We describe here the characterization of the rat m4 muscarinic acetylcholine receptor gene and the identification of its regulatory region. Two 5′-noncoding exons are located approximately 5 kilobases upstream from the coding exon, and at least two alternatively spliced variants of m4 mRNA are expressed in the neuronal cell line PC12D. There are two transcription initiation sites. The promoter region is GC-rich, contains no TATA-box, but has two potential CAAT boxes and several putative binding sites for transcription factors Sp1 and AP-2. We assessed the m4 promoter activity functionally in transient expression assays using luciferase as a reporter. The proximal 435-basepair (bp) sequence of the 5′-flanking region produced luciferase activity in both m4-expressing neuronal cell lines (PC12D and NG108-15) and non-neuronal cell lines (L6 and 3Y1B). A longer fragment containing an additional 638-bp sequence produced luciferase activity only in m4-expressing neuronal cell lines. These data suggest that the proximal 435-bp sequence contains a constitutive promoter and that a 638-bp sequence farther upstream contains a cell type-specific silencer element. A consensus sequence for the neural-restrictive silencer element is found within this 638-bp segment.

Muscarinic acetylcholine receptors (mAChRs) are members of the superfamily of G-protein-coupled receptors, which are characterized by the presence of seven putative transmembrane domains. mAChRs are widely expressed in the central and peripheral nervous system. In the brain, mAChRs are thought to play critical roles in higher functions, including attention, regulation of movement, learning, and memory (1, 2). Five subtypes of mAChR (m1-m5) have been identified by molecular cloning (3). Each subtype of mAChRs shows a distinct and complicated distribution in peripheral tissues and brain, suggesting subtype-specific functions (4–7). Although the coding region of each mAChR subtype has been cloned and sequenced, the precise structures of their 5′-noncoding regions and regulatory regions have not been reported. Cloning and analysis of the genetic regulatory elements of these genes should yield important insights into the mechanisms that underlie the tissue- and site-specific expression of each subtype of mAChR.

In this study, we focused on defining the sequences that regulate m4 mAChR gene expression. In mammals, the m4 mAChR gene is expressed predominantly in the central nervous system, although its expression has been detected in some peripheral tissues such as rat lung (8). In rat brain, m4 mRNA is present in the cerebral cortex, striatum, olfactory bulb, and pyramidal cell layer of the hippocampus (4, 7, 10, 11). m4 mAChR has been suggested to function not only postsynaptically but also presynaptically in some regions (12, 13). As a first step toward elucidating the regulation mechanism of m4 mAChR gene expression, we have characterized the rat m4 mAChR gene and identified its regulatory region.

**EXPERIMENTAL PROCEDURES**

Cell Culture—PC12D cells (a spontaneously arising derivative of the rat pheochromocytoma-derived cell line PC12 (14), a gift from Dr. M. Sano (Institute for Development Research, Aichi Prefectural Colony, Aichi, Japan)) were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, and NG108–15 cells (a hybrid cell line derived from mouse neuroblastoma N18 and rat glioma C6) in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and HAT medium (10 μM hypoxanthine, 40 μM aminopterin, and 1.6 mM thymidine, Life Technologies, Inc.). All culture media contained 100 units/ml penicillin G and 100 μg/ml streptomycin.

RNA Isolation and Amplification of the 5′-Noncoding Region—Total cellular RNA was isolated by the method of Chomczynski and Sacchi (15). Poly (A)⁺ RNA was isolated from total cellular RNA using Oligo-dT 30 (Super) (Takara). The 5′-noncoding region of m4 mRNA was amplified from 2 μg of PC12D poly(A)⁺ RNA by single-strand ligation to single-stranded cDNA-PCR using the 5′-Ampli FINDER™ rapid amplification of cDNA ends kit (Clonetech). First strand cDNA was synthesized using an m4 mAChR gene-specific primer m4primer1 (5′-ggaaatctgatcatagaggctg-3′), complementary to sequence located 432–456 bp downstream from the translation initiation site (lowercase characters denote the linker sequence). An anchor oligonucleotide (supplied with the kit) was ligated to the cDNA, which was then amplified twice by PCR using a primer complementary to the anchor and one of two gene-specific primers: m4primer1 (5′-ggaaatctgatcatagaggctg-3′) and m4primer2 (5′-ggaaatctgatcatagaggctg-3′). PCR products were cloned in pBluescript SK(+) and sequenced.

