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Molecular cloning of a putative vesicular transporter for acetylcholine

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ABSTRACT **Classical neurotransmitters such as acetylcholine (ACh) require transport into synaptic vesicles for regulated exocytotic release. The *Caenorhabditis elegans* gene *unc-17* encodes a protein with homology to mammalian transporters that concentrate monoamine neurotransmitters into synaptic vesicles. Mutations in *unc-17* protect against organophosphorus toxicity, indicating a role in cholinergic neurotransmission. Using the relationship of unc-17 to the vesicular amine transporters, we first isolated a related sequence from the electric ray *Torpedo californica* [*Torpedo* vesicular ACh transporter (TorVAChT)] that is expressed by the electric lobe but not by peripheral tissues. Using the relationship of the *Torpedo* sequence to unc-17, we then isolated the cDNA for a rat homologue (rVAChT). Northern blot analysis shows expression of these sequences in the basal forebrain, basal ganglia, and spinal cord but not cerebellum or peripheral tissues. *In situ* hybridization shows expression of rVAChT mRNA in all cholinergic cell groups, including those in the basal forebrain, brainstem, and spinal cord that previously have been shown to express choline acetyltransferase mRNA. The human VAChT gene also localizes to chromosome 10 near the gene for choline acetyltransferase. Taken together, these observations support a role for rVAChT in vesicular ACh transport and indicate its potential as a novel marker for cholinergic neurons.**

Neurotransmitters are stored in synaptic vesicles so that their release can be regulated by neural activity. For classical transmitters such as the monoamines and acetylcholine (ACh), synthesis occurs in the cytoplasm. Packaging into vesicles therefore requires specific transport from the cytoplasm into the lumen of the vesicle. Study of the tissue distribution and the competition for transport indicates four distinct vesicular neurotransmitter transport activities, one for monoamines, another for ACh, a third for glutamate, and a fourth for γ-aminobutyric acid and glycine (1, 2).

Using selection in the toxin *N*-methyl-4-phenylpyridinium (MPP^+), we previously isolated the cDNA clones for two vesicular monoamine transporters, VMAT1 and VMAT2 (3, 4). MPP^+ is the active metabolite of the potent neurotoxin *N*-methyl-1,2,3,6-tetrahydropyridine (MPTP) that produces an excellent model of Parkinson disease (5). The transporters appear to protect against MPP^+ toxicity by sequestering the toxin in vesicles, away from its primary site of action in mitochondria (3). The sequence of the VMAT1 and VMAT2 cDNAs predicts proteins with 12 putative transmembrane domains and similarity only to several bacterial antibiotic resistance proteins (4). The two VMATs, one expressed in the adrenal gland and the other in the brain (4, 6, 7), represent the first members of a mammalian gene family that presumably includes the other vesicular neurotransmitter transporters.

Genetic studies in *Caenorhabditis elegans* have identified a putative vesicular ACh transporter (VAChT) (8). Mutations in the gene *unc-17* protect against the toxicity of organophosphorus compounds, which act by inhibiting the degradative enzyme acetylcholinesterase and so result in lethal synaptic concentrations of ACh. *unc-17* mutations presumably confer resistance by reducing the efficiency of cholinergic synaptic transmission. Sequence analysis of the unc-17 cDNA predicts a protein with significant homology to the VMATs (8), suggesting that the mutation protects against organophosphorus toxicity by reducing vesicular ACh transport. Localization of the unc-17 protein to cholinergic neurons in *C. elegans* further supported a role for the unc-17 protein in vesicular ACh transport. We now report the isolation of a rat cDNA and its sequence†† and the localization of the human gene to chromosome 10.

