Neural Specific Expression of the m4 Muscarinic Acetylcholine Receptor Gene Is Mediated by a RE1/NRSE-type Silencing Element*

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Muscarinic receptor genes are members of the G-protein receptor superfamily that, with the inclusion of the odorant receptors, is believed to contain over a thousand members. Each member of this superfamily, which has been studied to date, appears to have a distinct pattern of expression, but little work has been done on the regulation of these complex expression patterns. We have recently isolated the rat m4 muscarinic receptor gene and identified a genomic 1520-nucleotide sequence that appeared capable of directing cell-specific expression (Wood, I. C., Roopra, A., Harrington, C., and Buckley, N. J. (1995) J. Biol. Chem. 270, 30933-30940). In the present study we have constructed a set of deletion promoters constructs to more closely define the DNA elements that are responsible for m4 gene expression. We have found that deletion of a RE1/NRSE silencer element between nucleotides 574 and –550, similar to that found in other neural specific genes, results in activation of reporter expression in non-m4-expressing cells. Gel mobility shift analysis has shown that a protein present in nonexpressing cells is capable of binding to this element and is probably the recently identified neural silencer, REST/NRSE. Of the constitutively active proximal promoter only a tandem Sp-1 site appears to be capable of driving cell-specific expression in vitro (1). This study we have identified DNA elements responsible for regulating cell-specific expression of the m4 gene. We have also used a gel mobility shift assay to identify genomic elements in the m4 promoter that are capable of recruiting DNA binding proteins.

MATERIALS AND METHODS

Cell Culture—Cell lines were cultured in 5% CO2 at 37 °C in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) containing 6 g/liter penicillin, 10 g/liter streptomycin, and 2 mM l-glutamine, 0.1 mM pyruvate, supplemented with either 10% fetal calf serum, (NG108–15, CHO, and 3T3 cells) or 5% fetal calf serum and 5% horse serum (PC12 cells).

Reporter Plasmid Construction—Reporter plasmids were constructed using a modified version of pGL3 basic (Promega), pGL3c.l. Primers to sequences of the m4 promoter, containing KpnI linkers were used in conjunction with Rm4x6a containing a BglII linker in polymerase chain reaction to amplify fragments of the m4 promoter ranging in size from 130 to 1520 bp. These were cloned into a KpnI/BglII-cut pGL3c to generate the pGL3c –1440/+80 to pGL3c –50/+80 series of constructs. In order to construct the pGL3c –151/+80 RE1s and pGL3c –151/+80 RE1a, two complimentary oligonucleotides were synthesized to the region between –574 and –350 of the m4 promoter and constructed such that when annealed they contain Acc51 cohesive ends. These oligonucleotides were cloned into the Acc51 site of pGL3c –150/+80 and recombinants screened for orientation.

Cell Transfections—Qiagen column-purified DNA was transfected into cells using lipofectamine (Life Technologies, Inc.) as described previously (1). 2–3 days after transfection, cells were harvested into reporter lysis buffer (Promega), and the lysate was freeze/thawed and stored at –80 °C. Luciferase measurements were carried out using the Promega luciferase assay system, according to manufacturers instructions in a Turner TD-20e luminometer. β-galactosidase measurements were carried out using an o-nitrophenyl-β-D-galactopyranoside assay (13) and used to normalize luciferase result.

Protein Isolation—Nuclear protein was extracted from tissue culture cell lines as described by Dent and Latchman (14). Aliquots of proteins were stored in liquid nitrogen until use. The concentration of proteins in the extracts was determined using a DC protein assay kit (Bio-Rad).

Gel Mobility Shift Assays—Radiolabeled DNA probes were produced either by Klenow fill-in of an EcoRI- or HindIII-cut DNA fragment using [γ-32P]ATP or by polymerase chain reaction with an oligonucleotide labeled using [γ-32P]ATP. Probes were run on an acrylamide gel and purified using standard protocols (13). For the assays, 2–20 μg of nuclear protein was preincubated on ice, with or without competitor

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1 The abbreviations used are: bp, base pair(s); CHO, Chinese hamster ovary.
DNA, for 20 min in 19 μl of solution containing 20 mM HEPES (pH 7.9), 100 mM KCl, 5 mM MgCl2, 8% glycerol, and 1 μg of poly(dI-dC). Approximately 10–20,000 cpm of radioactive probe was added to each reaction, and incubation continued at room temperature for a further 20 min. For supershift experiments, 1 μl of Sp-1 antibody (1 mg/ml; Santa Cruz) was added, and the reactions were incubated for a further 30 min at room temperature. Reactions were run on 0.5 × Tris borate/EDTA polyacrylamide gels, fixed, dried, and exposed to x-ray film. Complimentary oligonucleotides with the following sequence were annealed and used for competition experiments: RE1, 5′-GTACGGAGCTGTCGGAGTTGCTGAATGTCCTC-3′; m4 Sp-1, 5′-GATCCCCTTTAGGAGGGCGGAGGCGG-3′; consensus Sp-1, 5′-GACTCCGATCGGGCGGAGGCGG-3′; and Non Spec, 5′-AATTCGCGGAGGGCCGATCG-3′.

