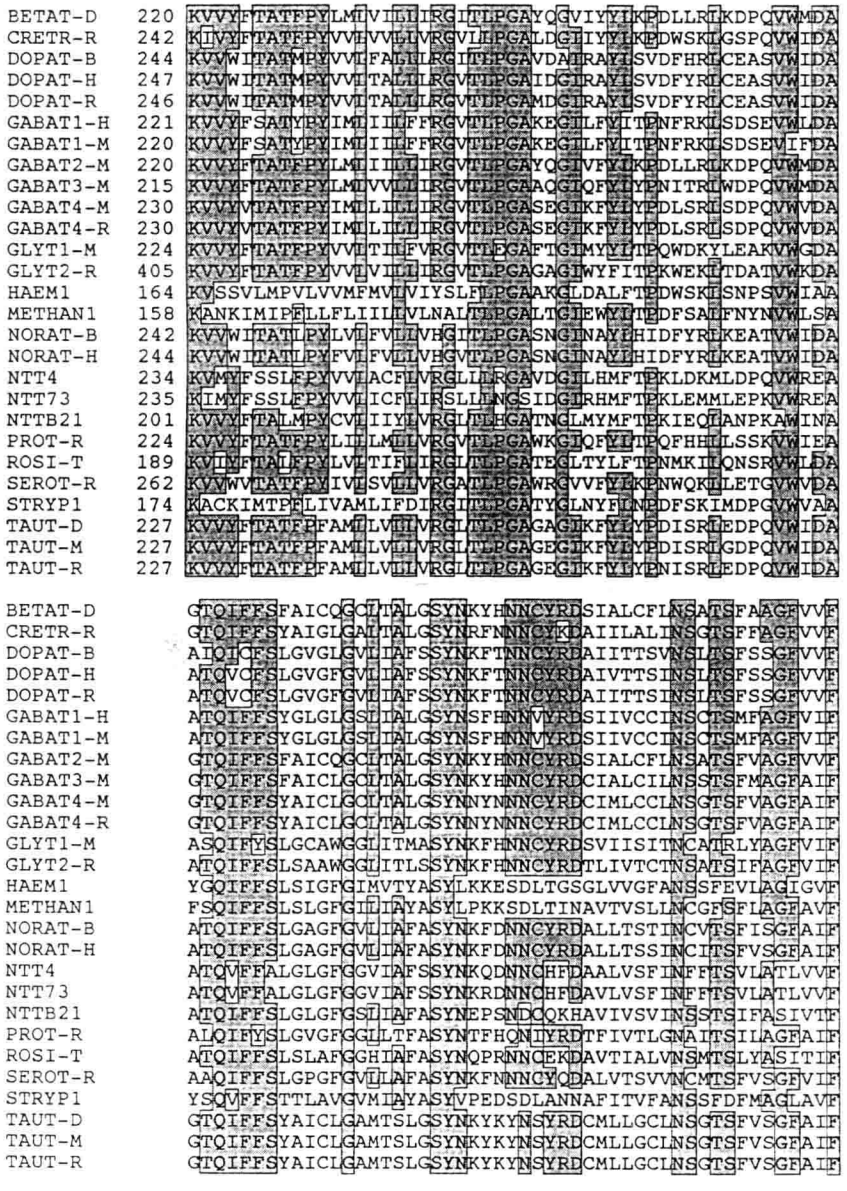

FIG. 1. (*continued*)