MATERIALS AND METHODS

Polymerase Chain Reaction (PCR). One microgram of $poly(A)^+$ RNA from the electric lobe of *Torpedo californica* was reverse-transcribed in 20 μl of 50 mM Tris chloride, pH 8.3/75 mM KCl/3 mM $MgCl_2$/20 mM dithiothreitol/1 mM dNTPs/0.75 μg of RNasin at 37°C for 60 min by using random primers and Moloney murine leukemia virus reverse transcriptase. PCR amplification was carried out in 10 mM Tris chloride, pH 8.3/50 mM KCl/1.5 mM $MgCl_2$/0.3 mM dNTPs containing at 3 μM degenerate primers (5′-GCXYTXYTX-YTXGAXAAXATGYT-3′ and 5′-ZTCXGCPATXGCZT-AXAC-3′, where X = A, C, G, and T; Y = C and T; Z = A and G; and P = A, G, and T) and *Taq* polymerase under the following conditions: 4 min at 94°C and then 35 cycles of 1 min at 94°C, 2 min at 55°C, and 3 min at 72°C. The products were separated by electrophoresis, and a 1.1-kb fragment was subcloned and sequenced on both strands by the method of chain termination (9). For PCR amplification of rat sequences, 1 μg of $poly(A)^+$ rat spinal cord RNA was reverse-transcribed and amplified as above by using the degenerate primers 5′-GCXTTYYTXGAZCCXACXAT-3′ and 5′-AY-XAZXGCPATXCCZAAZCA-3′. This reaction produced a 260-bp fragment that was then excised from a low-melting temperature agarose gel, subcloned, and sequenced.

Northern Blot Analysis. $Poly(A)^+$ RNA was separated by electrophoresis through formaldehyde-agarose; blotted to nitrocellulose; hybridized overnight at 42°C in 50% formamide containing 5× SSPE (0.18 M NaCl/10 mM phosphate,

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Abbreviations: ACh, acetylcholine; VAChT, vesicular ACh transporter; TorVAChT, *Torpedo* VAChT; rVAChT, rat VAChT; ChATase, choline acetyltransferase; VMAT, vesicular monoamine transporter.

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††The rVAChT sequence reported in this paper has been deposited in the GenBank data base (accession no. U09838).

pH 7.4/1 mM EDTA), 5× Denhardt's solution (0.02% Ficoll/0.02% bovine serum albumin/0.02% polyvinylpyrrolidone), 0.5% SDS, and 100 μg of denatured salmon sperm DNA per ml; washed in 0.1× SSPE at 65°C; and exposed to film for 4–12 hr at −70°C with an enhancing screen.

Library Screening. One million plaques from a λgt10 rat brainstem cDNA library were screened with the 260-bp rat spinal cord PCR product by using an aqueous hybridization procedure (10), hybridization at 60°C, and washes at 68°C. The phage were purified, and inserts were subcloned and sequenced on both strands (9).

***In Situ* Hybridization.** The *in situ* hybridization procedure is described in detail elsewhere (11). After hybridization at 55°C with 0.1 ng of strand-specific ^{35}S-labeled UTP-containing rat VAChT (rVAChT) and choline acetyltransferase (ChATase) (12) RNA probes prepared by using phage T3, T7, or SP6 RNA polymerases, the brain sections were washed in 4× SSC (0.15 M NaCl/0.015 M sodium citrate, pH 7) containing 50 mM 2-mercaptoethanol, treated with 50 μg of RNase A per ml for 30 min at 37°C, and washed in 2× SSC at room temperature, then in 0.1× SSC at 60°C, and finally in 0.1× SSC at room temperature overnight. The sections were mounted and exposed to film for 4–15 days.

RESULTS

The sequence of the protein predicted by the unc-17 cDNA provided important information about additional members of the vesicular neurotransmitter transporter family and in particular about putative mammalian VAChTs. To circumvent the problem of low abundance in mammalian tissues, we first used reverse-transcribed poly(A)$^+$ RNA from the electric lobe of *T. californica*. The electric lobe contains numerous cholinergic cell bodies and projects to the electric organ where high levels of ACh transport occur in synaptic vesicles (13–15). PCR amplification of electric lobe cDNA with degenerate oligonucleotide primers based on the amino acid sequences shown in Fig. 1 produced a fragment of the expected size (TorVAChT). Sequence analysis of the subcloned fragment showed substantial homology to unc-17 (Fig. 1). Northern analysis of *Torpedo* tissues showed expression of the RNA by the electric lobe but not by the electric organ target or by the liver (Fig. 2 *Left*), supporting a role in cholinergic neurotransmission.

To identify a mammalian VAChT, we used the relationship between unc-17 and TorVAChT to design additional degenerate oligonucleotide primers relatively specific for the VAChT subfamily. We used reverse-transcribed poly(A)$^+$ RNA from the spinal cord as template, since the ventral horn contains large numbers of cholinergic motor neurons. PCR amplification with degenerate primers based on the sequence shown in Fig. 1 yielded a fragment of the expected size, and sequence analysis of the subcloned product verified the substantial similarity to both unc-17 and TorVAChT. We then used this fragment as a probe to isolate two cDNA clones from a rat brainstem library by hybridization under moderate stringency.

