

# Structure and Transcription of the Human m3 Muscarinic Receptor Gene

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We have isolated and characterized the human m3 muscarinic receptor gene and its promoter. Using 5' rapid amplification of cDNA ends (RACE), internal polymerase chain reaction (PCR), and homology searching to identify EST clones, we determined that the cDNA encoding the m3 receptor comprises 4,559 bp in 8 exons, which are alternatively spliced to exclude exons 2, 4, 6, and/or 7; the receptor coding sequence occurs within exon 8. Analysis of P1 artificial chromosome (PAC) and bacterial artificial chromosome (BAC) clones and of PCR-amplified genomic DNA, and homology searching of human chromosome 1 sequence provided from the Sanger Centre (Hinxton, Cambridge, UK) revealed that the m3 muscarinic receptor gene spans at least 285 kb. A promoter fragment containing bp -1240 to +101 (relative to the most 5' transcription start site) exhibited considerable transcriptional activity during transient transfection in cultured subconfluent, serum-fed canine tracheal myocytes, and 5' deletion analysis of promoter function revealed the presence of positive transcriptional regulatory elements between bp -526 and -269. Sequence analysis disclosed three potential AP-2 binding sites in this region; five more AP-2 consensus binding motifs occur between bp -269 and +101. Cotransfection with a plasmid expressing human AP-2 $\alpha$  substantially increased transcription from m3 receptor promoter constructs containing 526 or 269 bp of 5' flanking DNA. Furthermore, m3 receptor promoter activity was enhanced by long-term serum deprivation of canine tracheal myocytes, a treatment that is known to increase AP-2 transcription-promoting activity in these cells. Together, these data suggest that expression of the human m3 muscarinic receptor gene is regulated in part by AP-2 in airway smooth muscle.

Muscarinic acetylcholine receptors are a family of G-protein linked seven transmembrane receptors. They are present throughout the body in smooth muscle, cardiac muscle, exocrine glands, and neurons of the central and peripheral nervous systems. Five receptor subtypes, termed m1-m5, have been identified, and these are encoded by distinct genes (1-3). The m1, m3, and m5 receptors interact with pertussis toxin-insensitive Gq proteins, thereby activating phospholipase C, whereas the m2 and m4 receptors preferentially interact with pertussis toxin-sensitive Gi proteins, and so inhibit adenylyl cyclase.

Both m2 and m3 muscarinic receptors are present on airway smooth muscle cells, with m2 receptors comprising the majority (4-6). However, the m3 receptor is the predom-

inant muscarinic subtype mediating acetylcholine-induced airway smooth muscle contraction, as demonstrated in competitive binding studies (4, 7, 8) and in genetically altered mice (9). The m3 receptor subtype also plays an important role in the regulation of intracellular calcium in bronchial epithelium (10).

To date, the cDNA sequence encoding the m3 muscarinic receptor has been identified in pig (GenBank X12712) (11), cow (GenBank U08286) (12), rat (GenBank AB017656) (2, 13), chicken (GenBank L10617) (14), mouse (GenBank AF264050), monkey (GenBank AF148140), sheep (GenBank AJ131184), dog (GenBank AF056305), rabbit (GenBank AF079113), and human (GenBank HSU29589 or X15266) (1, 3). There is substantial nucleotide homology among species, and between the m3 receptor and other muscarinic receptor subtypes within species. As with the m1, m2, and m4 receptor subtypes, the coding sequence is found in a single large exon, but neither the m3 receptor gene structure nor its promoter region has been isolated for any species. Accordingly, nothing is known of its transcriptional regulation. In this study, we identified the structure of the human m3 muscarinic receptor gene, which contains 8 exons and comprises at least 285 kb on chromosome 1, and analyzed its 5'-flanking DNA, which contains a number of potential transcription factor binding sites, and exhibits AP-2-modulated transcriptional activity in cultured airway smooth muscle.

## Materials and Methods

### Identification of the 5' Untranslated Region of cDNA

We used 5' RACE (rapid amplification of cDNA ends) to identify the 5' untranslated region of the human m3 receptor cDNA and its splicing variants. We performed 5' RACE using two approaches. In the first, 1  $\mu$ g poly-A+ RNA (Poly A Pure; Ambion, Austin, TX) from 1HAEo- human bronchial epithelial cells were reverse transcribed using Superscript II reverse transcriptase (Life Technologies/Invitrogen, Carlsbad, CA). The reaction was performed at 42°C for 60 min and terminated at 70°C for 15 min, and then RNA was digested with 1  $\mu$ l of RNase H mix (Life Technologies/Invitrogen) at 37°C for 30 min. The first strand cDNA was purified using the QIAquick PCR Purification Kit (QIAGEN, Valencia, CA) and recovered in sterile water. Next, a homopolymeric dC tail was added using terminal deoxynucleotidyl transferase (Life Technologies/Invitrogen) by 10 min incubation at 37°C, and the reaction stopped at 65°C for 10 min. Five microliters of this reaction were used as template to perform polymerase chain reaction (PCR) using the 5' RACE Abridged Anchor Primer (5'-GGCCACGCGTCTCGACTAGTACGGGIIGGGIIGGGIIG-3'; Life Technologies/Invitrogen) and one of three different gene specific antisense primers corresponding to what was later determined to be exon 8 (5'-GCAAGGTCATTGTGACTCTCTG-3' or 5'-TGC CAGCTGCTCGAGAAACATTGTAGC-3') or exon 7 (5'-CTAC CAGGAAGTCTGAGTTATGTAACAAGG-3'). After an initial de-

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Abbreviations: bacterial artificial chromosome, BAC; Dulbecco's modified Eagle's medium, DMEM; expressed sequence tag, EST; P1 artificial chromosome, PAC; polymerase chain reaction, PCR; rapid amplification of cDNA ends, RACE.

