Expression of the Rat m4 Muscarinic Acetylcholine Receptor Gene Is Regulated by the Neuron-restrictive Silencer Element/Repressor Element 1*

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Neuronal cell-specific expression of the rat m4 muscarinic acetylcholine receptor (mAChR) is regulated by a silencer element. A likely mediator of this silencing is the neuron-restrictive silencer element/repressor element 1 (NRSE/RE1), which is present 837 base pairs (bp) upstream from the transcription initiation site of the m4 mAChR gene (Wood, I. C., Roopra, A., Harrington, C., and Buckley, N. J. (1995) J. Biol. Chem. 270, 30933–30940; Mieda, M., Haga, T., and Saffen, D. W. (1996) J. Biol. Chem. 271, 5177–5182). In the present study, we examined whether this putative NRSE/RE1 functions as a silencer. Transient expression assays using m4 mAChR promoter/luciferase expression vectors showed that the m4 NRSE/RE1 is necessary and sufficient to repress m4 promoter activity in non-neuronal L6 cells. m4 promoter activity was only partially repressed, however, in neuronal NG108-15 cells exogenously expressing the neuronal-restrictive silencer factor/RE1-silencing transcription factor (NRSF/REST). By contrast, the promoter activity of the type II sodium channel (NaII) gene was nearly completely repressed in NRSF/REST-expressing NG108-15 cells. Experiments with expression vectors containing chimeric promoters revealed that the NRSE/RE1 elements derived from both the m4 and NaII genes are independently sufficient to silence NaII gene promoter activity, but only partially repress m4 mAChR gene promoter activity in NRSF/REST-expressing NG108-15 cells. Thus, the repression activity of NRSE/REST depends upon the species of promoter to which it is linked. Gel-shift assays showed that the NRSF/REST is the only protein that binds to a 92-bp segment from the m4 mAChR promoter containing NRSE/RE1. This and the fact that m4 promoter activity was completely repressed in L6 cells suggest that the proteins that bind to the m4 constitutive promoter may be different from those in NG108-15 cells. Deletion analysis of the m4 constitutive promoter revealed that a 90-bp segment immediately upstream from the transcription initiation site contains significant promoter activity. Gel-shift assays revealed that several proteins in nuclear extracts prepared from L6 and NG108-15 cells bind to this 90-bp segment and that some of these proteins are L6 or NG108-15 cell-specific. These data support the idea that the repression activity of NRSF/REST depends upon the species of promoter to which it is linked and upon the proteins that bind to those promoters.

Muscarinic acetylcholine receptors (mAChRs) are the members of the G-protein-coupled receptor superfamily. Five subtypes of mAChR (m1–m5) have been identified by molecular cloning (1). Each subtype of mAChR shows a unique distribution in peripheral tissues and brain (2, 3), but mechanisms that underlie differential expression of each subtype have not yet been determined.

Recently, we and others isolated the promoter region of the rat m4 mAChR gene and demonstrated that the neuronal cell-specific expression of this gene is regulated by a silencer element (4, 5). The segment of the promoter region required for this silencing contains a neuron-restrictive silencer element/repressor element 1 (NRSE/RE1). The existence of this element is consistent with the exclusive expression of the m4 gene in neurons, although its expression has also been detected in smooth muscle of rabbit lung (but not human or pig lung) (6–8).

The NRSE/RE1 was initially identified as a silencer element that regulates neuron-specific expression of the rat SCG10 (9) and rat sodium channel type II (NaII) genes (10). In addition to these two genes, the NRSE/RE1 is known to function in neuron-specific expression of the human synapsin I gene (11, 12), rat Na,K-ATPase α3 subunit gene (13), and chick neuron-glial cell adhesion molecule gene (14). Recently, a zinc finger protein termed neuron-restrictive silencer factor/RE1-silencing transcription factor (NRSF/REST) was cloned and shown to repress the activities of the constitutive promoters of the rat SCG10 and NaII genes by binding to the NRSE/RE1 sequence in non-neuronal cell lines (15, 16).

In the present study, we have shown that the NRSE/RE1 sequence regulates the neuronal cell-specific expression of this gene and that NRSF/REST significantly represses its expression. We also discuss the mechanism underlying NRSF/REST function in terms of the constitutive promoter region to which it is linked.

