Specific Vesicular Acetylcholine Transporter Promoters Lie within the First Intron of the Rat Choline Acetyltransferase Gene*

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The sequence encoding the vesicular acetylcholine transporter (VACHT) has recently been localized within the first intron of the choline acetyltransferase (ChAT) gene in various species. In rat, we previously identified a class of VACHT mRNAs that may originate from the same promoter region as two ChAT mRNAs. Here, we demonstrate by a detailed analysis of the 5′-noncoding region of the VACHT gene, that two specific VACHT promoters lie within the first intron of the ChAT gene. Two VACHT mRNAs are generated from these promoters. These results demonstrate that the promoter regions of these two genes are intermingled, which highlights the unique organization of the ChAT/VACHT gene locus.

Functional presynaptic expression of the neurotransmitter acetylcholine (ACh) in cholinergic neurons requires the activity of particular proteins: (i) a high affinity choline transporter on the plasma membrane, which controls the supply of extracellular choline; (ii) a vesicular acetylcholine transporter (VACHT), which translocates cytoplasmic ACh to the interior of synaptic vesicles; and (iii) choline acetyltransferase (ChAT; acetyl-CoA:choline O-acetyltransferase, EC 2.3.1.6), which synthesizes ACh from choline and acetyl coenzyme A. cDNAs encoding ChAT have been cloned and this led to the subsequent isolation and characterization of the ChAT gene (for review see Ref. 1). In rodent, ChAT is encoded by several mRNAs with different 5′-untranslated sequences. They are generated by differential promoter utilization and alternative splicing events (2, 3). Recently, cDNA and genomic sequences encoding VACHT have been identified and the VACHT gene was thereby localized to the first intron of the ChAT gene (4–7). This gene organization is well conserved between nematode and mammals, including man (4, 5, 7), and thus may have functional significance. Both ChAT and VACHT genes are in the same transcriptional orientation and both are required to express the cholinergic phenotype.

To our knowledge, this organization is unique in mammals. There are two classes of VACHT mRNAs in the rat, encoding the same VACHT protein, that we designate as R- and V-types. First, the R-type VACHT mRNAs contain common 5′-noncoding sequences (exon R) with two ChAT mRNAs and may therefore be transcribed from the same promoter (Ref. 4 and Fig. 1). In nematodes, this seems to be the only mechanism for generating VACHT mRNAs (7). Second, V-type mRNA species differs from the R-type mRNAs by the 5′-noncoding sequences (Refs. 4 and 5, Fig. 1). However, the molecular mechanisms by which the V-type mRNA species is produced has not been clearly elucidated.

In this study, we demonstrate that the first intron of the ChAT gene contains specific VACHT promoter regions. These regions give rise to two V-type VACHT mRNAs of 2.6 and 3 kb, previously detected by Northern blotting (4). We report a detailed analysis of the 5′-molecular diversity of VACHT mRNAs. These data clarify the transcription pattern of the rat ChAT/VACHT gene locus.

EXPERIMENTAL PROCEDURES

RNA Isolation—Total and poly(A)⁺ RNAs were purified as described previously (8, 4).

Ribonuclease (RNase) Protection Assays—cRNA probes were synthesized from DNA templates obtained by amplification of particular gene regions. Sequences were amplified with primer pairs A/A₁, B/B₁, or C/C₁ (Fig. 2B). The corresponding PCR products A, B, and C, were subcloned (TA cloning, Invitrogen) downstream from the SP6 promoter. Linear templates for cRNA synthesis (Fig. 2B) were prepared from the subcloned PCR products either by digestion (Bsu36I for the B2 probe, HindIII for the C1 probe) or by amplification with a reverse SP6 primer and a forward specific primer (AF, AF, and BF for the probes A1, A2, and B1, respectively). Antisense cRNA probes were synthesized (9) using SP6 RNA polymerase (Promega) in the presence of (α-³²P)jGTp (3000 Ci/mmol, Amersham Corp.). After digestion with DNase, full-length cRNAs were purified by electrophoresis on a 4% polyacrylamide, 8.3 M urea gel. RNase protection assays were performed as described (9), except that these RNAs were hybridized to cRNA probes for 16 h at 63 °C and digested with 25 μg/ml RNaseA and 1 μg/ml RNaseT1 at 30 °C for 50 min.

Northern Analysis—Northern blot experiments were performed as described (10). DNA probes (see Fig. 3A) were ³²P-labeled by nick translation (Life Technologies, Inc., probe 3) or random priming (Amersham, other probes).

Plasmid Constructions—The restriction fragments Xbal/SphI and HindII/Xbal (Fig. 4A) were inserted into the plasmid KS Luc, upstream from the luciferase reporter gene and downstream from a transcription terminator. KS Luc was constructed by W. Faust and A. M. Catherin.2 Cloning junctions were verified by sequencing.