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naturing step (94°C for 3 min), PCR was performed for 35 cycles, with an annealing temperature of 55°C and an extension time of 2 min. The PCR products were analyzed on a 0.8% agarose gel and individual bands cloned into pCR2.1-TOPO using a T/A cloning system (Life Technologies/Invitrogen). In a second approach, we performed 5' RACE using 1 µg human tracheal poly A+ RNA (Clontech, Palo Alto, CA) as template and the SMART cDNA Library Construction Kit (Clontech) according to manufacturer's instructions, with the exception that gene-specific primer (5'-TGCCAGCTGCTCGAGAAACATTGTAGC-3') designed from the coding region was used in the first-strand RT reaction, rather than the suggested oligo dT primer. Library amplification was performed by PCR between the gene-specific primer listed above and an anchor-specific primer supplied by the manufacturer. To establish the 5' extent of the m3 receptor cDNA, and to search for alternative splicing variants in the 5' untranslated region, we performed PCR using this 5' RACE library as template with the following primer pairs: (i) sense anchor primer + antisense 5'-GCCGCCTTCAGTTCATCGCCCGTC-3' (derived from sequence later determined to be exon 1); (ii) sense 5'-CAGCGCTTCTGGGAA GACGG-3' (exon 1) + antisense 5'-AGGAGCATCAAACCAA TACAATGTGTCAG-3' (exon 5); (iii) sense 5'-GAAGGAC TTTGCTGCTTTGGG-3' (exon 2) + antisense 5'-GCAAGGT CATTGTGACTCTGA-3' (coding exon 8); and (iv) sense 5'-CTGTGGCGTGGCACCTGGTCTC 3' (exon 5) + antisense 5'-CTACCAGGAAGTGAAGTATGTAACAAGG-3' (exon 7). PCR products were cloned into pCR2.1-TOPO using a T/A cloning system (Invitrogen), and sequenced.

### Primer Extension

Primer extension was performed to identify the principal transcription start sites. Because exon 1 proved to be a G/C-rich region, primer extension was performed using Thermostable Reverse Transcriptase (Perkin-Elmer Biosystems, Boston, MA), 25 ng human tracheal poly A+ RNA (Clontech) as template, and an antisense primer designed from what was later determined to be bp 79 to 101 of exon 1 (5'-GCGGACTGATGAGGAGCCTG GAG-3'). Primer extension was performed at 65°C or 75°C for 15 min, in the presence of [ $\alpha$ -<sup>32</sup>P]dCTP, and radiolabeled products were resolved on a 6% sequencing gel then autoradiographed.

### Isolation of Noncoding Exons and 5' Flanking DNA

To isolate genomic DNA containing 5' flanking DNA and exons upstream of the protein coding exon, we screened a human genomic P1 artificial chromosome (PAC) library (Incyte Genomics, St. Louis, MO) by PCR, using Taq polymerase (Perkin-Elmer), 35 amplification cycles, annealing temperature of 60°C, and 30 s extension at 72°C. Primers were designed from the known human m3 sequence corresponding to the third intracellular loop (sense 5'-AGCTGCAGCAGTTACGAACTTCAACAG-3'; antisense 5'-CACCTGCAGGTTGTCCGATGAGGG-3'). Positive (human genomic DNA as template) and negative (no polymerase) controls were included. PCR products were analyzed on a 1% agarose gel and two positive clones were demonstrated (269N9 and 132H1). These clones were purchased and amplified using the QIAGEN Plasmid Maxi Kit. The 5' end of the human m3 muscarinic receptor gene could not be identified in either PAC clone. Therefore, we purchased a human genomic bacterial artificial chromosome (BAC) clone (215P14) that contained the 5' end of this gene, including exons 1 and 2, from Research Genetics/Invitrogen (Huntsville, AL); this clone was isolated by Research Genetics through PCR screening of their BAC library, using primers derived from exon 1 and intron 1 (sense primer: 5'-CAGCGCT TCTGGGAAGACGG-3'; antisense primer: 5'-AGGACGCTAT GCTGAGGAAGTG-3'). Southern blots made from these PAC and BAC clones were probed with a series of [ $\alpha$ -<sup>32</sup>P]dCTP-

labeled PCR products from exons of interest. Hybridizing bands were identified, large-scale digestions were performed, and the products of digestion resolved and extracted from 0.8% agarose gels, and ligated into pBluescript II vector (Stratagene, La Jolla, CA).

Intron sequences that could not be determined by genomic subcloning or comparison with known sequences were obtained using the GenomeWalker Kit (Clontech). Nested gene-specific primers were designed from our cDNA and genomic DNA sequences. This kit includes four sets of human genomic DNA fragments obtained by digestion with one of four different restriction enzymes followed by ligation to adaptors of known sequence. Nested gene specific and nested anchor primers were used in sequential PCR to amplify genomic DNA within the region of interest. PCR products were T/A cloned and sequenced as above. Additional intron sequence was revealed by searching of the human chromosome 1 sequence available from the Sanger Centre (<http://www.sanger.ac.uk>).

### Expressed Sequence Tag Clones

Results from the 5' RACE and genomic cloning were compared for sequence homology with deposits in the GenBank nonredundant and expressed sequence tag (EST) databanks. Significant homology was identified with two human EST clones (GenBank AI362457 and AW131645), which were purchased from IMAGE Consortium and sequenced. Homology searching also revealed significant similarity with the sequence in GenBank AF279779, which is a cDNA transcribed from the antisense promoter of the human L1 retrotransposon.

### Promoter Constructs and Plasmids

A 5' deletion series of promoter fragments was obtained by PCR, using the human GenomeWalker libraries as template. 5' primers were chosen to generate DNA fragments with 5' ends corresponding to bp -526, -269, or -139, with an XhoI restriction site added at the 5' end; the 3' primer was chosen to generate products with 3' end corresponding to bp +101, plus a HindIII restriction site added at the 3' end. PCR products were digested with XhoI and HindIII, and cloned into similarly digested pGL3-basic luciferase reporter vector (Promega). A longer promoter fragment was generated by subcloning PCR-amplified genomic DNA containing bp -1404 to +101 into pCR2.1-TOPO, then digesting the resultant plasmid with XhoI and HindIII, to yield a fragment that includes bp -1240 to +101 of the m3 muscarinic receptor gene, plus 3' DNA sequence from the T/A cloning vector; this was then ligated into pGL3-basic as above. All PCR-generated promoter constructs were confirmed by sequencing. We previously described psmMHC-luc (15), which contains 3.3 kb of promoter from the human smooth muscle myosin heavy chain (sm-MHC) gene driving luciferase expression in pGL3-basic, and pMSV- $\beta$ gal (16, 17), in which the viral MSV-LTR promoter drives expression of the *lacZ* gene. Finally, pAP-2 $\alpha$ HA, which expresses AP-2 $\alpha$  (with a C-terminal hemagglutinin tag), was constructed by ligation of the cDNA encoding full-length human AP-2 $\alpha$  into EcoRI- and NotI-digested pMH expression vector (Roche) using Pfu polymerase (Stratagene). The construct sequence was then verified.

