Neuron-restrictive Silencer Factor (NRSF) Functions as a Repressor in Neuronal Cells to Regulate the μ Opioid Receptor Gene*

Received for publication, April 1, 2004, and in revised form, August 9, 2004
Published, JBC Papers in Press, August 18, 2004, DOI 10.1074/jbc.M403633200

Chun Sung Kim‡, Cheol Kyu Hwang, Hack Sun Choi, Kyu Young Song, Ping-Yee Law, Li-Na Wei, and Horace H. Loh

From the Department of Pharmacology, University of Minnesota Medical School, Minneapolis, Minnesota 55455

The μ opioid receptor (MOR) is expressed in the central nervous system and specific cell lines with varying expression levels perhaps playing important roles. One of the neuronal-specific transcription regulators, neuron-restrictive silencer factor (NRSF), has been shown to repress the expression of neuron-specific genes in non-neuronal cells. However, we showed here that the neuron-restrictive silencer element (NRSE) of MOR functions as a critical regulator to repress the MOR gene expression in specific neuronal cells depending on NRSF expression level. Using co-transfection studies, we showed that the NRSE of the MOR promoter is functional in NRSF-positive cells (NS20Y and HeLa) but not in NRSF-negative cells (PC12). NRSF binds to the NRSE of the MOR gene in a sequence-specific manner confirmed by supershift and chromatin immunoprecipitation assays, respectively. The suppression of NRSF activity with either trichostatin A or a dominant-negative NRSF induced MOR promoter activity and transcription of the MOR gene. When the NRSE was disrupted in NS20Y and HeLa cells using small interfering RNA, the transcription of the endogenous target MOR gene increased significantly. This provides direct evidence the role of NRSF in the cells and also indicates that NRSF expression is regulated by post-translational modification in neuronal NMB cells. Our data suggested that NRSF can function as a repressor of MOR transcription in specific cells, via a mechanism dependent on the MOR NRSE.

Three major types of opioid receptors, μ, δ, and κ, have been cloned and shown to belong to the G-protein-coupled receptor superfamily (1). The activation of opioid receptors results in analgesia, sedation, euphoria, respiratory depression, and other functions. Subsequently, chronic administration of an agonist promotes tolerance and the development of physical dependence (1–3). Based largely on pharmacological and clinical observations, the μ opioid receptor (MOR) has traditionally been considered the main site of interaction of the major clinically used analgesics, particularly morphine (4). MOR is mainly expressed in the central nervous system with receptors varying in densities in different regions and perhaps playing different roles (5–8). Thus, the pharmacological properties of analgesic drugs such as morphine will depend on how MOR expression is regulated in the central nervous system. Hence the structure and the transcriptional control regions within the MOR gene that result in this tightly controlled expression could determine the tissue-specific responses to these drugs.

The mouse MOR gene spans over 100 kb and consists of multiple exons (9, 10). Several isoforms have been found (9–11), and among them MOR-1 is the major form that is encoded by exons 1, 2, 3, and 4 (9). The temporal and spatial expressions of the MOR gene, as well as the presence of MOR isoforms, suggest that the control of the transcriptional regulation plays an important role in MOR gene expression.

The presence of both distal and proximal promoter regions in the MOR gene has been reported previously (12). The proximal promoter of the mouse MOR gene is known to be regulated via various cis-acting elements and trans-acting factors, all of which are important for the proximal promoter activity (12–14). In addition, MOR can be detected during the ontogenesis of the mammalian central nervous system, indicating its active participation in neural development. Thus μ opioid binding sites are present in the developing rat brain by the 14th day of gestation, whereas mRNA can be detected as early as E11.5 with in situ hybridization. These observations suggest that the MOR is actively involved in the developing mammalian nervous system. We are interested in determining how these expression profiles are established (i.e. what determines the receptor repertoire of specific neurons or cell lines?). There is very little known about the gene structure and the transcriptional control regions that result in this tightly controlled expression.

The objective of the present study was to characterize the mechanisms of the cell-specific expression of the MOR gene. The presence of MOR transcripts in specific neuronal cells was correlated with the absence in these cell types of a transcription factor named NRSF (neuron-restrictive silencer factor). A 21-bp DNA element named NRSE for neural-restrictive suppressor element, regulates expression of neural specific genes through the binding of NRSF (15, 16). The repression effected by NRSF required the interaction of NRSF with the histone deacetylase (HDAC), which induces hypoacetylation of histone (16). This transcription factor has been proposed to play an important role in establishing and maintaining expression of neuron-specific genes. Recently studies have shown that NRSEs were found in some non-neuronal genes (17), suggesting that NRSF may regulate small interfering RNA; SOX, Sry-like HMG box; ChIP, chromatin immunoprecipitation; HDAC, histone deacetylase.