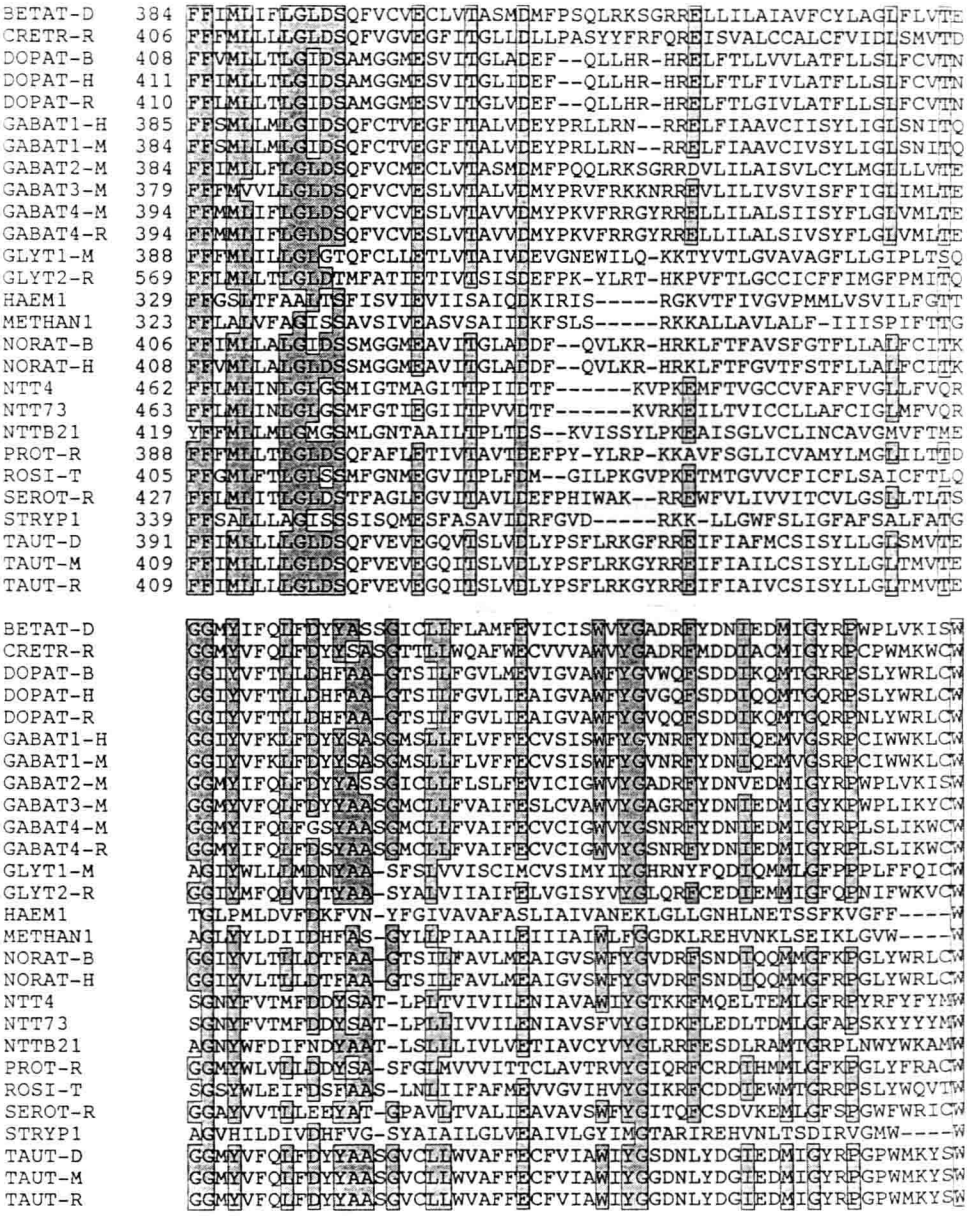

FIG. 1. (*continued*)