Screening of Genomic Library—A rat genomic library (cloned in bacteriophage λ vector, a gift from Dr. Richard Mains, Johns Hopkins University, School of Medicine) was screened using a 392-bp XhoI-Smal fragment of rat m4 mAChR gene coding region (sequence located 787–1179 bp downstream from the translation initiation site) as a probe. Approximately 1 × 10⁸ plaques were screened, and three overlapping clones were isolated. One of these clones (JH1411) was subjected to restriction map analysis and was partially sequenced.

S1 Nuclease Mapping—A Smal-HinfI fragment from the m4 genomic clone, which turned out to correspond to residues 90 to 194 (with the...
transcription initiation site designated as +1), was partially filled in at the HinfI site with dA and subcloned in the Xbal and EcoRV sites of pT7Blue vector (Novagen) following partial fill in of the XbaI site with dC and dt. Single-stranded DNA prepared from this construct was used as a template for preparation of single-stranded probe by the "prime cut" method (16). To anneal the primer to the template DNA, 0.2 pmol of the primer (Fig. 3, C, lane 2) and the template DNA, which corresponds to residues 811 to 137, was denatured individually (105 cells per well) in 20 mM HEPES (pH 7.4), 100 mM NaCl, 1 mM EDTA, and 0.05% SDS at 95°C for 5 min. After cooling slowly to room temperature, 0.5 µl of 3 mM (each) dGTP, dATP, and dTTP, 5 µl of [α-32P]dtCTP (3000 mCi/mmol) (Amersham), and 2 µl of Klenow fragment (1 unit/µl, New England Biolabs) were added and incubated at 25°C for 1 h. After addition of 0.75 µl of 1X E3 buffer, dATP, dCTP, and dTTP and further incubation for 30 min, the enzyme was heat inactivated at 70°C for 5 min. After digestion with BamHI, which cuts the multicloning site of the vector, the labeled product was extracted with phenol/chloroform and then ethanol precipitated. After denaturation at 95°C for 5 min in 95% formamide, 5X EDTA, 0.05% bromphenol blue, and xylene cyanol, the probe was purified by denaturing polyacrylamide gel electrophoresis in 8 X urea. Northern mapping was performed essentially as described previously (17). The probe was hybridized to 2 µg of poly(A)+ RNA from PC12D cells or rat kidney at 52°C overnight. 1000 units/ml S1 nuclease (Boehringer Mannheim) was used for digestion at 37°C for 30 min. The S1-resistant products were analyzed using 6% denaturing polyacrylamide gels containing 8 X urea.

Primer Extension—The primer was prepared as follows. A HindII-Apal fragment (333 to 337) was subcloned in the HindII and Apal sites of pBluescript SK(+). Using this construct as a template, the genomic fragment (333 to 157) was amplified by PCR using 41-1 primer (5′-ggaattCTGCTTCTGTCTTTCTCTGCT-3′) and 137 to +157) and the T3 primer. After digestion with SacI and EcoRI, the amplified fragment was cloned in the SacI and EcoRI sites of pBluescript SK(+), and single-stranded DNA was prepared. The antisense strand was synthesized and labeled as described above using a T7 primer. The labeled product was digested with HindIII and EcoRI, and the double-stranded DNA, which corresponds to residues +94 to +157, was purified. The 435-bp fragment from the 5′-noncoding region served as a primer template (Fig. 1). A consensus splice acceptor sequence was found immediately 5′ to the position where the sequences of the cDNA diverged (the sequence “cctccag” with the underline in Fig. 2). Two upstream exons (exons 1 and 2) were mapped to positions approximately 5.3 and 4.4 kb upstream from the transcription initiation codon by hybridization analysis (data not shown).