Southwestern Analysis—Nuclear protein (100 μg) was denatured in SDS-PAGE loading buffer, and proteins were resolved on a 5% SDS-PAGE gel (13). After running the proteins, they were transferred to nitrocellulose using a Trans-blot cell (Bio-Rad). The proteins were reanimated by exposing them to decreasing concentrations of guanidine as described in Ref. 15. Blocking of the membrane was achieved using a 5% non-fat dried milk solution, and the blot was probed with 1 μl of Sp-1 antibody (1 mg/ml; Santa Cruz) was added, and the reactions were incubated for a further 30 min at room temperature. Reactions were run on 0.5 × Tris borate/EDTA polyacrylamide gels, fixed, dried, and exposed to x-ray film. Complimentary oligonucleotides with the following sequence were annealed and used for competition experiments: RE1, 5′-GTACGGAGCTGTCGGAGTTGCTGAATGTCCTC-3′; m4 Sp-1, 5′-GATCCCCTTTAGGAGGGCGGAGGCGG-3′; consensus Sp-1, 5′-GACTCCGATCGGGCGGAGGCGG-3′; and Non Spec, 5′-AATTCGCGGAGGGCCGATCG-3′.

Western Analysis—After the Southwestern analysis, the presence of Sp-1 protein was detected using an antibody to Sp-1 and an ECL detection kit (Amersham Corp.) as per manufacturer’s instructions.

RESULTS

m4-specific Expression Is Confirmed through Repression of a Constitutive Activator—Transfection assays with deletion constructs of the m4 promoter driving expression of the luciferase gene are shown in Fig. 1. Of the four cell lines tested, NG108–15 and PC12 cells both express the m4 gene, whereas CHO and 3T3 cells do not. The two larger constructs, pGL3lc−1440/+80 and pGL3lc−677/+80 are both capable of driving expression in the two m4-expressing cell lines but are silent in the nonexpressing cell lines (Fig. 1). Deletion of the region of the m4 promoter between −677 and −271, to give pGL3lc−271/+80, resulted in constitutive expression in all four cell lines, suggesting that there is a silencing element within this region. Sequence analysis of this region identified a 28-bp sequence with homology to a RE1/NRSE repressor element that has been implicated in repression of other neuronal-specific genes such as the sodium type II channel gene (16), the SCG 10 gene (17), the dopamine β-hydroxylase gene (18), the Na,K-ATPase α3 subunit (19), and the synapsin I gene (20, 21) (see Fig. 2 for a comparison). The proximal m4 promoter appears to be constitutively active, implicating the ability of ubiquitous factors to bind to this region and activate transcription.

To ascertain whether the RE1/NRSE homologous sequence was indeed responsible for repression of m4 expression in these cell lines, 2 constructs were made that contained 28 bp of promoter sequence corresponding to nucleotides −574 to −550 ligated immediately upstream of the proximal promoter region (plasmids pGL3lc−151/+80 RE1a and pGL3lc−151/+80 RE1a). Both of these plasmids, when transfected into cell lines, show repression of reporter activity in 3T3 and CHO cells to basal levels comparable with the levels obtained with pGL3lc−1440/+80. pGL3lc−151/+80 RE1a both show reporter expression in NG108 and PC12 cells, although the levels appear slightly reduced when compared with reporter activity driven by pGL3lc−151/+80. The reason for this effect is not completely clear, although it may simply be an artifact of the juxtaposition of the RE1 site to the −151/+80 fragment.

The RE1/NRSE Element in the m4 Promoter Binds to Proteins Present in Nonexpressing but Not Expressing Cells—The region of the m4 promoter between nucleotides −693 and −332 was labeled and used in a gel mobility shift assay to identify cell extracts that contain proteins capable of binding to this region. The results are shown in Fig. 3 and can be summarized thus. Both CHO and 3T3 cells contain a protein(s) that is/are capable of binding to this DNA fragment, and this binding is competed

Fig. 1. Expression of luciferase reporter constructs in transient transfections of cell lines. All data are normalized to β-galactosidase expression driven by cotransfected pCMVβ. Numbers represent promoter activity as fold over basal, as defined by pGL3lc. Data represent the mean of three individual experiments, each performed in triplicate.
Repression of m4 Promoter