The sequence of the longer cDNA (1.9 kb) predicts a protein (rVAChT) of 530 amino acids with 12 transmembrane domains. Like the vesicular amine transporters, a large lumenal loop is predicted to occur between the first two transmembrane domains (Fig. 3). The primary amino acid sequence shows strong resemblance to both TorVAChT (82% similarity, 70% identity) and unc-17 (69% similarity, 48% identity), with somewhat less but still substantial resemblance to the rat VMATs (≈65% similarity, ≈40% identity). The highest degree of sequence conservation occurs in transmembrane domains 1, 4, and 11, with the large lumenal loop and both N and C termini most divergent. In particular, aspartate residues in transmembrane domains 1, 6, 10, and 11

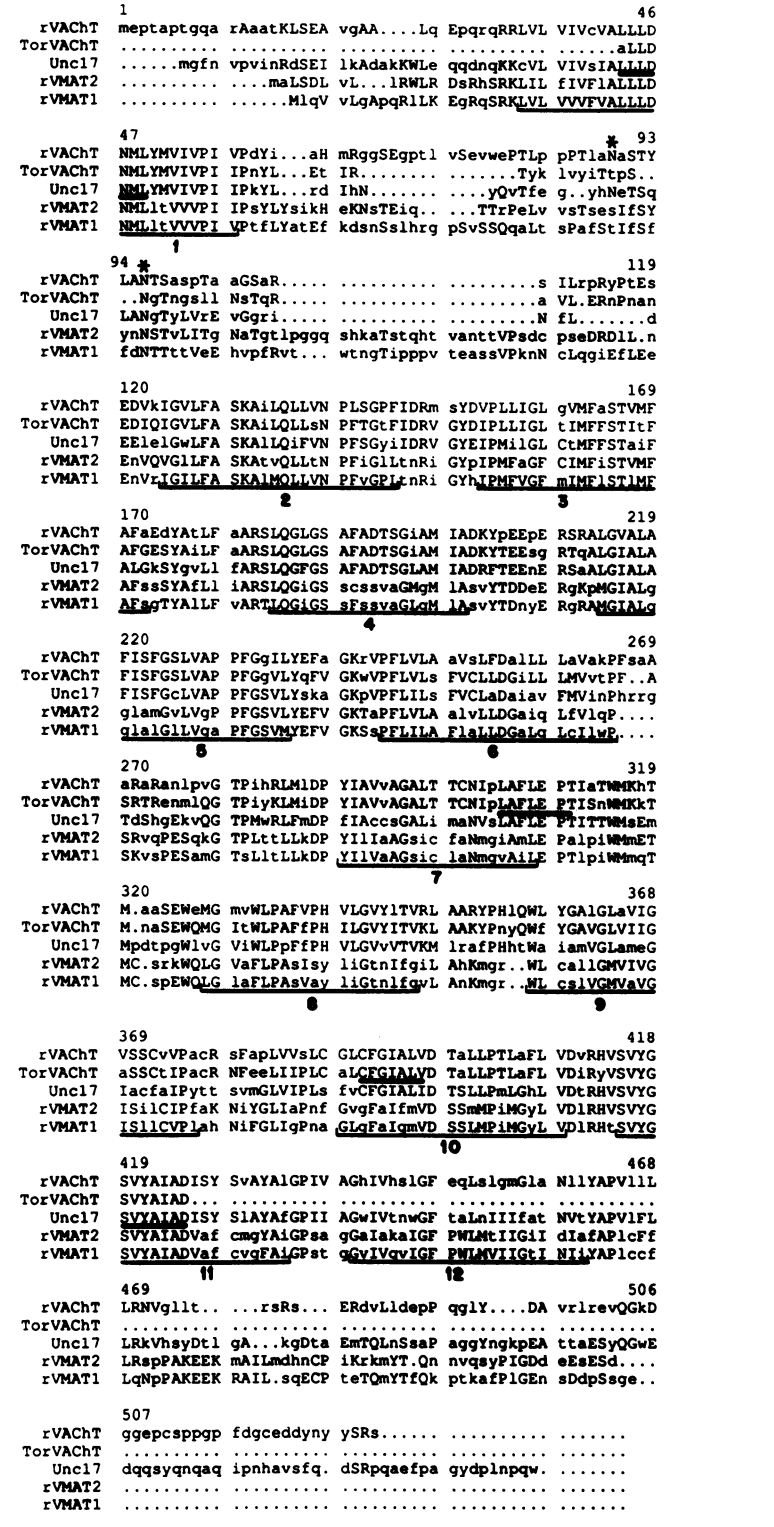

FIG. 1. Homology of the VMATs and putative VAChTs. Alignment of the amino acid sequences for the two rat VMATs (rVMAT1 and rVMAT2) with the putative VAChTs from *C. elegans* ("Unc17"), *T. californica* (TorVAChT), and rat (rVAChT). Uppercase letters indicate the consensus residues, and lowercase letters indicate the divergent residues. The underlined amino acids in unc-17 are the regions used to design degenerate oligonucleotides for PCR amplification of the *Torpedo* sequence. The underlined residues in TorVAChT indicate the regions used to design primers for amplification of the rat sequence. Brackets surround putative transmembrane domains, and asterisks denote the potential sites for N-linked glycosylation in rVAChT. A leucine-rich region extends from transmembrane domain 12 into the C terminus of rVAChT. The numbers refer to residues in rVAChT.

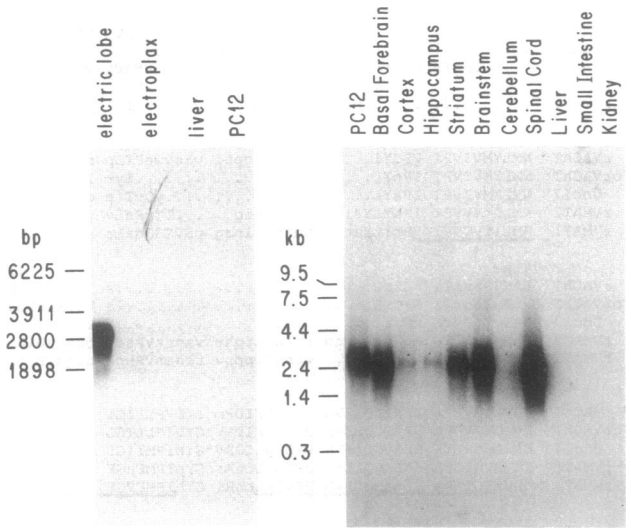