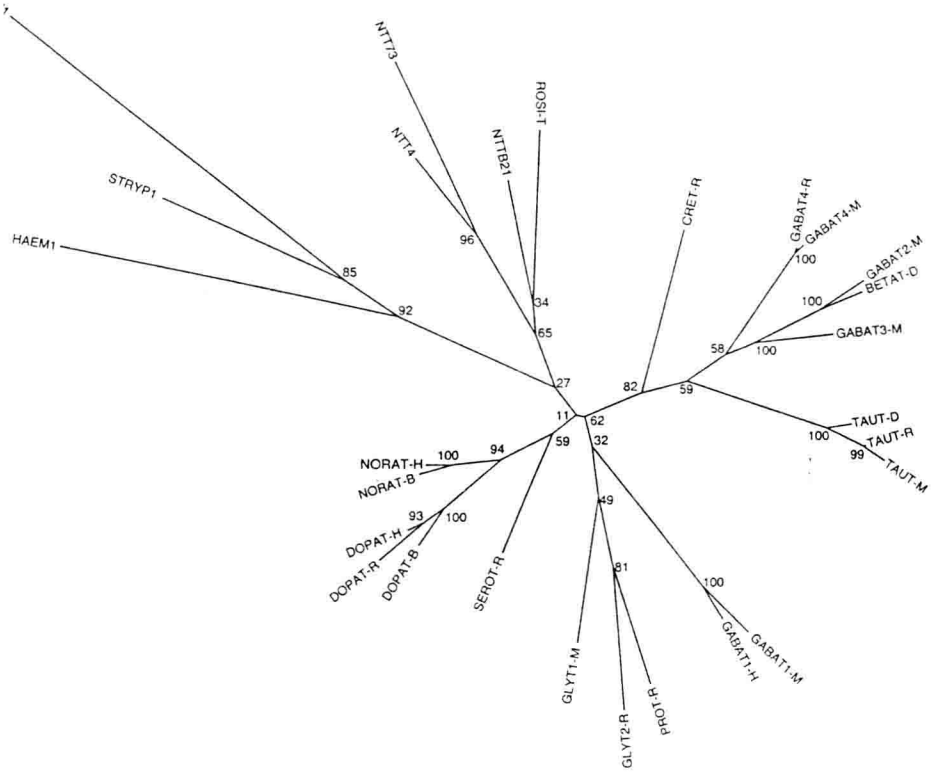

FIG. 2. Three evolutionary trees constructed from the amino acid sequences shown in Fig. 1.

included in the package builds trees according to the maximum likelihood algorithm[45] with branch lengths proportional to the relative number of base changes. Eight runs of the program were carried out on each dataset, always jumbling the input order of the sequences according to random numbers and thereby avoiding the possibility of introducing artifacts by an unfavorable order of sequence input. After finishing a run, the program was directed to perform global rearrangements of the trees found (i.e., to remove subtrees from the tree and put them back on in all possible ways so as to have a better chance of finding an optimal tree). Out of the eight results, the tree with the best likelihood score was finally selected. We also performed a statistical analysis on the same data sets. The sequences were first bootstrapped by the SEQBOOT program, creating 100 new data sets by sam-

[45] J. Felsenstein, *J. Molecular Evolution* **17**, 368 (1981).

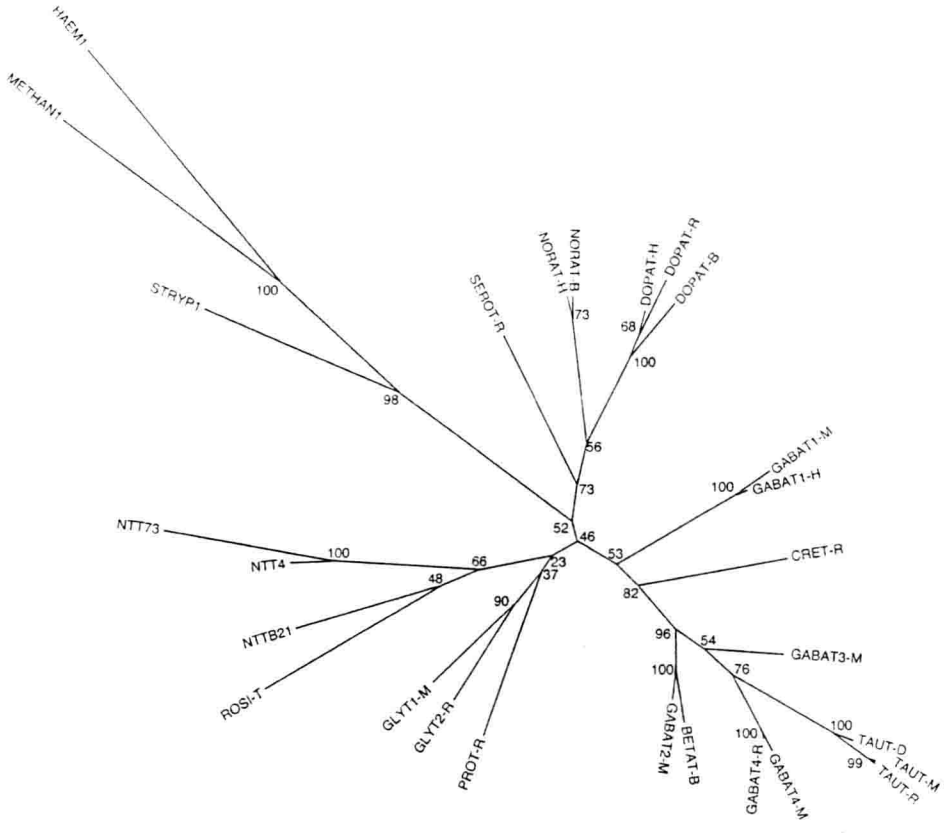

FIG. 2. (*continued*)

pling the characters randomly with replacement. The resulting data sets have the same size as the original, but some characters have been left out and others have been duplicated. The random variation of the results from analyzing these bootstrapped data sets can be shown statistically to be typical of the variation that one would get from collecting new data sets.[46] The 100 sets were used as input to DNAPARS, a program calculating evolutionary trees according to the parsimony algorithm.[47] The 100 trees resulting from the DNAPARS runs were used as input to the CONSENSE program to calculate one consensus tree and to examine the statistical relevance of the branchings. We were pleased to learn that the trees showed