Cell Cultures and Transfection—The cell line 293 (human embryonic kidney) was maintained in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) supplemented with 10% fetal calf serum. The cell lines PC-12 and PC-G2 (rat pheochromocytomas) were grown in RPMI 1640 medium (Life Technologies, Inc.) supplemented with 10% horse serum with 5% or 10% fetal calf serum, respectively. Exponentially growing cells were transfected by electroporation with a single electrical pulse at 200 V using a Bio-Rad Gene Pulser as in Ref. 11. Cells (10⁶) were transfected with a mixture of 1 pmol of the plasmid to be tested.

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2 W. Faust and A. M. Catherin, unpublished results.
1 μg of a Rous sarcoma virus-chloramphenicol acetyltransferase (CAT) vector (used to assess the transfection efficiency) and a carrier DNA (Bluescript) to give a total of 12 μg of DNA. Luciferase activity was normalized with CAT activity determined in the same extract (11). Each transfection was carried out in triplicate.

RESULTS AND DISCUSSION

Molecular Diversity of VAcH T mRNAs—A V-type mRNA species was previously identified by primer extension. The longest fragment contained 426 bp of the gene sequence upstream from the translation initiation codon (4). A similar VAcH T cDNA with a longer 5' noncoding sequence (856 bp) has also been isolated (5). Thus, several V-type VAcH T mRNAs with different 5' lengths might be produced, and we analyzed this potential diversity.

RNase protection experiments were performed to localize the transcription initiation site(s) of the V-type VAcH T mRNA(s). Two transcription start sites at positions -402 and -426 (Fig. 2A) were mapped with both radiolabeled antisense riboprobes A1 and A2 (Fig. 2B). In spinal cord extracts both probes yielded a similar pattern of protected fragments (Fig. 2C). First, a cluster of up to 7 protected fragments of 115–121 nucleotides, whose sizes differ by one nucleotide, was clearly revealed. The 5' ends of these fragments lie between positions -425 and -431, within an A-rich sequence (-433, 5'-AAAGAAAAAAA-3', -421). The presence of these A residues may explain why the RNase protection assay gave multiple bands rather than a single band. The 5' end of the amplified primer extension product obtained previously (Ref. 4, position -426) maps to this region, confirming that

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**Fig. 1.** Schematic representation of the rat VAcH T gene and of the R- and V-type VAcH T mRNAs. Black and white boxes indicate coding and noncoding sequences, respectively. Position 1 corresponds to the translation initiation codon. Vertical bars represent two donor splice sites in the exon R (-1435 and -1357) and an acceptor splice site (-309) used to generate the R-type VAcH T mRNAs (4). Vertical dotted lines represent the positions of the 5' end of a VAcH T cDNA (-856, Ref. 5) and of a primer extension product (-426, Ref. 4). VAcH T mRNAs not subjected to the splicing of the genomic region between the acceptor and donor splice sites indicated above are designated as V-type.

**Fig. 2.** RNase protection analysis of the 5' region of the rat VAcH T gene. A, schematic representation of the rat VAcH T gene as in Fig. 1. Additional vertical bars represent the regions containing the 5' ends of V2 (-888/-863) and V1 (-426/-402) mRNAs and the 3' end of the VAcH T mRNAs (+198). Abbreviations: B, Bsu36I; H, HindIII. B, representation and positions of the cDNA probes (A1, A2, B1, B2, C1) and of the amplified DNA fragments (A, B, C) from which these probes were produced (see "Experimental Procedures"). The sequences of the PCR primers used are: AF, 5'-CATCCTGGGCATCTCAGA; AR, 5'-ACGGCCTCTCTGCACCGCAG; AF9, 5'-GAGACTCACCGGTATA; BF, 5'-TGCAAGACTTTCTGCCTAAGGGC; BR, 5'-GTTCCTCCCACTGCTCAGCCATC; BF9, 5'-TTGGCGTCAGAGCCTCTTG; CF, 5'-CAGAGGCTGATCTGT-TCAGCCGT; CR, 5'-CCTCCTCTCA GTCCATACCCTC. C, RNase protection analysis. Total RNA from spinal cord (SC) or, as a negative control, liver (L), where VAcH T mRNAs could not be detected (4, 6), was hybridized with the probes A1, A2, B1, B2, C1 and tested for protection from RNases. The amounts of RNA hybridized to the probes are indicated at the top of each lane. Note that the probe A1 was hybridized with up to 50 μg of liver RNA. Pr+RNases, probe with RNases (subjected to each experimental step); Pr+RNases, probe without RNases. Open arrows indicate the fragments completely protected by the probe. Filled arrows show the other fragments specifically protected in spinal cord. Sizes were determined by comparison with known sequencing reaction products electrophoresed in separate lanes of the same gel.