EXPERIMENTAL PROCEDURES

Cell Culture—L6 (a rat skeletal muscle myoblast cell line) and NG108-15 cells (a hybrid cell line derived from mouse neuroblastoma N18 and rat glioma C6) were cultured as described previously (4).

Constructions—A series of constructs, in which the NRSE/RE1 se-
NRSE/RE1 Regulates m4 mAChR Gene Expression

NRSE/RE1 was cloned in the BamHI site of the plasmid pUC119 and then re-isolated by digestion with BamHI and KpnI. The sequence was digested with PstI and KpnI. The largest fragment that contains the constitutive promoter region of the rat m4 mAChR gene and the luciferase gene was isolated. This fragment was ligated to the pSt-Kpn1 fragment containing the NRSE/RE1 sequence. pGL2-SaP90 (Fig. 4) was deleted from the SaP1-Sau fragment. This fragment was digested with SaP1 and Kpn1, and the short fragment was isolated and cloned in the largest of the fragments derived by digestion of pGL2-ScP1600 with SaP1 and Kpn1. In constructs pCAT-P435 and pCAT-P1074, promoter regions contained in pGL2-P1074 were cloned upstream of the chloramphenicol acetyltransferase (CAT) gene. These constructs were generated as follows. pGL2-P1074 was digested with XhoI and partially with XhoI, and the fragment containing appropriate m4 promoter regions were cloned in the SaP1 and XhoI sites of the pBLCAT2 (obtained from ATCC). These constructs were digested with XhoI following by blunt-end, and then partially digested with HindIII. Resultant fragments containing m4 promoter regions were ligated to the CAT-containing fragment obtained by digestion of pSDK7 (10) (a gift from Dr. G. Mandel) with Pacl and HindIII, in which the NaI/Na2 fragment was directly linked to the constitutive promoter region of the m4 mAChR gene, was generated as follows. The SaP1 site was blunt-ended. Construct pCAT-SauP92P435, in which the Sa3AI fragment (residues 895 to 803, containing the NRSE/RE1 sequence) was directly linked to the constitutive promoter region of the m4 mAChR gene, was obtained as follows. The Sa3AI fragment was cloned in the BamHI site of the plasmid pBluescript SK(+) (pBS-Sau92). pBS-Sau92 was digested with XhoI, blunt-ended, and then digested with PstI to re-isolate the Sa3AI fragment. This fragment was ligated to pGL2-P1074 and pGL2-P1074, respectively, and digested with Spal and HindIII, in which the Sa3AI and Spal sites were blunt-ended. Construct pCAT-Nam4, in which the NRSE/RE1 containing HindIII-BglII fragment derived from the NaI gene (10) was fused to the constitutive promoter region of the m4 mAChR gene, was generated as follows. The Pacl site was blunt-ended. Construct pCAT-SauP92P435, in which the Sa3AI fragment (residues 895 to 803, containing the NRSE/RE1 sequence) was directly linked to the constitutive promoter region of the NaI gene, was constructed as follows. The Sa3AI fragment was cloned in the BglII site of the pGL2-Basic vector and then re-isolated by digestion with BamHI and PstI, and partially with PstI. This fragment was ligated to the pSDK7 digested with BglII and PstI, pCAT-Sau92Na, in which the Sa3AI fragment derived from m4 mAChR gene (residues 895 to 803, containing the NRSE/RE1 sequence) was directly linked to the constitutive promoter region of the NaI gene, was constructed as follows. The Sa3AI fragment was cloned in the BglII site of the pGL2-Basic vector and then re-isolated by digestion with BamHI and PstI, and partially with PstI. This fragment was ligated to pSDK7 digested with HindIII and BglII. pEF-REST, in which expression of NRSF/REST is regulated by the human elongation factor 1α promoter, was obtained as follows. REST-Express (16) (a gift from Dr. G. Mandel) was digested with HindIII and XhoI, and the fragment encoding NRSF/REST was isolated and cloned between the HindIII and SaI sites of pUC119. The fragment encoding NRSF/REST was then isolated again in the presence of KpnI and HindIII and cloned in pBluescript SK(+) (pBS-ScP90). Finally, the fragment encoding NRSF/REST was isolated by digestion with XbaI and was cloned in the XbaI site of mammalian expression vector pEF-BOS (18). Constructs pGL2-NP376, pGL2-NP206, pGL2-NP143, and pGL2-NP1 (Fig. 4) were generated by cloning appropriate NheI-Xhol fragments derived from pGL2-P1074 in the SmaI and XhoI sites of the pGL2-Basic vector. pGL2-NP296 (Fig. 4) was obtained by deleting the 1.3-kb KpnI-NruI fragment from pGL2-ScP1600. pGL2-SmP90 (Fig. 4) was obtained by deleting the 1.5-kb SmaI fragment from pGL2-ScP1600.