FIG. 2. Localization of VAChT sequences. (*Left*) Northern analysis of 3 μg of poly(A)$^+$ RNA from *Torpedo* electric lobe, electric organ (electroplax), liver, and rat pheochromocytoma PC12 cells under high stringency show expression of an ≈3-kb TorVAChT transcript in the electric lobe but not in other tissues, consistent with a presynaptic role in cholinergic transmission. (*Right*) Analysis of 4 μg of poly(A)$^+$ RNA from rat PC12 cells, different regions of dissected rat brain, spinal cord, and peripheral tissues shows expression of an ≈3-kb transcript in PC12 cells and selected tissues that contain cholinergic cell bodies. Cerebellum and peripheral tissues show no hybridization, and the cortex and hippocampus show only a weak signal.

and a lysine in transmembrane domain 2 show conservation. An additional asparate occurs in transmembrane domain 4 in VAChT but not in the amine transporters, and a glutamate occurs in the last predicted transmembrane domain of only the rat protein. Two sites for N-linked glycosylation occur in the large lumenal loop, and a leucine-rich region near the C terminus may indicate interaction with another vesicle protein. Several potential sites for phosphorylation by casein kinase II and protein kinase C also occur near the C terminus of rVAChT at Ser-478 and Ser-480, respectively, indicating the potential for regulation.

To determine the tissue distribution of rVAChT, we have used both Northern blot analysis and *in situ* hybridization. Northern analysis of poly(A)$^+$ RNA shows expression of the sequences in PC12 cells and in the basal forebrain, basal ganglia, brainstem, and spinal cord, regions that contain ChATase mRNA-expressing cell bodies (Fig. 2 *Right*). Cerebellum and peripheral tissues that do not contain ChATase mRNA-positive neurons do not express detectable rVAChT sequences. The presence of ChATase-immunoreactive cell bodies in the rodent cortex and hippocampus remains controversial (16), and these tissues show low levels of rVAChT mRNA expression, possibly as a result of contamination by the adjacent basal ganglia.