* This work was supported by National Institutes of Health Research Grants DA00564, DA01583, K05-DA70554, DA11190, and DA13926 and by the A&F Stark Fund of the Minnesota Medical Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
‡ To whom correspondence should be addressed: Dept. of Pharmacology, University of Minnesota Medical School, 6-120 Jackson Hall, 321 Church St. S.E., Minneapolis, MN 55455. Tel.: 612-626-6539; Fax: 612-625-8408; E-mail: kimxx313@umn.edu.
† The abbreviations used are: MOR, μ opioid receptor; NRSF, neuron-restrictive silencer element factor; RT-PCR, reverse transcription PCR; TSA, trichostatin A; siRNA, small interfering RNA; SOX, Sry-like HMG box; ChIP, chromatin immunoprecipitation; HDAC, histone deacetylase.
neuronal as well as non-neuronal genes. Although the regulation of neuronal genes has been widely studied, little information is available on the biological role that the interaction of NRSE-NRSF has in genes inside and outside the nervous system as well as the regulation of NRSE expression in neuronal cells. Previous study (18) has shown that the activity of the NRSF promoter functioned equally in neuronal and non-neuronal cells. This result indicated that the post-translational modification of NRSF is important to regulate its target gene.

Here we identified a conserved NRSE sequence in the promoter of mouse MOR gene, and we demonstrated that NRSF specifically bound to this mouse NRSE. Finally, we showed that the transcriptional repressor NRSF controlled MOR gene expression in specific neuronal cells.

**Experimental Procedures**

**Plasmid Constructs**—Constructs encoding a luciferase gene driven by the proximal promoter of the mouse MOR gene (pNRSE) or proximal promoter with mutated NRSE (pNRSEm) were generated by PCR using genomic DNA from mouse NS20Y cells, and an upstream sense oligonucleotide (5'-CTCTTCCCCGAGTCTGCTGC-3'), which incorporates a SacI site at the 5'-end and a downstream antisense oligonucleotide (for pNRSE, 5'-GCTGCTGGTACCCATGGTTCTGCTGCTGTCCATGGTTCTGCTGCTGTCCATGGTTCT-3' and for pNRSEm, 5'-CTTGACAGAAGATCTCATAAGTGCAGGGAGAGGACTCCATT-3') that were generated blunt-ended at the 3'-end, were used to amplify the relevant fragments from -452 to +12 (designated +1 at the translation start codon) of the mouse MOR gene. The amplified products were then cloned into the SacI/SmaI sites of a luciferase reporter plasmid gpl3 basic vector. Plasmids encoding NRSF (REEX1) and a dominant-negative form of NRSF (p73), designated here as DN-NRSF, were synthesized and scrambled primers (sense and antisense) as negative controls were used. The human MOR transcript was amplified with primer set P2Ss (5'-CACCAAGAGGATTTTCTTCATGCTC-3'). After 35 cycles of amplification, 10 μl of the PCR products were analyzed on a 1.5% agarose gel with Tris borate-EDTA buffer containing 10 mM Tris, pH 8.0. PCR reaction mixtures contained 4 μl of the immunoprecipitated chromatin sample with the following primers spanning the human MOR promoter region: 5'-AAGTTGAGCCAGGAGCCAGGT-3' and for pNRSEm, 5'-ACCTCA-3'.

**Cell Culture, Transfection, and Reporter Gene Assay**—Human neuronal cells were cultured as described previously (19). NS20Y and HEK cells were routinely grown in Dulbecco's minimal essential medium supplemented with 10% heat-inactivated fetal bovine serum at 37 °C in a humidified atmosphere of 5% CO2. PC12 cells were cultured in 10% CO2 in Dulbecco's minimal essential medium with 10% horse serum and 5% fetal bovine serum. Cells were plated in 6-well dishes at a concentration of 1 × 106 cells/well and cultured overnight before transfection. Various plasmids at concentrations indicated in each figure were used with the Effectene Transfection reagent (Qiagen) as described by the manufacturer. Briefly, for luciferase reporter analysis of each promoter construct, 1 μg of the reporter plasmid was mixed with the Effectene transfection reagent for 10 min before being added to various cells. Forty-eight hours after transfection, cells were washed three times with PBS and the media were removed and replaced with fresh media containing 30 μM tritiated thymidine (5'-GAGATCCAGGCTGGAAGCAGTGAC-3') and 3% fetal bovine serum. The DNA sequences of PCR products were confirmed by automated sequencing. Quantitative analyses were done using ImageQuant 5.2 (Amersham Biosciences) software.