### Cell Culture and Transient Transfection Analysis

Canine tracheal myocytes of passage 1–3 were grown to 50–70% confluence on uncoated plastic dishes, and maintained in Dulbecco's modified Eagle's medium (DMEM):F-12 (1:1) plus 10% FBS, 0.1 mM nonessential amino acids (NEAA), 50 units/ml penicillin, and 50 µg/ml streptomycin as previously described (15, 17). Myocytes in 6-well dishes were transiently transfected in serum free Optimem (Life Technologies) using 12 µg Lipofectamine (Life Technologies) and 1.2 µg total DNA per well. In each well,

0.6  $\mu$ g luciferase promoter construct were cotransfected with 0.6  $\mu$ g pMSV- $\beta$ gal to normalize for transfection efficiency. In some wells, pAP-2 $\alpha$ HA (60 ng) or the corresponding empty vector (pMH) was also included. After 5 h, medium was replaced with DMEM:F-12 plus 10% FBS, 0.1 mM NEAA, 50 units/ml penicillin, and 50  $\mu$ g/ml streptomycin. Serum-deprived myocytes were grown to confluence, then maintained in serum free DMEM:F-12 supplemented with 5  $\mu$ g/ml insulin, 5  $\mu$ g/ml transferrin, and 5 ng/ml selenium (ITS), plus NEAA and antibiotics, with fresh medium provided every 2–3 d as previously described (15). Seven-day serum-deprived myocytes were transfected in 6-well dishes using 12  $\mu$ g Lipofectamine and 1.2  $\mu$ g total DNA for 5 h in Optimem, after which cells were returned to serum free DMEM:F-12/ITS (15). Under these conditions, transfection efficiency is  $\sim$  6% for serum-fed myocytes and is just less than 1% for serum-deprived myocytes. Serum-fed or serum-deprived cells were harvested 48 h after transfection, and luciferase and  $\beta$ -galactosidase activities measured (15, 17). Results from triplicate wells were averaged to provide the data for each experiment; mean  $\pm$  SEM from 3–7 experiments are shown.

## Results

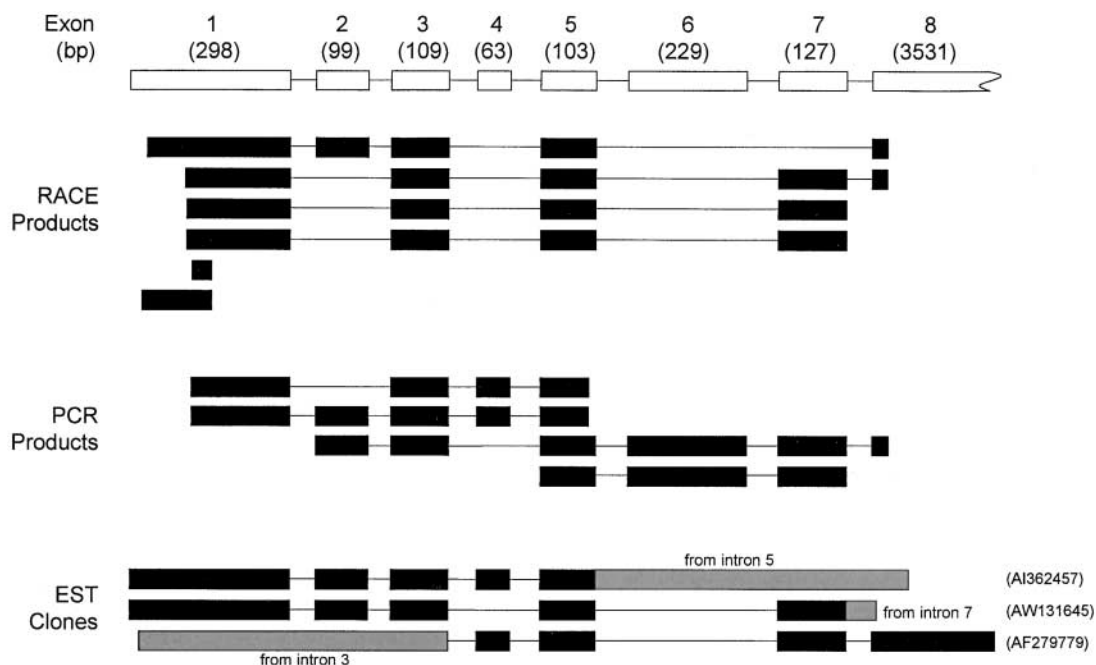
### Identification of the 5' Untranslated Region

5' RACE was performed using gene-specific antisense primers derived from the previously known protein coding sequence of the human m3 receptor (GenBank HSU29589) or from exon 7, learned by analysis of initial RACE products. Additional variation in the cDNA 5' untranslated region was sought by PCR amplification of a 5' RACE cDNA library, as described above. Figure 1 summarizes our results, and demonstrates that the m3 receptor mRNA is encoded by 8 exons. It is alternatively spliced, with vari-

ants that exclude exons 2, 4, 6, and/or 7. Using DNA sequence derived from these RACE and PCR products, we searched the GenBank human EST database and found two homologous clones (AI362457 and AW131645); these were obtained and sequenced, and determined to contain exons 1–5 and exons 1–3 and 5, respectively (Figure 1), plus downstream intron sequence at the 3' end (the molecular basis of which is unknown). Another recently deposited sequence within GenBank (AF279779) represents an m3 muscarinic receptor cDNA variant transcribed from an antisense retrotransposon promoter, and includes a portion of intron 3 as well as exons 4, 5, and 7, and a portion of coding exon 8. Based on all these results, and on the previously reported human m3 receptor protein coding sequence (GenBank HSU29589), a consensus sequence for the full-length m3 receptor cDNA is provided in Figure 2. Note that the sequence shown includes all exons, even though this longest possible cDNA was not actually isolated in our studies, and that the most 5' base in exon 1 is identified from the EST sequences in GenBank AI362457 and AW131645, which extend 35 bp further upstream than our longest 5' RACE clone. Additional homology searching revealed that exons 4 and 5 are 80% homologous to the known bovine m3 receptor cDNA (BTU08286, bp 543–708).