To test the possibility that PC12D cells express m4 mRNA that contains both of the two upstream exons, we carried out reverse transcription-PCR using primers specific to each exon. As shown in Fig. 1, we obtained a cDNA clone that contains sequences from both exons 1 and 2 (cDNA 3). The length of exon 2 was determined to be 158 bp, and a consensus sequence for a splice acceptor site was found next to the 5′-end of the exon. Taken together with the data shown in Figs. 1 and 2, respectively. A consensus splice acceptor sequence was found immediately 5′ to the position where the sequences of the cDNA diverged (the sequence “cctccag” with the underline in Fig. 2). Two upstream exons (exons 1 and 2) were mapped to positions approximately 5.3 and 4.4 kb upstream from the transcription initiation codon by hybridization analysis (data not shown).

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Fig. 1. Schematic representation of the rat m4 mAChR gene (the phage clone JH1411) and isolated cDNAs. Exons are indicated by boxes (open boxes represent the coding sequence). cDNAs 1 and 2 were isolated by single-strand ligation to single-stranded cDNA-PCR using primers complementary to sequences within the coding region. cDNA 3 was isolated by reverse transcription-PCR using primers complementary to sequences within exon 1 (5'-CTCTGGCTTGGCCGGCTCAGT-3') and exon 2 (5'-ATCTCCCCGCTTCACAGCTC-3'). Splicing patterns for each cDNA and predicted structures of each mRNA are shown. A, Apal; H, HindIII; K, KpnI; P, PstI; Sc, Sall; Sm, SmaI restriction cutting sites.

Fig. 2. Nucleotide sequence of the 5'-flanking and 5'-noncoding region of the rat m4 mAChR gene. Arrow heads indicate transcription initiation sites determined by primer extension analysis and S1 nuclease mapping. Numbers are relative to the upstream transcription initiation site (G). The initiation codon (ATG) is shown in bold and double-underlined. Asterisks represent the location of the 5'-end of cDNA1 and cDNA2. Consensus sequences for Sp1, AP-1, AP-2, Zif268 transcription factors, the NRSE and CAAT-box are shown in bold. Lower-case characters denote the intron sequences. Consensus sequences for splice acceptor and donor sites, three PstI sites and two SmaI sites, are underlined.
m4 mAChR Gene Promoter

**Fig. 4.** Alignment of NRSEs between rat m4 mAChR, rat SCG10 (35), rat type II sodium channel (NaII) (36), and human synapsin I (37) genes. For m4 mAChR, the sense strand is written at the top and in the orientation 3'→5'. The numbering of the bases is as described in Fig. 2. Asterisks show nucleotides that are not seen in other genes.

Functional Analysis of the m4 Promoter—We assessed the m4 promoter activity functionally by transient expression assay using luciferase as a reporter (Fig. 5). We inserted different portions of the 5'-flanking sequence of the m4 gene in the pGL2-Basic vector (Promega) and transfected these into PC12D cells, NG108-15 cells, L6 cells, and 3Y1B cells. For m4 mAChR, the sense strand is written at the top and in the orientation 3'→5'. The numbering of the bases is as described in Fig. 2. Asterisks show nucleotides that are not seen in other genes.