**Small Interfering RNA-based (siRNA) Experiments**—Small interfering RNA (siRNA) experiments were performed using the siRNA Target Designer from Ambion. The primers for mouse MOR, nRSF and NRSF, were 5'-GAGATCCAGGCTGGAAGCAGTGAC-3' and NRSF reverse, 5'-AAGTTGAGCCAGGAGCCAGGT-3'. After 35 cycles of amplification, 10 μl of the PCR product was analyzed on a 2% agarose gel.

**Total RNA Preparation and RT-PCR Analysis**—Total RNA was isolated according to the supplier's protocol (TRI Reagent, Molecular Research Center, Inc.). For reverse transcription-PCR (RT-PCR), 5 μg of total RNA was reverse transcribed with superscript II reverse transcriptase (Invitrogen) and random primers (Promega). The PCR products were then separated on a 1.5% agarose gel with Tris borate-EDTA buffer (0.01% SDS, 1% Triton X-100, 1 μM EDTA) and stained with ethidium bromide and visualized using the Klenow fragment of DNA polymerase I in the presence of [γ-32P]dATP. Free nucleotides were separated by centrifugation through a G-50 column (Roche Applied Science). DNA-protein binding reactions were carried out in a 20-μl final volume of reaction buffer containing 10 μM Tris, pH 7.6, 50 mM NaCl, 1 mM dithiothreitol, 1 mM EDTA, 1 mM MgCl2, 5% glycerol, and 250 μg of poly(dI-dC)/ml. The nuclear extract (10 μg of protein) was added to the reaction buffer in the absence or presence of unlabeled cold competitor and nonspecific oligonucleotide. For the supershift assay, antibodies or preimmune serum were pre-incubated for 30 min on ice before adding the labeled probe. A radiolabeled probe was then added, and the mixture was incubated for a further 20 min on ice. Electrophoresis to resolve DNA-protein complexes was performed in 4% non-denaturing polyacrylamide gels in 0.5× Tris borate-EDTA buffer at 150 V for 2–3 h. The monoclonal antibody against NRSF was obtained from Dr. D. J. Anderson. Anti-interferon regulatory factor 4 antibody was purchased from Santa Cruz Biotechnology.

**Chromatin Immunoprecipitation Assay (ChIP)—**NS20Y cells were used for ChIP assays. ChIPs were performed using a modified protocol from Upstate Biotechnology (Lake Placid, NY). Cells in a 10-cm dish (70% confluent) were treated for 10 min with 1% formaldehyde at 37 °C. The cells were lysed in cell lysis buffer (5 mM HEPES, pH 8.1, 85 mM KCl, 0.5% Triton X-100), and formaldehyde-fixed nuclei were washed with TE buffer (50 mM Tris-HCl, pH 8.1, 10 mM EDTA, 1% SDS). The lysate was sonicated under conditions yielding fragments ranging from 200 to 1,000 bp. One-tenth of the diluted lysate was used for input, and the residual lysate was subjected to the following immunoprecipitation. Samples were subsequently preincubated at 4 °C with recombinant protein A-agarose beads coated with salmon sperm DNA. Precoated lysate (100 μl) diluted in immunoprecipitation buffer (0.01% SDS, 1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl, pH 8.1, 167 mM NaCl) was used for overnight immunoprecipitation with 10 and 5 μg of NRSF antibody at 4 °C. Complexes were collected for 4 h by incubation at 4 °C with recombinant protein A-agarose beads coated with salmon sperm DNA. After washing and elution, formaldehyde cross-linking was reversed using 0.1 M glycine in 2× saline and lysed with TE buffer. DNA samples were subjected to PCR analysis of each promoter construct, 1 μl of the PCR product was analyzed on a 2% agarose gel.