A Tandem Sp-1 Site Within the Proximal Region Is Capable of Recruiting a Host of DNA Binding Proteins—Having identified a repressor region in the m4 promoter that appears responsible for silencing m4 expression in nonneuronal cells, we were interested in identifying which elements in the proximal promoter confer constitutive activation. A gel mobility shift assay using the −151 to +80 region of the m4 promoter was performed. The tandem Sp-1 motif, between nucleotides −67 and −55, was found to be solely responsible for the binding seen with this fragment and was found to recruit a selection of DNA binding proteins in each of the cell lines tested. To more closely examine this binding, two complimentary oligos, covering the sequence −68 to −48, were used in gel mobility shift assays, the results of which are shown in Fig. 4. At least six shifted bands can be seen when the −68/−48 region is incubated with protein from CHO or NG108−15 cells (Fig. 4, lanes 2 and 8). Incubation of the proteins with an antibody to Sp-1 (Fig. 4, lanes 3 and 9) results in a diminished intensity of band a and a supershifted band in lane 3 (+Ab, open arrow). None of the other bands appear to be altered by the presence of the antibody, indicating that these bands are the result of binding of proteins other than Sp-1. In accordance with this, gel mobility shift assays using a consensus Sp-1 probe resulted in a single shifted band that migrated the same distance as band a (data not shown). Competition studies show that each of the bands a−f can be competed with excess unlabeled oligonucleotides containing the m4 tandem Sp-1 sites (lanes 4, 5, 10, and 11). The consensus Sp-1 oligonucleotides compete effectively for bands b and c (Fig. 4, lanes 6, 7, 12, and 13), but do not completely compete for bands c−f. This inefficient competition may be due to a greater affinity of the complex for a tandem Sp-1 site. It is interesting to note that the m4 tandem Sp-1 oligonucleotides appear to compete for binding of bands b−f more completely than for band a. The significance of this, however, is unclear.

The Tandem Sp-1 Sites Bind to a Variety of Proteins Present in Different Tissues—Gel mobility shift assays were performed on the tandem Sp-1 region of the m4 promoter using nuclear protein extracted from various primary tissues. A variety of tissues were selected and included cortex and striatum (two neuronal tissues where m4 is expressed), cerebellum (a neuronal tissue where m4 is not expressed), liver (a tissue that does not express any muscarinic receptor subtype), and lung (a tissue that does not express m4 but does express another muscarinic receptor subtype). The results of the gel mobility shift assay are shown in Fig. 5. Of the seven shifted bands observed (g−m), bands k−m are thought to be due to nonspecific binding even though it does appear that they can be competed to some extent. Protein from the cortex and striatum (lanes 1 and 7), both show the greatest number of shifted bands and both have the same pattern of bands (the low levels of intensity of bands g−j in lane 7 may be due to the quality of the protein purified from the striatum). Band g is probably the result of binding of Sp-1 to the probe as it migrates at the same speed as band a from Fig. 4, and it can be supershifted with an Sp-1 antibody. The most interesting band is band h, as this is produced by cortex and striatum extracts, where m4 is found to be expressed, but not in any of the other extracts where m4 is not expressed. This band is also inefficiently competed by oligonucleotides containing the m4 tandem Sp-1 sites (lanes 1−6). Data were obtained from Refs. 1, 18–23, and 28–31.
deletions containing a single Sp-1 site, suggesting a higher affinity of this DNA binding protein for a tandem Sp-1 site.

The m4 Promoter Interacts with the Larger Isoform of Sp-1—To identify the sizes of proteins that can interact with the m4 proximal promoter, a Southwestern analysis was performed using protein isolated from CHO, NG108–15, and PC12 cell lines and probed with the −149 to +80 region of the m4 promoter. Fig. 6 shows that two different proteins present in the cell lines were found to bind to this region. The larger protein, running at approximately 98 kDa is thought to be Sp-1, as it is the same size as a protein recognized by an Sp-1 antibody. The identity of the smaller, approximately 32 kDa, protein that binds to the m4 promoter region is unknown. A second protein gel was run to better resolve the two Sp-1 isoforms. It can be seen that the m4 promoter only binds to the larger, 106-kDa, isoform of Sp-1 in the three cell lines.