Results

The NRSE/RE1 Regulates Neuronal Cell-specific Expression of the m4 mAChR Gene—Previously we found that neuronal cell-specific expression of the rat m4 mAChR gene is regulated by a silencer element and that the promoter region contains a putative NRSE/RE1 in an inverted orientation. In this study, we used transient transfection assays to determine whether the NRSE/RE1 of the m4 mAChR gene is a functional silencer. For this purpose, we constructed luciferase reporter plasmids in which the NRSE/RE1 is directly linked to the constitutive promoter region of the m4 mAChR gene (the proximal 435 bp sequence of the 5'-flanking region; Ref. 4) (Fig. 1A). Fusion of the NRSE/RE1 derived from the m4 mAChR gene repressed luciferase induction by the m4 mAChR constitutive promoter approximately 10-fold in L6 myoblast cells, which do not express the endogenous m4 mAChR gene. This repression was independent of the orientation of the NRSE/RE1 (m4-f and m4-r, respectively), contain the NRSE/RE1 in the same and reversed orientation compared to that in the genome). Similar repression was shown with the NRSE/RE1 derived from the NaI gene (Na-f and Na-r). By contrast, luciferase activities were not changed by the presence of NRSE/RE1 sequences in NG108-15 cells, which express the endogenous m4 mAChR gene. We also examined the effect of the mutant form of the NRSE/RE1 (constructs m4m-f and m4m-r). In the mutant form, two cytosine residues of the m4 NRSE/RE1 were substituted with adenine residues (Fig. 1B). Homologous mutations in the NRSE/RE1 of the rat SCG10 and human synapsin I genes are known to abolish the binding of a repressor protein and its
silencer activity (9, 11). As expected, this mutant did not have silencer activity.

Deletion of a Sau3AI fragment (residues −895 to −803) from pGL2-P1074, pGL2-P1074dSau92, resulted in recovery of luciferase activities in L6 cells (Fig. 1A). The extent of derepression was more than 10-fold compared to pGL2-P1074, and activities were comparable to ones observed in NG108-15 cells. These data indicate that the NRSE/RE1 sequence of the m4 mAChR gene functions as a silencer in a way similar to NRSE/RE1 sequences found in other neuron-specific genes and that the NRSE/RE1 is necessary and sufficient to repress the expression of the m4 mAChR gene in non-neuronal cells.

Nuclear Extracts from Non-neuronal Cells Contain a Protein That Binds to the NRSE/RE1 of the m4 mAChR Gene—To determine if there is any nuclear protein that binds to this silencer element, we carried out gel-shift assays using nuclear extracts from L6 cells and NG108-15 cells. We detected a nuclear protein that bound to a 92-bp Sau3AI fragment containing the NRSE/RE1 (residues −895 to −803) only in L6 nuclear extracts (Fig. 2A). This binding was inhibited by addition of an excess of the NRSE/RE1 sequences derived from the m4 mAChR gene, but was not inhibited by the mutant form of the m4 mAChR NRSE/RE1 (the same mutant as described above) or by a Sp1 binding sequence (as a negative control), showing that binding is specific to the NRSE/RE1. Addition of an excess of the NRSE/RE1 derived from the NaII gene also inhibited this binding, suggesting that the same protein, probably NRSF/REST, binds to the NRSE/RE1 derived from both m4 mAChR and NaII genes. The extent of the mobility shift of this band was about the same as that obtained with the HindIII-BglII fragment derived from the rat NaII gene promoter (residues −1051 to −937; Ref. 10) containing the NRSE/RE1 sequence (data not shown). When the Sp1 consensus binding sequence was used as a probe, we found binding proteins in both L6 and NG108-15 extracts, suggesting that the cell type-specific binding activity to the NRSE/RE1 is not due to a failure in preparing the nuclear extracts (Fig. 2B). These data and results of transient expression assays described above indicate that a repressor protein binds to the NRSE/RE1 and represses the expression of the m4 mAChR gene in non-neuronal cells.