In situ hybridization shows strong expression of rVAChT sequences in the forebrain, brainstem, and spinal cord. In the forebrain, hybridization occurs in the olfactory tubercle, the caudate-putamen, the medial septal nucleus, the vertical and horizontal limbs of the diagonal band, and the nucleus basalis (Fig. 4). Fig. 5 shows expression of rVAChT sequences in the brainstem, with strong hybridization by motor nuclei of the trigeminal, facial, vagal, and hypoglossal cranial nerves. Fainter hybridization also appears in other nuclei such as the laterodorsal tegmental nucleus in the floor of the fourth ventricle (Fig. 5*A*) and the nucleus ambiguus, which is ventral and lateral to the vagal and hypoglossal nuclei (Fig. 5*C*). In the spinal cord, reactivity occurs in the ventral horn (Fig. 5*D*) and in the intermediolateral cell column (data not shown). These regions all contain cholinergic cell groups, and hybridization of adjacent sections with an anti-sense probe for ChATase (12) showed an identical pattern (Fig. 4*C*), indicating that cells in these structures express both rVAChT and ChATase mRNAs. Sense-strand probes for rVAChT showed no hybridization signal (data not shown).

To determine the chromosome localization of the human VAChT gene, we have used the rat cDNA as a probe to hybridize with a panel of human–mouse somatic cell hybrids. Analysis of the hybridization pattern shows localization of the gene to human chromosome 10 with no discordancies and no ambiguities (data not shown).

DISCUSSION

Two main lines of evidence support the conclusion that rVAChT participates in vesicular ACh transport. First, the predicted amino acid sequence shows substantial similarity to known vesicular transporters for monoamine transmitters (4, 6). Second, expression of the sequences is restricted to ChATase mRNA-containing cell populations in the brain and spinal cord by both Northern analysis and *in situ* hybridization. Relative to the amine transporters, the rVAChT cDNA bears a closer relationship to unc-17 and TorVAChT as well

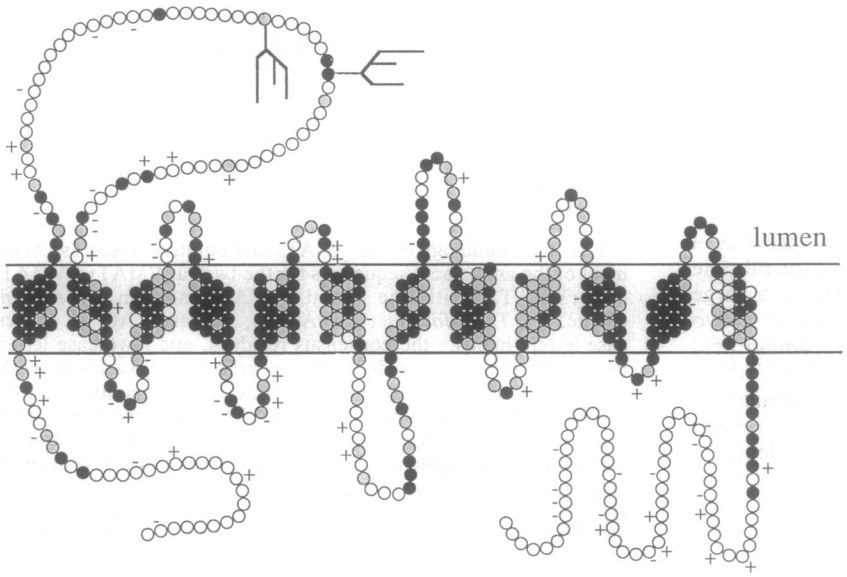

FIG. 3. Predicted structure of rVAChT. The sequence of the cDNA predicts a protein with 12 transmembrane domains and strong resemblance to the other vesicular neurotransmitter transporters. The lumen of the vesicle is above the membrane, the cytoplasm is below, and each sphere represents an amino acid, with basic and acid residues denoted by + and −, respectively. Residues identical in rVAChT, TorVAChT, and unc-17 are shown in black; residues with conservative changes, in gray; and divergent amino acids, in white. The branched structures in the first lumenal loop indicate potential sites for N-linked glycosylation.