### Identification of the Transcriptional Start Site

We performed primer extension to identify the principal transcriptional start sites, using poly A<sup>+</sup> mRNA from human trachea as template. As shown in Figure 3, primer extension revealed a cluster of start sites between bp 51 and



**Figure 1.** Structure and alternative splicing of the human m3 muscarinic receptor cDNA. Comparison of DNA sequences of 5' RACE products, of internal PCR products, and of EST/cDNA clones revealed that the cDNA variably includes sequence from eight exons. Exons 2, 4, 6, and 7 are variably omitted; the m3 receptor protein coding region is in exon 8.

Exon 1  
 1 GCGGCCGCGG GCGGAGCGCT CTCAGACCCC GGAGCGCACA CCGCGGGGGCC  
 61 ATCGGTGCCA TCGGGATCTT CCAGGCTCTC CATCAGTCCG CCGGGCGCGC AGCAGCGCTT  
 121 CTGGGAAGAC GGGCGATGAA CTGAAGGGCG GCTCCGGGCA GGGGGGACAG ATCTTAAGGA  
 181 CAGTCCGCTCC CTGAACCGGG AGCCGGAGGA GACGAAGGGA AGGTGGAGCG GACGCCACCC  
 241 GCGCACCGGG CAGGGCGGGA GACCGGCGTG GCACAGCCAC CTGGAGCGCA GCTCCGAG

Exon 2  
 299 AGAAGGACTT TGCTGCTTTG GGCCAGGATC TGAACCTTAG TGTAAACCAT TGCCTTGGCA  
 301 GAGGGAACCT ACCCAGTCCA TTGCTGCTCG CTACAAG

Exon 3  
 398 TTTGGTTATG AGTCACTCAG TGGACTGTGG ATTGAATGAA CTGTATCCAT CCCCATCATG  
 421 ATGTACAGAA CCAAGTCTCT TCACTA

Exon 4  
 507 AGTCAGAACT TCAGCTAAGG TACAATAAG

Exon 5  
 570 CTAGAAGGAA AGTTCAACAT ACAGACAAT TCTGGACACA TTGTATTGGT TTGATGCTCC  
 601 TACCTGGAAC AG

Exon 6  
 673 GCCAACAC TAAACATAAG TTGAGAGCT GGCCTGTGGT GGAACCTACA  
 721 AGTCTTATTG GCAATATATG TGAGGAGACA AAAAGCAACA TAGGGAGAAA TACAAAAATA  
 781 TACTCCCTAG AGATACTGCC TCTTCAGATG TCAAGAAAAA CTTGGATGAA ATGAAGCAAG  
 841 AGTGGCACCG TGCAAGATTA CAAATTCAGG GCCACCCACA GAACCAACA TTAACACTTA  
 901 G

Exon 7  
 902 GTGGAGCTG GTCTCTGGG CAGCCTGACA TCTGGTCCAC TCCTCTGCCC TATGCCGGGA  
 961 TCATCATGAC CGTAGAGATT ATGTCACCTG TTTGCATCCT TGTATACATA CTCAGTTCCT  
 1021 GGTAGATT

Exon 8  
 1029 AC TATGTCAGAG AGTCAACATG ACCTTGACA ATAACAGTAC AACCTGCGCT  
 1081 TTGTTTCCAA ACATCAGCTC CTCCTGGATA CACAGCCCTC CCGATCGAGG GCTGCCCGCG  
 1141 GGAACCGTCA CTCAATTTCG CAGCTAGCAAT GTTCTTCGAG CAGCTGGCAA TTCTCCTCT  
 1201 CCAGACGGTA CCACCGATCA CCTCTGGGA GGTCAATCCG TCTGGCAAGT GGTCCTCATC  
 1261 GCTTTC'TTAA CCGGACTCCT GGCCCTGGTG ACCATCATCG GCAACATCCT GGAATATTGG  
 1321 TCATTTAAGG TCACAAGCA GCTGAAGACG GTCAACAACCT ACTTCTCTCT AAGCCTGGCC  
 1381 TGTGCCGATC TGATATCCGG GGCATTTTCA ATGAATCTGT TTACGACCTA CATCATCATG  
 1441 AATCGATGGG CCTTAGGGAA CTTGGCCTGT GACCTCTGGC TTGCCATGGA CTACGTAGCC  
 1501 AGCAATGCCT CTGTTATGAA TCTTCTGGTC ATCAGCTTTG ACAGATACTT TCCCATCACG  
 1561 AGGCCGCTCA CGTATCCGAG CAAAAGCAAC ACAAGAGAG CCGSTGTGAT GATCGGCTCG  
 1621 GCTTGGGTCA TCTCCTTTGT CTTTGGGCTC CTTGCCATCT TGTTCCTGGCA ATACTTTGTT  
 1681 GGAAAGAGAA CTGTGCCCTCC GSGAGAGTGC TTCAATCAGT TCCTCAGTGA GCCACCATT  
 1741 ACTTTTGGCA CAGCCATCCG TGCTTTTAT ATGCCTGTCA CCATTATGAC TATTTATAC  
 1801 TGGAGGATCT ATAAGGAAC TGAAGAAGCT ACCAAAGAGC TTGCTGGCTC GCAAGCCTCT