[46] J. Felsenstein, *Evolution* **39,** 783 (1985).
[47] A. G. Kluge and J. S. Farris, *Systematic Zoology* **18,** 1 (1969).

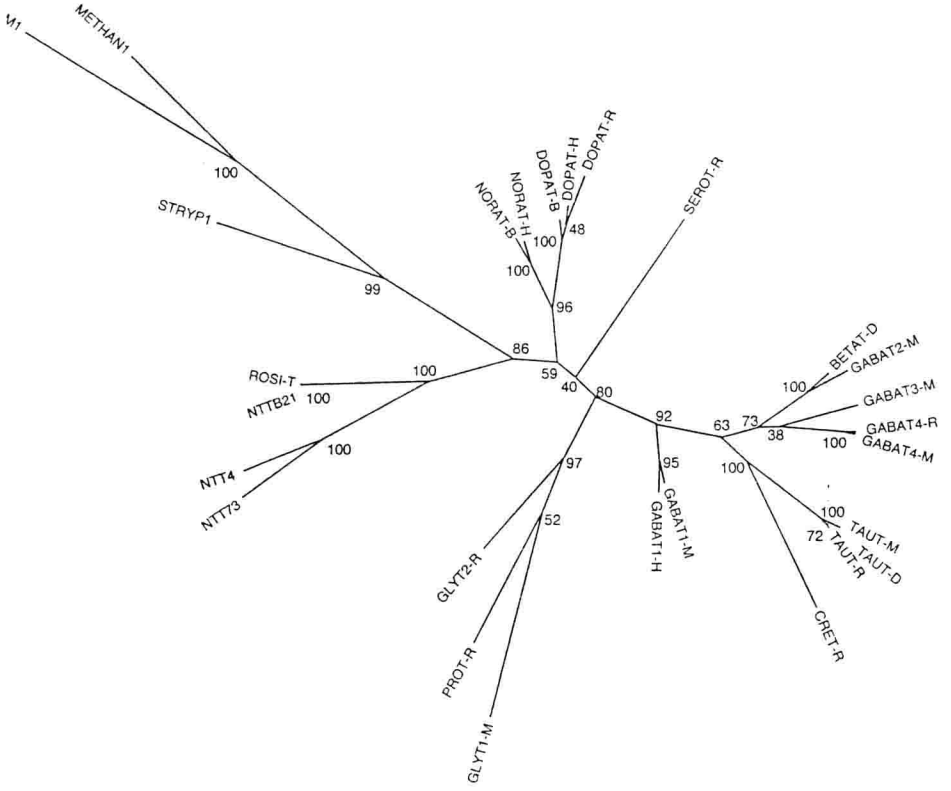

FIG. 2. (*continued*)

very high similarity. Only minor differences occurred in the length of certain branches of trees calculated by the maximum likelihood method, and the six trees obtained with the maximum likelihood as well as with the bootstrapping method differed only slightly in the innermost branching order. The trees obtained with the three fragments are shown in Fig. 2. The branch lengths were taken from the maximum likelihood analysis and show the relative genetic diversity between species. The numbers depict the results of the consensus analysis, indicating how many times the group which consists of the species to the right of the fork occurred.

By dividing the various transporter sequences into three fragments that contain the most homologous sequences, not only did we put the method to a rigorous test, but we also obtained additional insight into the relations among the various transporters. In general, the four subfamilies of neurotransmitter transporters are maintained. This is quite remarkable because

three bacterial transporters were added to the calculation of the evolutionary trees. The bacterial transporters are loosely grouped with the "orphan" (NTT4) transporters. The only striking deviation was recorded with the GABA transporter GAT1, which in fragment 1 is grouped with the amino acid transporters of glycine and proline. It should be noted that the amino acid sequence of GAT1 is the most distant of all the other members of the subfamily of GABA transporters. This property of GAT1 is in line with its substrate specificity, which is unique among all the other members of this subfamily.