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it corresponds to the same 5' end of a VACHT mRNA. A less intense 93-nucleotide fragment was also protected, demonstrating an additional 5' end at position -402. Thus, these two sites are used to generate a first type of VACHT mRNA designated as V1. In addition, both A1 and A2 probes were completely protected, indicating that a mRNA extending further upstream was also present. The transcription initiation site for this transcript was determined with the riboprobes B1 and B2 (Fig. 2B). Several identical protected fragments were visualized with both probes (Fig. 2C). Three major fragments of 223, 231, and 248 nucleotides were obtained, indicating three prevalent transcription initiation sites at positions -863, -871, and -888, respectively. Thus, the cDNA isolated previously (5) was nearly full-length, and the corresponding mRNA is referred to as V2. Therefore, two clusters of transcription start sites separated by about 450 bp are used for V-type VACHT mRNA synthesis, confirming a diversity in the 5' region of the V-VACHT mRNAs. Surprisingly, the probes B1 and B2 were also completely protected from RNases, evidence for an additional mRNA species. Using the riboprobe C1 (Fig. 2B), which covers a sequence further upstream, as far as exon R, a single protected fragment was detected, corresponding to the complete protection of the probe (Fig. 2C). Thus, the sequence of this last mRNA, designated V3, extends at least to 16 nucleotides from the 3' end of the exon R.

These results show the existence of several V-type VACHT mRNAs. In addition, for VACHT mRNAs no diversity in the 3' region was detected (data not shown). A single 3' end, corresponding to position +1998, was found, which suggests that they derive from the use of a specific polyadenylation signal located 18 bp upstream. This indicates that VACHT mRNAs differ only by the length of their 5'-noncoding sequences.

Northern blot analysis of VACHT mRNAs—Brainstem or, as a negative control, liver poly(A)+ RNAs were analyzed by Northern blot using probes specific to different regions of the VACHT mRNAs (Fig. 3A). By hybridization with a probe complementary to the VACHT coding region (Fig. 3A, probe 4), two mRNAs of 2.6 and 3 kb were detected, as previously reported in spinal cord and brainstem (4). Moreover, a diffuse band between 3.9 and 3 kb was also visualized (Fig. 3B, lanes b and 4). This result was confirmed in four independent experiments performed with different RNA preparations from spinal cord or brainstem (not shown) and by using cytoplasmic poly(A)+ RNA (Fig. 3B, lane a), thus ruling out possible contamination with nuclear primary transcripts. The hybridization pattern obtained with probe 3 (lane 3), whose sequence is specific to all V-type mRNAs, is identical to that of probe 4. A 7-day exposure of the blot was required to detect in brainstem samples a weak band near the upper point of the smear. Probe 2, specific to the V2- and V3-type mRNAs, hybridized to the 3-kb mRNA species and also revealed the diffuse band between 3.9 and 3 kb (lane 2). Probe 1, specific to V3-type mRNA, specifically recognized this diffuse band (lane 1). However, it is not clear if the presence of the smear is due to poor stability of the corresponding mRNA species.

These results show that the VACHT mRNAs of 3 and 2.6 kb correspond to the V2- and V1-type mRNAs, respectively, which is in agreement with the sizes calculated from their sequences. Moreover, the V3-type mRNA encodes VACHT. This mRNA is probably produced from the same promoter as the R-type mRNAs and results from the nonexcision of the region between the exon R and the acceptor splice site at position -309.

Promoter Activities and Sequence Analysis in the Region 5' to the Transcription Start Sites—V1- and V2-type mRNAs differ by the length of their 5' sequences. They may result from transcription either from a common promoter but initiating at different sites or from different promoter regions. These possibilities were tested by transient expression assays. The upstream regions of the V1 (XS) and V2 (HX) transcription initiation sites (Fig. 4A) were cloned upstream from the luciferase reporter gene and used to transfect three cell lines: PC-12, which expresses the endogenous VACHT gene (5), and PC-G2...
The transcription initiation sites lack TATA and CAAT boxes or the VAChT specific promoters. The sequences in and around both the ChAT and the VAChT genes. However, this remains to be determined.

We can now propose a model for the transcription of the rat ChAT/VAChT gene locus. First, VAChT and ChAT genes may be transcribed from a promoter region located upstream from exon R. VAChT mRNAs are then generated when the transcription is stopped at position +1998. These VAChT mRNAs either are spliced to generate the R-type mRNAs, or remain unspliced (V3-type). Alternatively, transcription of the VAChT/ChAT genes may continue until the ChAT polyadenylation signal. R-ChAT mRNAs are then obtained by splicing out a 7-kb fragment containing the VAChT open reading frame. Second, the VAChT gene may be transcribed from the two promoters localized in the first intron of the ChAT gene to produce the VAChT mRNAs V1 and V2. Finally, the ChAT gene may be transcribed from promoters located downstream from the VAChT gene, giving rise to the ChAT N- and M-type mRNAs. Note that all VAChT mRNAs we detected have the same 3' end, and thus there appears to be no bicistronic mRNA.

To conclude, we provide evidence that promoter regions are contained within the first intron of the ChAT gene. The organization of the rat ChAT/VAChT locus is both complex and unusual, with the promoters of these two genes intermingled. The analysis of this organization may reveal novel mechanisms involved in the expression of eukaryotic genes.

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