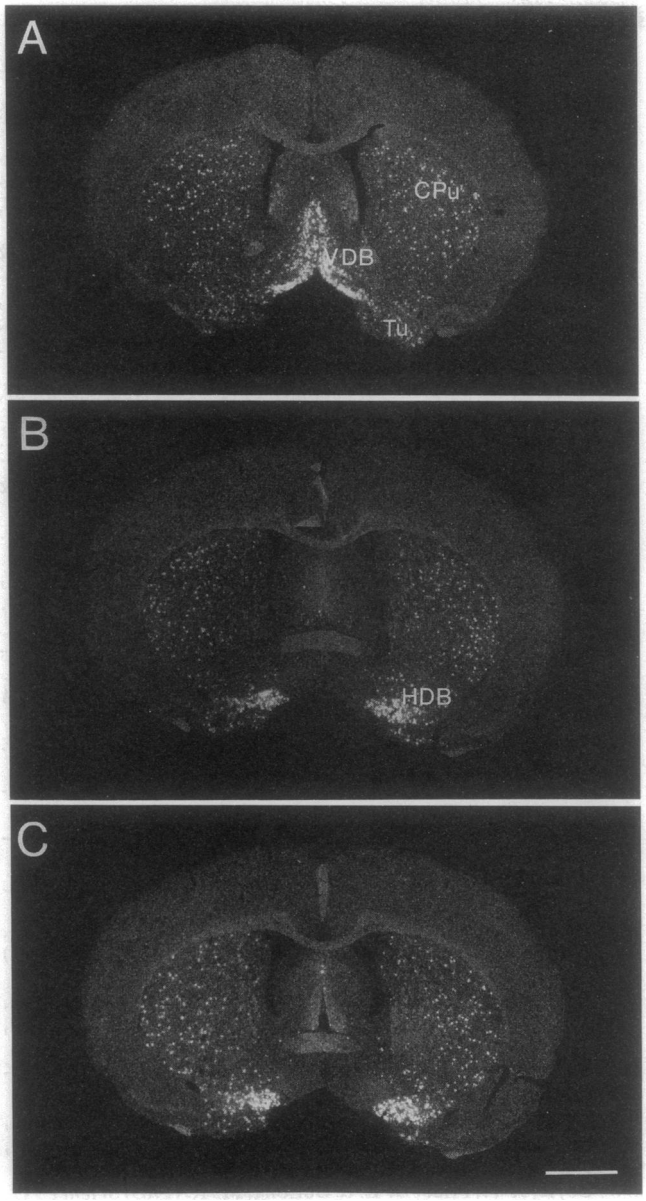

FIG. 4. Colocalization of rVAChT transcripts with ChATase in cholinergic cell groups of the forebrain. *In situ* hybridization with a 1.9-kb antisense RNA probe to rVAChT (*A* and *B*) detects cell bodies in the caudate and putamen (CPu), vertical limb of the diagonal band (VDB), medial septal nucleus, olfactory tubercle (Tu), and horizontal limb of the diagonal band (HDB). (*C*) A section adjacent to *B* was hybridized with a 0.6-kb antisense probe to ChATase and shows an identical pattern. Additional sections hybridized with a 1.9-kb sense probe for rVAChT revealed no signal (data not shown). (Bar = 1 mm.)