Exogenous Expression of NRSF/REST Partially Represses Promoter Activity of the m4 mAChR Gene—A strong candidate for this repressor protein is NRSF/REST, which was recently cloned and shown to repress the activities of the constitutive promoters of the rat SCG10 and NaII genes in non-neuronal cell lines by binding to the NRSE/RE1 sequence (15, 16). NRSF/REST is also known to be expressed in L6 cells (16). To examine whether NRSF/REST can repress m4 mAChR promoter activity, we co-transfected a NRSF/REST expression plasmid with CAT reporter plasmids into NG108-15 cells (Fig. 3). Exogenous
as probe. Sequences of these oligonucleotides are shown in “Experimen-
tal Procedures” and Fig. 1. Panel B, gel-shift assay using the Sp1
consensus binding sequence as a probe.

expression of NRSF/REST repressed promoter activity of
NRSE/RE1-containing reporter plasmid pCAT-Sau92P435
(residues −895 to −803 and −435 to +19) approximately 2-fold
(Fig. 3A). Activity of pCAT-P435 (residues −435 to +19), which
does not contain the NRSE/RE1, was not repressed by exoge-
 nous expression of NRSF/REST, suggesting that NRSF/REST
functions via the NRSE/RE1. We carried out the same co-
transfection assay with reporter constructs containing the pro-
moter of the NaII gene as a positive control. The promoter
activity of the rat NaII gene was repressed approximately
10-fold by exogenously expressing NRSF/REST protein in
NRSE/RE1-dependent manner (Fig. 3A, pSDK7 and pSDK7/
dHB). Since promoter activity of the m4 mAChR gene is approxi-
mately 15-fold higher than that of the NaII gene (pCAT-
P435 versus pSDK7/dHB), we cannot directly compare the
effect of exogenous NRSF/REST expression on the m4 mAChR
promoter to that on the NaII promoter. But when pCAT-
Sau92P435 or pSDK7 were introduced into L6 cells, their ac-
tivities were repressed almost completely (Fig. 3B), although
the difference of activities between pCAT-P435 and pSDK7/
dHB was more than that in NG108-15 cells (approximately
20-fold). These data suggest that exogenous expression of
NRSF/REST represses promoter activity of the m4 mAChR
gene to a much lesser extent than that of NaII gene and is not
sufficient for the repression of the m4 mAChR gene expression.
There are two possible explanations for this difference be-
 tween NaII and m4 mAChR genes. One is that NRSF/REST
binds to the NRSE/RE1 of the m4 mAChR gene with lower affinity
than that of NaII gene. The other is that NRSF/REST can bind to
the NRSE/RE1 effectively but other factor(s) are also required for
full repression of the m4 mAChR gene. To discriminate be-
tween these two possibilities, we constructed chimera reporter
plasmids of m4 mAChR and NaII gene promoter regions and
co-transfected with the NRSF/REST expression plasmid (Fig.
3C). Exogenous expression of NRSF/REST repressed promoter
activity of pCAT-Sau92Na by approximately 90% but that of
pCAT-NaP435 only by 50%, showing that the NRSE/RE1 de-
ferred from the m4 mAChR gene represses the constitutive
promoter of the NaII gene to an extent similar to that of the
NRSE/RE1 derived from the NaII gene. These results suggest
that the character of the constitutive promoter is important for
NRSF/REST function, supporting the hypothesis that some
factor(s) other than NRSF/REST are required for silencing.