1861 GGGACAGAGS CAGAGACAGA AAAC'TTGTG CACCACCAGG GCAGTTCCTG AAGCTGCAGC  
 1921 AGTTACGAA TCAACAGCA AAGCATGAAA CGTCCCAACA GGAGGAAGTA TGGCGCTGCG  
 1981 CACTTCTGGT TCAACAACAA GAGCTGGAAA CCCAGTCTCG AGCAGATGGA CCAGAGCCAC  
 2041 AGCAGCAGTC ACAGTTGGAA CAACAATGAT GCTGCTGCTC CCTGGAGAAA CCGCCCTCC  
 2101 TCCGACGAGS AGGACATTTG CTCCGAGAGC AGAGCCATCT ACTCCATCTG GCTCAAGCTT  
 2161 CCGGGTCACA GCACCATCCT CACTCCACC AAGT'TACCT CATCGGACAA CCGTCAAGTG  
 2221 CCTGAGGAGT AGCTGGGGAT GGTGGACTTG GAGAGGAAAG CCGACAAGCT GCAGGCCAG  
 2281 AAGAGCGTGG ACGATGGAGG CAGTTTCCA AAAAGCTTCT CCAAGCTTCC CATCCGCTA  
 2341 GAGTCAGCCG TGGACACAGC TAAGACTTCT GAGTCAACT CCTCAGTGGG TAAGAGCAGC  
 2401 GCACCTCTAC CTCTGTCCTT CAAGGAAGCC ACTCTGGCCA AGAGGTTGC TCTGAAGACC  
 2461 AGAATCAGA TCACTAAGCG GAAAAGGATG TCCTTGCTCA AGGAGAAGAA AGCCGCCAG  
 2521 ACCCTCAGT CGATCTTGT TGCCTCATC ATCACTTTGA CCCCATACAA CATCATGGTT  
 2581 CTGGTGAACA CCTTTTGTGA CAGCTGCATA CCCAAAACCT TTTTGAATCT GGGCTACTGG  
 2641 CTGTGCTACA TCAACAGCAC CGTGAACCCC GTGTGCTATG CTCTGTGCAA CAAAACATTC  
 2701 AGAACCCACT TCAAGATGCT GCTGCTGTG CAGTGTGACA AAAAAAGAG GCGCAAGCAG  
 2761 CAGTACCAGG AGAGACAGTC GGTCAATTTT CACAAGCGCG CACCAGGACA GGCTCTTAGT  
 2821 AGTAGCGTGT TATCAATAGC AGTGAACAAA CGCACAAATC AACCCGACGA CCTTAGGAGT  
 2881 AGGAAGCGCA GGGCGGGGT ACTTCTGGTG ATGATAAAA TGGT'TTATC ACCCAGATAT  
 2941 GAAGAACAAC GCCTGTTTAC TGATCAATG AATAAAACA TTTTAAATA AAAAGCAAT  
 3001 ACCAATTCAG CAAAAAGAAA AAAAAAAGAT ACTCAAAAT ATAAAGAAAT T'ATTT'GAA  
 3061 ATGACTCTTA CGTGT'TTTT TCTTAAAGAG GAGAAAATA TCGCTTGAGG GCAATTATG  
 3121 ACCCAAAGT ATTTGCTGG GTCTTTAAT TCCCATTAGC TTGGGAATCT CAGATGAGC  
 3181 TAGCTGACCC AGTTCOCACA TTCTTCCAT GATGCAAAA TTGGGAATCC AGACCCGACA  
 3241 TGAACACACT CAGGCTTACG AATCTGTGT TCCAAAATTA TTTCACTAGT TGCAGAGCTG  
 3301 AATCTTCTAC TCCCAATAGA GCTTCTGTG TCCCTTTGG GTTGTGTA AACTCATATT  
 3361 GTGGACTTGA TTCTTGATC TTGCAAGTA CTGTTTGTG CAGTTCAGT TCTGTACAAA  
 3421 TAAAATACT AAGTATATAT ATATGTGTGA GTTCTGCAGC CACACACATA GGTATATAA  
 3481 TATCATGGGA AACACTGAAC TGSCAAATTA TTCTCAATC ATAGCTTTTC ATGTACTTTG  
 3541 TAATCGAAGT TCTTAGAGAT CCTAATGCAA CATTAACAGT AAAAAGGCC AGTGTAAATG  
 3601 TTTTGAACA CAGGGCTGTT TTCCACAGAG AGCAGCCAGG CCTTCCAGCG AGGCTGTGCG  
 3661 AGAGCGGACA GGCTCGTAG TCACTGAGC GCGCTGGCTC CCCCAGACTT GGTGTAAAGC  
 3721 AACCTCTCTT GTTGTATGCT CAACAGAGCT AAATCGGGGC CCCCCTGAGC TCAAAGAAAG  
 3781 AACCATATCC ACACGTTTGA ATTTAATCAT CTAAATCTGA ATGTTTCTGA ACAAAATTTT  
 3841 TGTATCTTAA ACTGCTTGA ACTCAATAAT ACTGCTCAGT TTGATATGTA TACACAGCAA  
 3901 TATATATATA TGTATATA TATATATAT GCAAGCAAAA AAAAAAACA TGGTAAAGAA  
 3961 TGAAGAAGGA GAACATTTG TTTGATCTCT GCTGAATGSC ACCTTCTCAA AGAAAATAGG  
 4021 GCTTGCACCT TTGTTAATCA GCTGTGGCCA GTGCTTCTG GTGTTCAGT TTAACACTTC  
 4081 ACCCAGGAAT AGGTGAGGTT TTAGGAAGTT ACAGTCTCTC TTAGGAAAGG ATGACTACTT  
 4141 GMAAGTAACT GCTTCAAAAT GATTTCTTGA CTTTGTGGGA AAAAAAATA ATGTTTTT  
 4201 TGCCATCTCC CTTGAATGTA CCAAAATGTT AACGT'TTCA GTTGGGAGG GATGSGGTTG  
 4261 TGCCCATCAT TGCTGTGTT GTTCTGCTG TAGCTTGTG GGTTCCTTTT CCTTGTCCCG  
 4321 GGCTGTGTT GGGAGAGGGA GGGAGGGAG AGCTGGAGGC CCGCGAGATA TCTTCCCTT  
 4381 TGACAGGGC ATCTGTGTT GTGAACCCAG AGCTGGGTAG AAGCTGCTTT TGTATTCACT  
 4441 GTGAGGTGST GTTTACAGAC GACTTTGACA ACAGTAGAAG TGTACTCAGT GGTGTCTGG  
 4501 TATCTGAAGT ATTTAATTTT GGTATTGTT TATATGCGA AATATTATG GATACTACA

Figure 2. Sequence of human m3 muscarinic receptor cDNA, including all exons. Note that the sequence of protein coding exon 8 before nucleotide 1142 is taken from GenBank HSU29589. Start (ATG) and stop (TAG) codons in exon 8 are boxed.

73 in exon 1. This finding parallels similar observations in other muscarinic receptor genes (18–21), and is consonant with the transcriptional start site implied by the longest RACE clone we isolated (Figure 3).