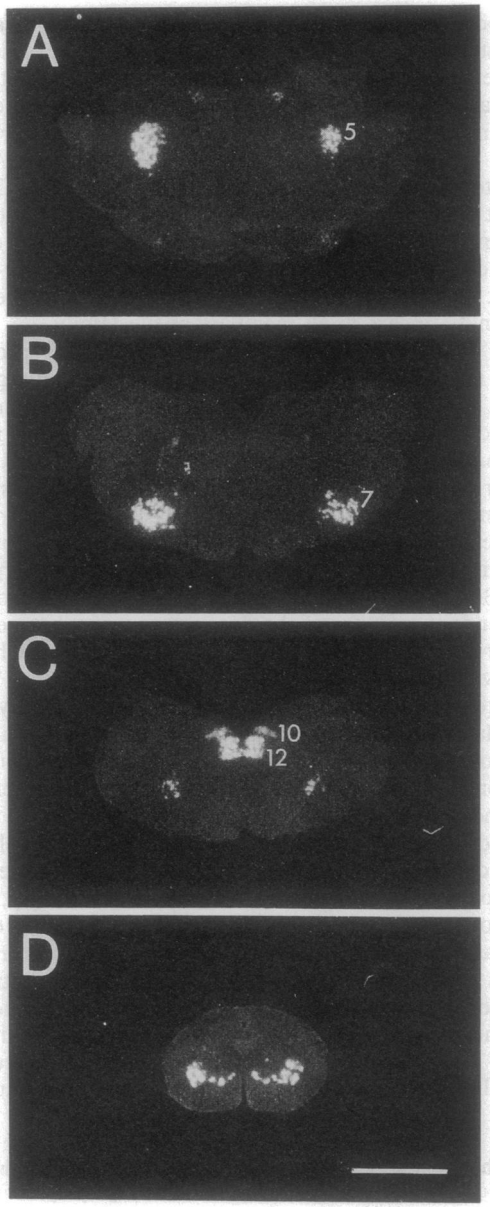

FIG. 5. Localization of rVAChT to motor nuclei in the brainstem and spinal cord. (*A* and *B*) *In situ* hybridization of a section through the mid (*A*) and lower (*B*) pons shows expression of rVAChT in the motor nucleus of the trigeminal nerve (5) and the facial nucleus (7) as well as additional scattered cholinergic nuclei (not marked). (*C*) A section through the medulla reveals expression by the dorsal motor nucleus of the vagal nerve (10) and by the hypoglossal nucleus (12) as well as the more ventral and lateral nucleus ambiguus (not marked). (*D*) In the spinal cord, expression occurs in the ventral horn. Adjacent sections hybridized with a probe for ChATase (17) showed the identical pattern, and an rVAChT sense-strand probe showed no signal (data not shown). (Bar = 1 mm.)

as to the sequences from other *Torpedo* species that have been reported recently (18). Since *unc-17* mutations protect against organophosphorus toxicity and the *Torpedo* electric lobe provides extremely dense cholinergic innervation to the electric organ, these relationships support a role for rVAChT in cholinergic neurotransmission. Interestingly, PC12 cells also express rVAChT mRNA and have previously been reported both to store ACh and to release the transmitter in response to stimulation (19–22).

The sequence of the rVAChT cDNA reveals a number of interesting differences from the VMATs (4, 6). The lumenal loop between the first two transmembrane domains of rVAChT is somewhat smaller than in the VMATs. An additional aspartate occurs in putative transmembrane domain 4, and an additional glutamate occurs in transmembrane domain 12. The aspartate in transmembrane domain 4 also appears in unc-17 and the *Torpedo* sequence, but the glutamate does not occur in the other putative VAChTs. The leucine-rich region at the C terminus includes part of the last transmembrane domain and suggests an interaction with other vesicle membrane proteins. Interestingly, the ACh transporter in synaptic vesicles of the *Torpedo* electric organ appears to associate with the proteoglycan SV2 (23), but SV2 lacks an obvious homologous leucine-rich region for the interaction (17, 24, 25). Although the possibility of interaction with another protein as a heteromultimer may be required for

transport activity, the vesicular amine transporters do not have such a requirement and function when expressed alone in a heterologous system (4, 6). In terms of interaction with the ligand, rVAChT contains the aspartate in transmembrane domain 1 that is presumed to bind to the positive charge of the amino group in both the amines (26) and perhaps ACh. As expected for a putative ACh transporter, rVAChT also lacks the set of serines in both the third and fourth transmembrane domains that may interact with the hydroxyls on the catechol and indole groups that are present in monoamines (26) but absent from ACh. However, one serine in transmembrane domain 4 appears in all members of the family.

The availability of the rVAChT cDNA now provides a useful marker for cholinergic neurons and cholinergic function in both physiologic and pathologic circumstances. Cholinergic neurons of the basal forebrain have a role in memory or attention (27, 28) and degenerate relatively early in Alzheimer disease (29, 30). Cholinergic motor neurons control movement and degenerate relatively selectively in amyotrophic lateral sclerosis. ChATase and the receptors for neurotrophic factors serve as markers for cholinergic neurons (31), but a probe for the VAChT can now provide an independent measure of the degenerative process, reflecting a different aspect of cholinergic transmission than ChATase. The availability of the rVAChT cDNA may also indicate ways to improve ACh release both physiologically and in transplants (32). The possibility that, like vesicular amine transport (3), vesicular ACh transport protects against certain forms of neural toxicity due to either endogenous or exogenous compounds should be explored.

To address the potential role in disease and in development of the cholinergic phenotype, we have localized the human gene for VAChT to chromosome 10. Interestingly, human chromosome 10 also contains the genes for VMAT2 (33) and ChATase (34). Fluorescent *in situ* hybridization further indicates localization of human VAChT to the subcentromeric region of the long arm (J.F., A.R., R.H.E., unpublished data) near the gene for ChATase and raises the possibility that, similar to *C. elegans* (35), the biosynthetic enzyme and VAChT undergo coordinate regulation from the same promoter.

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