Fig. 2. Detection of a nuclear protein in L6 nuclear extracts
that binds to the NRSE/RE1 sequence of the m4 promoter. Panel
A, gel-shift assay using the m4 NRSE/RE1 as a probe. A 92-bp Sau3AI
fragment derived from the m4 mAChR gene was labeled and used as a
probe to detect binding activities in nuclear extracts prepared from L6
and NG108-15 cells. For competition assays, an excess of unlabeled
oligonucleotides was added (at the molar ratios indicated). NaII, NRSE/
RE1 derived from the rat NaII gene; m4, NRSE/RE1 derived from the
rat m4 mAChR gene; m4m, mutant form of m4; Sp1, consensus binding
sequence for transcription factor Sp1; Sau92, the same fragment used
as probe. Sequences of these oligonucleotides are shown in “Experimen-
tal Procedures” and Fig. 1. Panel B, gel-shift assay using the Sp1
consensus binding sequence as a probe.

The 90-bp Segment Proximal to the Transcription Start Site
of the m4 mAChR Gene Produces Significant Promoter Activity—If the above explanation is correct, how is it that the m4
mAChR gene is completely repressed in L6 cells? We have
shown that the NRSE/RE1-containing 92-bp Sau3AI fragment
is sufficient to repress the promoter activity of the m4 mAChR
gene in L6 cells (Figs. 1 and 3B) and that NRSF/REST is
apparently the only protein that binds to that fragment (Fig. 2).
NRSF/REST is also the only protein that binds to the 92-bp
Sau3AI fragment in NG108-15 cells that express NRSF/REST
exogenously. These results suggested that the difference in
the ability of NRSF/REST to silence in L6 and NG108-15 cells may
depend upon differences in the proteins that bind to the con-
stitutive m4 promoter in these cell lines. We therefore decided
to analyze the constitutive promoter region of the m4 mAChR
gene to test this possibility. For this purpose we constructed
luciferase reporter plasmids containing various lengths of the
435-bp constitutive promoter region and carried out transient
transfection assays (Fig. 4). Serial deletions of pGL2-P435 from
the 5’ side gave similar profiles of luciferase activities in L6
and NG108-15 cells. In both cell lines, a reporter plasmid
containing a 90-bp fragment just upstream to the transcription
initiation site (pGL2-SmP90) produced significant luciferase
activities (more than 20-fold increase compared to pGL2-NP1).

Nuclear Proteins That Bind to the Proximal 90-bp Fragment
of the Promoter Region of the m4 mAChR Are Different between
L6 and NG108-15 Cells—We next used gel-shift assays to ex-
amine whether any nuclear proteins bind to this 90-bp frag-
ment. We detected several proteins that bind to the 109-bp
FIG. 3. Repression of m4 mAChR promoter activity by exogenous expression of NRSF/REST protein in NG108-15 cells. Panel A, NRSF/REST expression plasmid pEF-REST was co-transfected with reporter plasmids. In pEF-REST, NRSF/REST cDNA is cloned downstream of the human elongation factor 1α promoter. The CAT reporter plasmid pSDK7 (a gift from Dr. G. Mandel) includes a NRSE/RE1-containing HindIII-BglII fragment (residues 1051 to 933) and the constitutive promoter (residues −134 to +177) of the rat NaII gene (10). pSDK7/dHB, which contains only the NaII constitutive promoter region, was constructed by deleting the HindIII-BglII fragment from pSDK7. pCAT-P435 contains the constitutive promoter region of the m4 mAChR gene (residues −435 to +19). pCAT-Sau92P435 includes the constitutive promoter region and a NRSE/RE1-containing Sau3AI fragment from the m4 mAChR gene (residues −895 to −803). We measured the activity of pSDK7/dHP to determine background activity. pSDK7/dHP contains no promoter sequence and was obtained by deleting HindIII-PstI fragment from pSDK7. The activity of each reporter plasmid co-transfected with vector that does not contain NRSF/REST cDNA (pEF-BOS) was normalized to 100%. The results, expressed as mean ± S.E., represent data obtained in at least three independent experiments, in which each assay was performed in duplicate.