Isolation of Noncoding Exons and 5' Flanking DNA

The exon-intron organization of the human m3 receptor gene was revealed by comparing cDNA sequence with that of human genomic DNA, isolated from PAC and BAC clones, or by PCR amplification of human genomic DNA using the GeneWalker kit. Additional intron sequence was identified by homology searching of the chromosome 1 sequence available from the Sanger Centre.

Figure 4A shows the structure of the human m3 receptor gene. It includes eight exons and spans at least 285 kb. Sequence analysis of the 1.4 kb of 5'-flanking DNA reveals consensus binding sequences for multiple transcription factors, at the positions shown in Figure 4B. However, no neuron-restrictive silencer element/repressor element 1 (NRSE/RE1) motifs, which have been found to be important in neuronal restriction of other muscarinic receptor genes (22, 23), were identified, and no consensus CARG boxes, known to be important in smooth muscle specific gene transcription (24), were identified. Similar to other muscarinic genes, the 5' flanking region is GC rich and contains no TATA box or CAAT box near the transcrip-

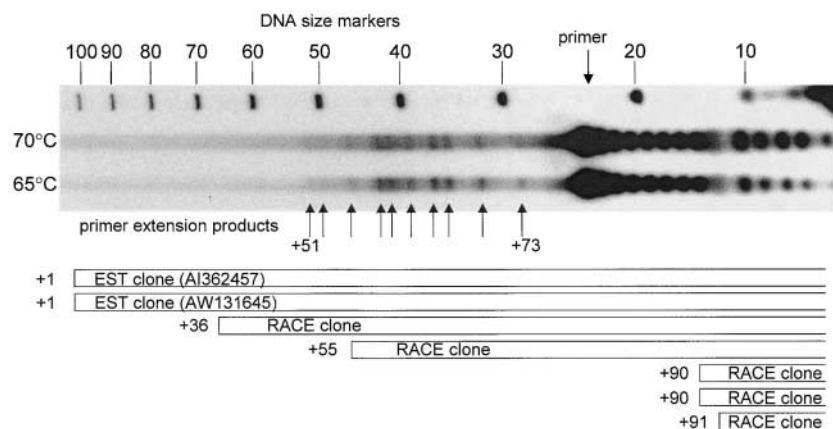


Figure 3. Identification of human m3 muscarinic receptor gene transcriptional start site through primer extension analysis. Primer extension was performed using human tracheal polyA+ mRNA and an antisense primer corresponding to what proved to be bases +79 to +101 of the cDNA. Multiple start sites between +51 and +73 were evident. The most 5' extents of two EST clones and of five 5'RACE clones are also shown.

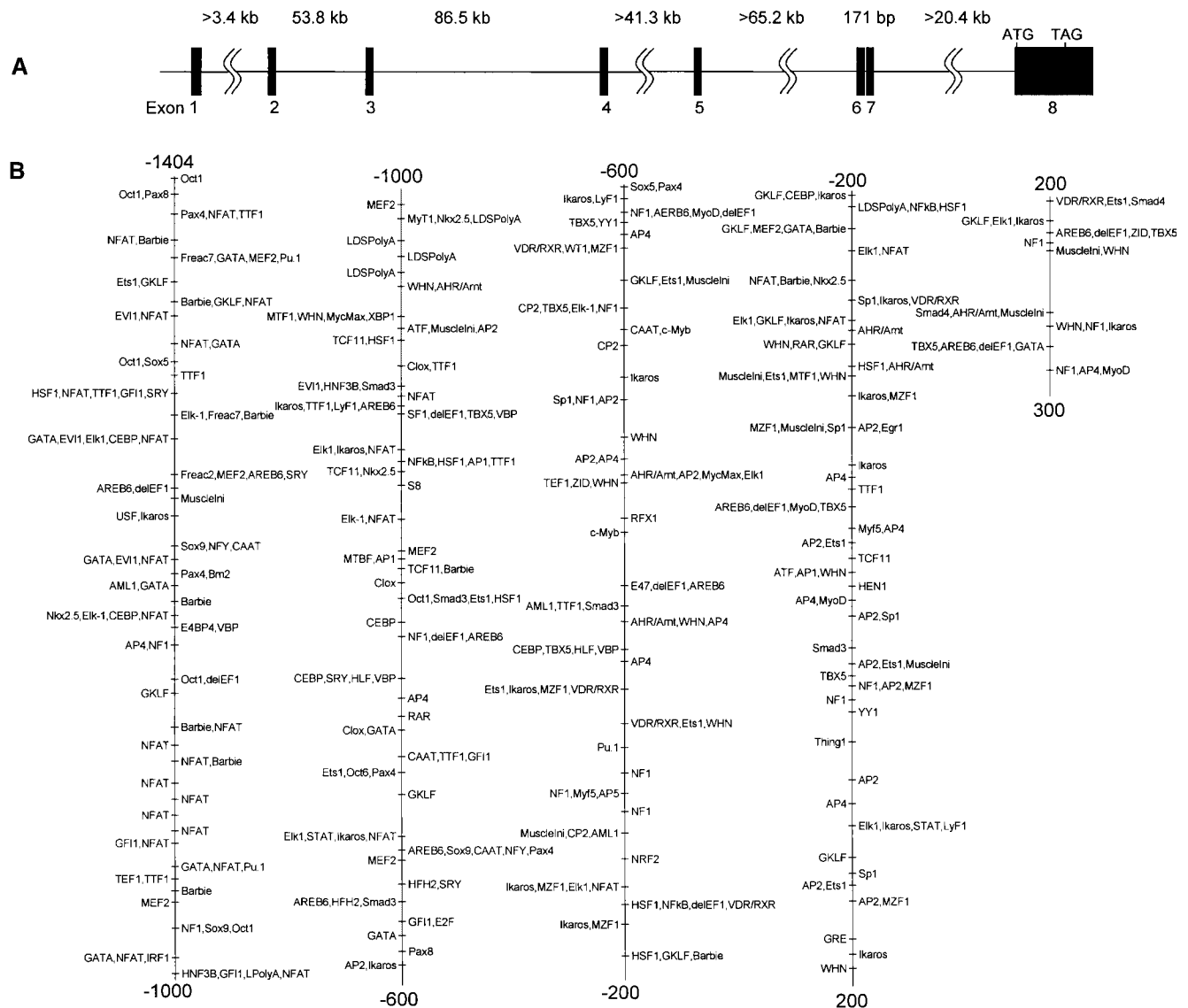


Figure 4. Structure of the human m3 muscarinic receptor gene and its promoter. (A) Exon-intron organization. Intron length is displayed above each intron. (B) Potential transcription factor binding sites within the promoter region and exon 1.