[29] Vaccinia Virus–T7 RNA Polymerase Expression System for Neurotransmitter Transporters

By SUE L. POVLOCK and SUSAN G. AMARA

Introduction

Biochemical analyses of transport activities in heterogeneous preparations such as brain slices, synaptosomes, and plasma membrane vesicles have been complicated by the presence of multiple carrier subtypes and the frequent overlap of their substrate specificities. Furthermore, the low abundance of many of the carrier subtypes and the difficulties encountered in protein purification have made efforts at isolating these activities a challenging undertaking. Ligand affinity and binding techniques have contributed to the successful purification of at least one transporter, the human platelet plasma membrane serotonin transporter (SERT).[1] Transporters for which high-affinity binding ligands are unavailable have required more difficult reconstitution assays for purification. Carriers purified by this approach include a rat brain γ-aminobutyric acid (GABA) transporter,[2] a porcine brain stem glycine transporter,[3,4] and a rat glial glutamate transporter.[5] As a way of circumventing the difficulties associated with the purification of carrier proteins, the cloning of genes encoding neurotransmitter transporters not only has offered insight into the diversity of carrier

[1] J.-M. Launay, C. Geoffroy, V. Mutel, M. Buckle, A. Cesura, J. E. Alouf, and M. Da Prada, *J. Biol. Chem.* **267,** 11344 (1992).
[2] R. Radian, A. Bendahan, and B. I. Kanner, *J. Biol. Chem.* **261,** 15437 (1986).
[3] B. Lopez-Corcuera, J. Vazquez, and C. Aragon, *J. Biol. Chem.* **266,** 24809 (1991).
[4] C. Aragon and B. Lopez-Corcuera, *Methods Enzymol.* **296,** [1], (1998) (this volume).
[5] N. C. Danbolt, G. Pines, and B. I. Kanner, *Biochemistry* **29,** 6734 (1990).

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subtypes, but also has allowed new approaches for examining their functional properties and pharmacological specificities.

The availability of cDNAs encoding neurotransmitter carriers offers the advantages of high level expression of single activities in heterologous cells, in the absence of vesicular storage compartments and free from the confounding influences of other transport pathways. A variety of heterologous expression systems have proven useful in characterizing neurotransmitter transporters. *Xenopus laevis* oocytes have been used both to study activities encoded by endogenous brain mRNAs and to identify and characterize transporter cDNAs by functional expression. To facilitate the investigations into the basic structural and functional properties of transporter proteins, cDNAs have been stably transfected into cell lines such as HeLa, LLC-PK_1 (porcine kidney), COS (monkey kidney), and MDCK (Madin–Darby canine kidney) using a variety of standard transfection and selection techniques. For transient expression of cloned transporter cDNAs, mammalian cell systems such as COS cells[6] offer significant advantages over *Xenopus laevis* oocytes in terms of ease and reproducibility. As will be considered in this chapter, a vaccinia virus/T7 polymerase expression system has also been used as a convenient system for characterizing cloned transporter cDNAs.[7–12]

The vaccinia virus/T7 polymerase expression system has been used to direct the expression of cDNAs encoding a variety of cellular genes in mammalian cells. In contrast to the more standard vaccinia virus expression systems, it does not require the generation of recombinant vaccinia virus containing the gene of interest. The only prerequisite is that the gene must be inserted into a plasmid vector under the control of a T7 promoter. The strain of vaccinia used in this approach has been engineered to express T7 RNA polymerase,[13] and when a cDNA driven by the T7 promoter is transfected into cells infected with this virus, the encoded protein is expressed at high levels. Vaccinia virus replicates in the cytoplasm and has a relatively broad host range, allowing cDNAs to be expressed in a variety of cellular environments. Furthermore, the gene of interest is transcribed

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[8] T. Pacholczyk, R. D. Blakely, and S. G. Amara, *Nature* **350,** 320 (1991).
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[10] J. Kilty, D. Lorang, and S. G. Amara, *Science* **254,** 578 (1991).
[11] K. J. Buck and S. G. Amara, *Proc. Natl. Acad. Sci. USA* **91,** 12584 (1994).
[12] T. T. Nguyen and S. G. Amara, *J. Neurochem.* **67,** 645 (1996).
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