**DISCUSSION**

The present study shows that the m4 muscarinic receptor core promoter is constitutively active in a number of cell lines and that cell-specific expression is mediated via transcriptional repression in nonexpressing cell lines. Deletional analysis of a series of reporter constructs has identified a RE1/NRSE silencer element responsible for this silencing in nonneural cells. This RE1/NRSE element was originally identified in the type II Na⁺ channel and SCG10 promoters (16, 17) and was shown to be responsible for silencing gene expression in nonneural cells. Since then DNA data base analysis has identified this element in 18 genes, most of which are selectively expressed in neural tissue. The sequence shows very high homology in various genes and is well conserved in species as diverse as human and chicken (Fig. 2). However, functional characterization of this element has only been demonstrated for promoters of the type II Na⁺ channel (16), SCG10 (17), synapsin I (21–23), dopamine β-hydroxylase (18), and Na,K-ATPase α3 subunit (19) genes. In the case of the human synapsin I gene, Schoch et al. (23) dissected the promoter and concluded that the REST/NRSF binding motif was solely responsible for the neural specific expression of this gene. In addition, a transgenic analysis of the RE1/NRSE site has been reported for one gene, the Na,K-ATPase α3 subunit (19), and, in this case, 210 bp of the sequence 5’ to the transcription initiation site drove tissue-specific expression. Mutation of the RE1/NRSE site within this region resulted in ectopic expression of the reporter gene in all
nonneuronal tissues tested. In this study, we have demonstrated that deletion of this RE1 element in the m4 gene leads to expression in both nonneuronal and neural cells. Furthermore, direct juxtaposition of the RE1 element (in either orientation) to the constitutively active promoter reestablishes silencing of the gene in nonexpressing cells. Gel mobility shift assays (Fig. 3) showed that the RE1/NRSE sequence in the m4 promoter bound to a protein present in nonexpressing cells but not in m4 expressing cells, consistent with the notion that this sequence recruits a transcriptional repressor.

Genes such as the type II Na+ channel, SGC10, and synapsin I, in which the RE1 silencing element has functional activity are all pan-neuronal genes that are widely expressed in most neuronal cells. In contrast, the m4 gene is expressed predominantly in a subset of telencephalic neurones (7) and autonomic neurones (9, 10). Since REST/NRSF is not expressed in any differentiated neuronal cells in the central nervous system (24, 25), it follows that there must be a set of neuronal cells that does not express REST/NRSF and that does not express the m4 gene. Therefore the m4 gene must be under the control of other regulatory factors in addition to REST/NRSF. Two possibilities arise. 1) Another repressor protein or set of proteins is responsible for silencing m4 expression in those cells that do not express m4. 2) In vivo the m4 proximal promoter may not be constitutively active in neuronal cells, and specific activators would drive expression in neuronal subpopulations. The latter possibility infers that in vitro transcriptional regulation may not accurately reflect gene regulation in vivo. Many examples exist of these disparities, such as the nicotinic α receptor gene where a proximal promoter drives myotube-specific expression in vitro but fails to recapitulate specific expression in transgenic mice (26).

In order to identify other DNA elements in the proximal promoter that are important in defining m4 gene expression, a series of gel shift assays was performed. All of the observed band shifts were found to be attributable to a tandem Sp1 site located between nucleotides –68 and –48. This DNA element was found to bind to several proteins, including Sp1, in both expressing and nonexpressing cell lines. Because of the limited phenotypic of the cell lines, protein isolated from a selection of different tissues was analyzed using this tandem Sp1 site. A similar pattern of shifted bands was seen in the neural tissues used with the exception of one of the bands, band h. This band was present in cortex and striatum, which both express m4 but absent in the other tissues. It is tempting to equate a specific DNA binding protein present in the tissues that express m4 with that of a transcriptional activator, but proof of this hypothesis awaits functional data.

Recently another group have published results of experiments characterizing the rat m4 promoter (27). The conclusions drawn by Mieda et al. (27) are very similar to our original work (1) except in the position of the transcription initiation site, which they claim is located 336 bp downstream from the initiation point defined previously by us. We have compared the expression levels obtained in NG108 and PC12 cell lines with our reporter constructs to the levels reported by Mieda et al. (27) and found them to be practically identical, despite the fact that their constructs include a further 270 bp of exon 1 as defined in Ref. 1. From this, we conclude that our constructs contain the major m4 transcription start site and sequences between positions +80 and +350 have no effect on expression levels in NG108 and PC12 cells. In fact, the nuclease protection data of Mieda et al. (27) do show evidence of a transcription start site upstream from the one that they define.

The use of two independent but complimentary approaches to analyzing the control of m4 expression has greatly aided us in the search for important transcriptional control elements. Transient transfections identified a repressor element that appears responsible for silencing m4 expression in nonneuronal cells. Gel mobility shift assays were used to identify other elements that are capable of recruiting DNA binding proteins. This approach has led us to identify a tandem Sp1 motif in the proximal region that interacts with a variety of protein species including one that is present only in those brain regions where m4 is expressed.

In summary, we have demonstrated that the proximal promoter of the m4 cholinergic receptor gene is constitutively active in both neural and nonneuronal cells and that a distal RE1/NRSE silencing element is responsible for restricting expression to neural cells. Clarification of the role of this element and others in restricting expression to neuronal subpopulations in vivo must await analysis in a transgenic mouse model.

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