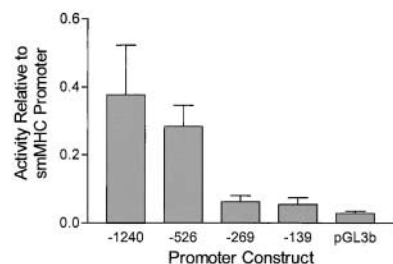
tional start site. The sequence of the promoter region and noncoding exons have been deposited in GenBank (AF331832-AF331838).

#### Analysis of Promoter Function

Figure 5 reveals that 1,240 bp of 5' flanking DNA (plus 101 bp of exon 1) exhibit considerable transcriptional activity in subconfluent, serum-fed cultured canine tracheal smooth muscle cells; activity was ~40% of that of the 3.3 kb human smooth muscle myosin heavy chain gene promoter. 5' deletion analysis demonstrated that the proximal 526 bp of 5' flanking DNA retained almost full transcriptional activity, but further truncation to 269 bp reduced promoter activity by three-quarters, suggesting that bp -526 to -269 contain functionally important positive regulatory elements. This region contains three consensus motifs for AP-2 binding, and bp -269 and +101 contain five

more potential AP-2 binding sites (Figure 4B). We therefore tested whether AP-2 $\alpha$  overexpression further enhances transcription from the -526 to +101 or -269 to +101 promoter fragments. As shown in Figure 6A, cotransfection with an AP-2 $\alpha$  expression plasmid increased transcription from these promoter constructs by 3.5-fold or 4.8-fold, respectively, while AP-2 $\alpha$  overexpression enhanced activity of the smooth muscle myosin heavy chain promoter by only 38%. This suggests that AP-2 $\alpha$  is an important activator of transcription from the human m3 muscarinic receptor gene promoter in airway smooth muscle cells.

Previously, we found that long-term serum deprivation increases m3 muscarinic receptor protein expression in cultured airway myocytes (25, 26), and restores acetylcholine-induced calcium mobilization (26) and myocyte contraction (25) in these cells. We also showed that long-term



**Figure 5.** 5' deletion analysis of human m3 muscarinic gene promoter function during transient transfection in cultured subconfluent, serum-fed canine tracheal myocytes. Activity of m3 muscarinic gene promoter is shown relative to that of 3.3 kb

of human smooth muscle myosin heavy chain (smMHC) promoter. Each m3 promoter fragment is identified by its 5' extent; bp +101 of exon 1 was the 3' terminus of each promoter fragment. Serial 5' truncation from bp -1240 to bp -526 results in only minor diminution of promoter activity, but further truncation to bp -269 results in loss of 78% of remaining transcriptional activity. pGL3b - promoterless pGL3basic plasmid.

serum deprivation redistributes AP-2 $\alpha$  from the cytoplasm to the nucleus of cultured canine tracheal myocytes, and that this redistribution is accompanied by substantially increased transcription from the AP-2 $\alpha$ -sensitive promoter contained in the luciferase reporter plasmid p4xAP-2luc (15). These findings, along with our present results implicating AP-2 as a positive regulator of transcription from the human m3 receptor gene promoter, suggested the hypothesis that long-term serum deprivation might transcriptionally activate m3 receptor expression. To test this possibility, we transfected 7 d serum-deprived canine tracheal myocytes with reporter plasmids in which luciferase expression is controlled by bp -526 to +101 or bp -269 to +101 of the human m3 receptor gene, or by 3.3 kb of the human smMHC promoter. As shown in Figure 6B, long-term serum deprivation substantially upregulated transcription from the longer or shorter m3 receptor promoter constructs, by 4.5-fold or 5.3-fold, respectively. This was not a nonspecific effect, as transcription from the smMHC promoter was substantially reduced (by 85%) in long-term serum-deprived myocytes. Previously, we demonstrated that downregulation of the smMHC promoter activity stems from reduced transcription promoting activity of serum response factor (SRF) in long-term serum-deprived airway myocytes, which appears attributable in part to redistribution of SRF out of the nucleus of these cells (15).

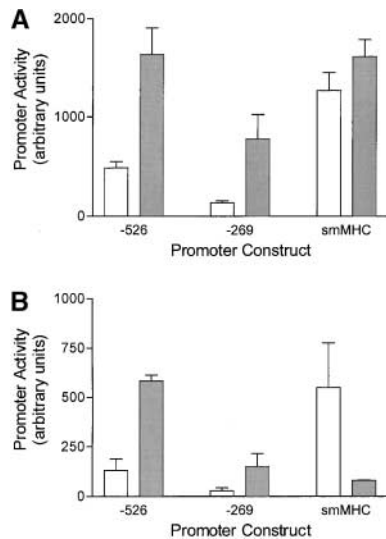
## Discussion

We have for the first time isolated the human m3 muscarinic receptor gene, which contains eight exons and comprises over 285 kb on chromosome 1, and analyzed its promoter region, which exhibits AP-2-regulated activity in cultured airway smooth muscle cells. Despite the physiologic importance of the m3 receptor in mediating cholinergic bronchoconstriction and airway secretion, the gene encoding this receptor has not been cloned previously in any species. The rat m1, chick m2, and rat m4 muscarinic receptor genes and their promoters have been isolated. They share some similarities with each other and with the human m3 muscarinic receptor gene, but there are also noteworthy differences.