Panel B, CAT reporter plasmids were introduced in L6 cells. Activities of pSDK7 and pCAT-Sau92P435 are expressed as percent of activities of pSDK7/dHB and pCAT-P435, respectively. The results represent data obtained in two independent experiments, in which each assay was performed in duplicate. Panel C, m4 mAChR promoter-NaII promoter chimera reporter plasmids, pCAT-Nam4 and pCAT-Sau92Na, were co-transfected with NRSF/REST expression plasmid pEF-REST. pCAT-Nam4 contains a HindIII-BglII fragment from the NaII gene and the constitutive promoter region of the m4 mAChR gene. pCAT-Sau92Na contains a Sau3AI fragment (residues −895 to −803) from the m4 mAChR gene and the constitutive promoter region of the NaII gene. The results, expressed as mean ± S.E., represent data obtained in three independent experiments, in which each assay was performed in duplicate.
SmaI-PstI fragment (residues −90 to +19) in both L6 and NG108-15 nuclear extracts (Fig. 5). Some of these were NG108-15-specific (bands a and b) or L6-specific (bands d–f), suggesting that different proteins bind to this SmaI-PstI fragment in NG108-15 and L6 cells. No band disappeared upon addition of an excess of Sp1 binding sequence, indicating that no bands are shifted-up bands due to Sp1 binding, even though this SmaI-PstI fragment contains Sp1 consensus binding sequences (residues −87 to −82 and −83 to −78). Bands c and f were weakly competed by an excess of unlabeled SmaI-PstI fragment but not by a Sp1 binding sequence, suggesting that these bands resulted from specific binding to the SmaI-PstI fragment.

DISCUSSION

In this study, we have shown that the NRSE/RE1 regulates neuronal cell-specific expression of the rat m4 mAChR gene (Fig. 1). Nuclear extracts from the non-neuronal cell line L6 contain a protein that binds to the NRSE/RE1 derived from the m4 mAChR gene and is not present in nuclear extracts of m4 mAChR-expressing NG108-15 cells (Fig. 2). The m4 mAChR gene is the first example of a NRSE/RE1-regulated gene among genes whose products are involved in neurotransmission, such as neurotransmitter-synthesizing enzymes, neuropeptide, and receptors. The choline acetyltransferase gene is a candidate for another such gene, since silencers have been implicated in neurotransmission, such as neurotransmitter-synthesizing enzymes, neuropeptide, and receptors. The choline acetyltransferase gene is a candidate for another such gene, since silencers have been implicated in its cholinergic neuron-specific expression, and a sequence highly homologous to the NRSE/RE1 is present in the 5′-flanking region of the rat gene (21–24). Although the NRSE/RE1 regulates the neuron-specific expression of m4 mAChR gene, an additional mechanism is necessary to explain the restricted expression of this gene to specific subsets of neurons. Such a mechanism remains to be elucidated.

We examined whether NRSF/REST mediates repression of the m4 mAChR gene via the NRSE/RE1 using co-transfection assays in NG108-15 cells with CAT reporter plasmids. Initially we tried these co-transfection assays with reporter plasmids containing a luciferase reporter gene such as pGL2-P435 and pGL2-P1074, but exogenous expression of NRSF/REST unexpectedly resulted in NRSE/RE1-independent repression of luciferase activities. In addition, exogenous expression of NRSF/REST repressed luciferase activities even when the reporter plasmids containing NaII constitutive promoter or SV40 early promoter were used (data not shown). Exogenous expression of NRSF/REST may result in its interaction with luciferase gene or luciferase and somehow down-regulate luciferase activity. For this reason, we used the CAT gene as a reporter gene in co-transfection assays. In previous studies, co-transfection assays with NRSF/REST expressing plasmids were also carried out.
out using CAT gene as a reporter (15, 16). Promoter independent down-regulation of the luciferase gene by $T_2$ and $T_3$ receptors has also been reported previously (25). The mechanism of this down-regulation is unknown.