**DISCUSSION**

We have determined the structure of the 5'-noncoding region of the rat m4 mAChR gene. Two 5'-noncoding exons are located approximately 5 kb upstream from the coding exon. PC12D cells express at least two alternatively spliced variants of mRNA (mRNAs 1 and 2 in Fig. 1), the latter is predicted based on the splicing patterns of cDNAs 2 and 3), which originate from these two upstream exons. Also, we have obtained cDNAs that correspond to cDNAs 1 and 2 (Fig. 1) using RNA from rat brain by reverse transcription-PCR (data not shown). Previous studies have shown that genes encoding rat m1, m3, m5, and porcine m2 mAChR contain at least one intron in their 5'-noncoding regions and that m4 mAChR gene also has a potential splice acceptor site in its 5'-noncoding region (21, 22). There are no introns in the coding or the 3'-noncoding regions of these genes (3). The porcine m2 mAChR gene has at least two alternatively spliced 5'-noncoding exons, although its precise gene structure is not known (23). Another group has reported...
that two 5'-noncoding exons are located about 5.5 kb upstream from the coding exon in the rat m4 gene (24).

The 5'-flanking region of the rat m4 mAChR gene lacks a TATA-box, is GC-rich, and contains several potential Sp1 binding sites. These characteristics are typical of promoters for housekeeping genes, which are constitutively expressed. However, recent studies have demonstrated that some highly regulated genes, including immediate early genes, developmentally regulated genes, and tissue-specific genes, also have promoters that lack TATA boxes (25). Genes encoding several genes that have promoters lacking TATA boxes, binding of Sp1 is GC-rich promoters that lack TATA boxes, also have adrenergic receptors, also have GC-rich promoters that lack TATA boxes. These promoters also contain several putative Sp1 binding sites, although the requirement of Sp1 binding for transcription of these genes is yet to be determined. In some genes that have promoters lacking TATA boxes, binding of Sp1 has been shown to play a critical role in transcription initiation (33, 34).

In mammals, the m4 mAChR gene is expressed primarily in neurons. We assessed whether or not the 5'-flanking region of the m4 mAChR gene functions as a cell type-specific promoter by transient expression assays using non-neuronal cell lines and m4 mAChR-expressing neuronal cell lines. The fragment containing 1073 bp of the 5'-flanking region (Fig. 5, construct B) was found to be sufficient for cell type-specific expression of the m4 mAChR-luciferase fusion gene. Deletion from the 5'-side to base −435 did not result in a loss of promoter activity but rather in a loss of specificity of expression, indicating that the region −1073 to −435 represses the expression of m4 mAChR gene in cell lines where the m4 receptor is not expressed. The existence of a putative NRSE between bases −857 and −837 is consistent with the repression of the m4 mAChR-luciferase fusion gene expression in non-neuronal cell lines, although the involvement of the NRSE in the neuron-specific expression of the m4 mAChR gene remains to be proven. NRSE is a silencer element that is known to regulate neuron-specific expression of several genes including the rat SCG10 (35), rat type II sodium channel (36), and human synapsin I genes (37). However, m4 mAChR is not expressed in all neurons and shows a unique, site-specific expression pattern in the brain. If we assume that NRSE is involved only in determining the neuron-specific expression of the m4 mAChR gene, an additional mechanism may be required to restrict the expression to only a subset of neurons. It remains to be determined whether the 5'-flanking region we have isolated contains some elements regulating the specificity of expression among different kinds of neurons. The mouse serotonin 2 receptor (5-HT2) gene is known to possess repressor domains that regulate its neuron-specific expression and an activator domain that allows gene expression in a glial cell line that expresses the 5-HT2 receptor (38, 39). The m4 mAChR is also known to be expressed in some glial cell lines (40), although there is no direct evidence for expression of the m4 mAChR in glia cells in vivo.

In summary, we have determined the structure of the rat m4 mAChR gene and identified its promoter region. It has two 5'-noncoding exons, from which at least two alternatively spliced variants of mRNA originate in PC12D cells. The fragment containing 1073 bp of the 5'-flanking region is sufficient for cell type-specific (at least neuron-specific) expression.

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Promoter Region of the Rat m4 Muscarinic Acetylcholine Receptor Gene Contains a Cell Type-specific Silencer Element
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