The rat m1 receptor was cloned independently by two groups (19, 20). Pepitoni and colleagues (19) identified one upstream exon separated from the single protein coding exon by a 13.5 kb intron; they found no TATA or CAAT box in the promoter region, and showed that 0.9 kb of 5' flanking region plus exon 1 exhibited transcriptional activity. These investigators (27) later demonstrated important transcription factor binding sites in exon 1. Another group (20) identified two upstream exons in the rat m1 gene separated from the coding exon by a 14 kb intron, and they found a different promoter (1.5 kb of 5' flanking DNA plus their exon 1) that had activity in neuronal cells. The rat m4 muscarinic receptor gene has also been isolated by two groups, again with slightly differing results. Wood and coworkers (21) identified one upstream exon separated from the single protein coding exon by a 4.8 kb intron, whereas Mieda and associates (23) identified two upstream exons with introns of 0.8 and 4.4 kb. Both groups (22, 28) identified an important neuron-restrictive silencer element (NRSE) at bp -550 or -837 (depending on the numbering system used) that repressed m4 receptor expression in non-neuronal cells. The chick m2 gene contains one upstream exon separated from the coding exon by a large (> 8 kb) intron. This gene has multiple transcription start sites and lacks a TATA box (18). There is at least one important GATA-6 site in the chick m2 receptor gene promoter, and cotransfection studies showed that GATA-6 transactivates a 786 bp m2 promoter in cardiac cells (29).

In common with these other muscarinic receptor genes, the human m3 muscarinic receptor gene has a large intron (at least 20.4 kb) immediately upstream of the single protein coding exon. Its promoter is GC rich, lacks a TATA box, and initiates transcription from a cluster of start sites (Figure 4). Unlike these other muscarinic receptors, though, the m3 receptor gene has 7 untranslated exons (some separated by very large introns), and there is considerable variability in exon splicing. Perhaps similar variation in splicing also occurs in m1 or m4 receptor transcripts and so explains some of the discrepancies of prior reports noted above (19–21, 23). Our studies demonstrate that the 5' flanking DNA in the region -1,240 to +101 exhibits substantial transcriptional activity in cultured tracheal smooth muscle cells. Although this region does not contain an NRSE motif, sequence analysis does disclose potential binding sites for various ubiquitous or tissue-restricted transcription factors.

Eight AP-2 consensus binding motifs occur in the m3 receptor gene promoter between bp -526 and +101, and 5' deletion of bp -526 to -269 to exclude three of these sites markedly reduced transcriptional activity in subconfluent smooth muscle (Figure 5). Conversely, cotransfection with an AP-2 $\alpha$  expression plasmid increased transcription from both -526 and -269 m3 receptor promoter constructs substantially (Figure 6A). Together, these results implicate AP-2 as an important transcriptional activator of m3 muscarinic receptor expression. Furthermore, airway myocytes deprived of serum for 7 d demonstrated a specific and marked increase in m3 receptor promoter activity (Figure 6B). Long-term serum deprivation is known to increase AP-2 transcription-promoting activity in these cells (15), and so it seems likely that some of the m3 receptor



**Figure 6.** Influence of AP-2 $\alpha$  overexpression or long-term serum deprivation on transcriptional activity of two human m3 muscarinic receptor gene promoter fragments during transient transfection analysis in cultured canine tracheal myocytes; -526 and -269 indicate m3 receptor promoter fragments containing bp -526 to +101 or -269 to +101, respectively. (A) In subconfluent, serum-fed myocytes, cotransfection with expression plasmid encoding human AP-2 $\alpha$  increases activity of the -526 or -269 m3 receptor promoter fragments by 3.5-fold or 4.8-fold, respectively, but has little effect on transcription from the 3.3 kb human smooth muscle myosin heavy chain promoter (smMHC) (38% increase). *Open bars*, empty vector; *shaded bars*, AP2  $\alpha$  expression vector. (B) Seven days serum deprivation enhances transcription from the -526 or -269 m3 receptor promoter fragments by 4.5-fold or 5.3-fold, respectively, but reduces smMHC promoter activity by 85%. *Open bars*, subconfluent serum-fed cells; *shaded bars*, 7 d serum-deprived cells.

increases activity of the -526 or -269 m3 receptor promoter fragments by 3.5-fold or 4.8-fold, respectively, but has little effect on transcription from the 3.3 kb human smooth muscle myosin heavy chain promoter (smMHC) (38% increase). *Open bars*, empty vector; *shaded bars*, AP2  $\alpha$  expression vector. (B) Seven days serum deprivation enhances transcription from the -526 or -269 m3 receptor promoter fragments by 4.5-fold or 5.3-fold, respectively, but reduces smMHC promoter activity by 85%. *Open bars*, subconfluent serum-fed cells; *shaded bars*, 7 d serum-deprived cells.

promoter upregulation observed reflects serum deprivation-induced enhancement of AP-2 activity. Because long-term serum deprivation also increases the abundance of m3 muscarinic receptor protein, restores functional coupling of cholinergic m3 receptor stimulation to intracellular calcium mobilization, and restores acetylcholine-induced contraction of cultured airway myocytes (25, 26), it is conceivable that transcriptional upregulation of the m3 muscarinic receptor gene is required for full expression of the contractile phenotype in airway smooth muscle. Long-term serum deprivation induces the contractile phenotype in a small proportion ( $\sim 1/6$ ) of cultured airway smooth muscle cells (25, 26), and immunostaining localizes most m3 receptor protein to this subpopulation (25). Previously, we showed that both contractile and non-contractile phenotype cells are transfected with approximately similar efficiency (15), but it remains conceivable that the m3 muscarinic receptor gene promoter is differentially activated among myocytes of different phenotypes in long-term serum-deprived cultures. Our study did not address other potential mechanisms that might also regulate m3 receptor transcription during long-term serum deprivation.

The human muscarinic m3 receptor gene promoter contains a number of other potential transcription factor binding sites, including eleven consensus GATA factor motifs. Because GATA factors have been implicated in the regulation of m2 receptor gene transcription in cultured cardiac myocytes, it is conceivable that GATA factors also transactivate the m3 receptor gene promoter in some cell types. Also interesting is the presence of many consensus motifs for lymphocyte-associated nuclear factors, includ-

ing those of the Ikaros family (30–33), LyF-1 (34, 35), and NF-AT (36–39). M3 muscarinic receptors are expressed on peripheral lymphocytes (where they are the most abundant muscarinic subtype) (40–44), and m3 receptor stimulation mediates calcium mobilization from intracellular stores in Jurkat T cells (44). Given that the m3 muscarinic receptor is expressed in a wide range of cell types, but is not ubiquitous, the rich structure of its promoter region may allow for its transcriptional activation through a variety of lineage-specific regulatory programs.

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