Exogenous expression of NRSF/REST in NG108-15 cells represses promoter activity of the m4 mAChR gene by approximately one-half in a NRSE/RE1-dependent manner (Fig. 3). Since the extent of repression was much less than that observed for the NaII gene, we concluded that repression of the m4 mAChR gene expression by exogenous expression of NRSF/REST is partial in NG108-15 cells. Co-transfection assays with m4 mAChR-NaII promoter chimera reporter plasmids showed that NRSF/REST can bind to the NRSE/RE1 derived from the m4 mAChR gene and represses the NaII gene promoter almost completely, but does not repress the m4 mAChR gene promoter completely. This result is consistent with data obtained by gel-shift assays suggesting that the same protein binds to the NRSE/RE1 derived from the m4 mAChR and NaII genes (Fig. 2). We therefore propose that the difference in repression by NRSF/REST between the m4 mAChR and NaII genes is a consequence of differences in their constitutive promoter regions. Three possible reasons may be given to explain the fact that the 92-bp Sau3AI fragment (residues -895 to -803) allows complete silencing in L6 cells, but not in NG108-15 cells (Fig. 3). First, another repressor protein, which is expressed in L6 cells but is not expressed in NG108-15 cells, binds to the 92-bp Sau3AI fragment at a site other than the NRSE/RE1. Second, a cofactor expressed only in L6 cells is required for NRSF/REST to repress the promoter of the m4 mAChR gene sufficiently. The first two cases do not seem likely, since data obtained by gel-shift assays suggest that only one species of protein binds to this 92-bp Sau3AI fragment (Fig. 2), although there might be other shifted-up bands undetectable in these conditions. The third possibility is that activator proteins that bind to the constitutive promoter region are different between L6 and NG108-15 cells and these are regulated differently by NRSF/REST. Analysis of the constitutive promoter region of the m4 mAChR gene showed that the proximal 90-bp region produces significant promoter activity (Fig. 4). Gel-shift assays showed that several nuclear proteins bind to this region (Fig. 5). Some of them were specific to nuclear extracts from L6 or NG108-15 cells. The existence of cell-specific nuclear proteins that bind to the promoter region is consistent with the third case described above. Gel-shift assays using the constitutive promoter region of the NaII gene as a probe showed that patterns of shifted-up bands were similar among nuclear extracts from both NaII-expressing and -nonexpressing cell lines (10). This fact may explain the difference in repression by NRSF/REST between the m4 mAChR and NaII genes. Further study will be required to prove this hypothesis.

The proximal 90-bp region contains an inverted CAAT box (residues -62 to -58), a putative CRE-like sequence (residues -46 to -39, 7 bases match 8 bases consensus sequence TGACGTCA), and consensus binding site for Sp1 (residues -87 to -78), although Sp1 does not seem to bind to this site (Fig. 5). Shifted-up bands may contain factors that bind these sequences and/or contribute to the transcription initiation complex. Further analyses will be required for the characterization of these nuclear proteins.

In summary, we have shown that the NRSE/RE1 regulates neuronal cell-specific expression of the m4 mAChR gene. Exogenous expression of NRSF/REST repressed its expression significantly but not completely. We propose that the ability of NRSF/REST to repress transcription depends on the species of promoter to which it is linked and activator proteins that bind there. We therefore analyzed the constitutive promoter region of the m4 mAChR gene and found that proximal 90-bp region produced significant promoter activity. Several nuclear proteins bind to this region, some of which are cell type-specific. These data support the idea that the repression ability of NRSF/REST depends on the species of promoter and promoter-binding proteins.

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Addendum—Previously, Wood et al. (5) detected a transcription initiation site at a location different from one we determined (at -293 in our numbering). During preparation of this manuscript, they showed that the NRSE/RE1 represses transcription of the m4 mAChR gene from their transcription initiation site in non-neuronal cells (26). They claimed that inclusion of our transcription initiation site had little effect on promoter activity. However, our data are inconsistent with theirs. Deletion of their transcription initiation site from the 5' side reduced, but left significant promoter activity (Fig. 4). Furthermore, deletion of our transcription initiation site from the 3' side reduced promoter activity to approximately one-third (data not shown). These data indicate that transcription from our transcription initiation site produces a substantial part of the total transcription of the m4 mAChR gene. Taken together, these data suggest that there are at least two basal promoters in the m4 mAChR gene. In our study, luciferase expression produced from reporter plasmids that contains both transcription initiation sites were shown to be repressed by the NRSE/RE1 (Fig. 1).

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