**Total RNA Preparation and RT-PCR Analysis**—Total RNA was isolated according to the supplier's protocol (TRI Reagent, Molecular Research Center, Inc.). For reverse transcription-PCR (RT-PCR), 5 μg of total RNA was reverse transcribed with superscript II reverse transcriptase (Invitrogen) and random primers (Promega). The PCR products were separated on a 1.5% agarose gel with Tris borate-EDTA buffer (0.01% SDS, 1% Triton X-100, 1 μM EDTA) and stained with ethidium bromide and visualized using the Klenow fragment of DNA polymerase I in the presence of [γ-32P]dATP. Free nucleotides were separated by centrifugation through a G-50 column (Roche Applied Science). DNA-protein binding reactions were carried out in a 20-μl final volume of reaction buffer containing 10 μM Tris, pH 7.6, 50 mM NaCl, 1 mM dithiothreitol, 1 mM EDTA, 1 mM MgCl2, 5% glycerol, and 250 μg of poly(dI-dC)/ml. The nuclear extract (10 μg of protein) was added to the reaction buffer in the absence or presence of unlabeled cold competitor and nonspecific oligonucleotide. For the supershift assay, antibodies or preimmune serum were pre-incubated for 30 min on ice before adding the labeled probe. A radiolabeled probe was then added, and the mixture was incubated for a further 20 min on ice. Electro-
siRNA control were also synthesized. The oligonucleotide-directed production of small RNA transcripts with T7 RNA polymerase, described previously (24, 25), was carried out using the T7 Ribomax Express RNAi system. Sense and antisense 21-nucleotide RNAs generated in separate reactions were annealed by mixing both transcription reactions and incubating at 70 °C for 10 min followed by 20 min at room temperature to obtain small interfering double-stranded RNA synthesized by T7 RNA polymerase. The mixture was then purified by isopropanol precipitation, washed with 70% ethanol, dried, and resuspended in the appropriate amount of nuclease-free water. The concentration of siRNAs used was 1–3 μg in each transfection for NS20Y and HeLa cells using RNAiFect transfection reagent (Qiagen). Forty-eight hours after the transfection, total RNA was prepared using TRI Reagent according to the manufacturer’s instructions and used to perform RT-PCR analysis. Quantitative analyses were done using ImageQuant 5.2 software.

RESULTS

Identification of the Mouse MOR Promoter Region—As shown in Fig. 1A, the 21-bp NRSE identified in human (from nucleotides -12 to +9) is highly homologous to mouse and rat MOR sequences and to the consensus NRSE sequence. Previous studies have shown that 22 more genes (17), 17 of which are expressed mainly in neurons, were found to contain NRSE-like sequences and the results of gel-shift assays indicated that at least 7 bases (Fig. 1A, lowercase letters) of the residues of the NRSE are not critical for NRSF binding activity. The 14 bases of the core sequence (Fig. 1A, capital letters) are likely to include residues that are relatively more important for function (17). The NRSE of mouse, rat, and human MOR have only one residue mismatched compared with functional known NRSE consensus sequences (Fig. 1A). Mori et al. (26) demonstrated that mutating two adjacent G residues (Fig. 1A, underlined in core region) to T residues drastically reduced both NRSF binding and silencing. The MOR NRSE in all three species also has these two adjacent G residues, warranting further studies to investigate a possible role of NRSE/NRPS in MOR gene regulation.

Expression of NRSE and MOR in Different Cell Lines—Neuronal NMB (MOR-positive) and NS20Y (MOR-negative) cell lines, and non-neuronal cervical carcinoma HeLa (MOR-negative) were used as models to study the mechanism controlling the cell-specific expression of the MOR gene. Total RNA was isolated and subjected to RT-PCR analysis to detect the expression of the NRSEF and MOR transcripts from those cells. As shown in Fig. 1B, MOR transcripts were detected in NMB cells but not in NS20Y and HeLa cell lines. All these cell lines express a high level of the NRSE mRNA in the order of HeLa > NS20Y > NMB cells. By contrast, the NRSE protein expression level was low in the MOR-positive NMB cells and was high in MOR-negative NS20Y and HeLa cells (Fig. 1C). Because NRSE may act in a concentration-dependent manner (15, 27), the low level of NRSEF in NMB cells may be insufficient for silencing activity.

Functional Analysis of the 5′-Flanking Region of the Mouse MOR Promoter—Studies in our laboratory show that expression of MOR is driven by two promoters, distal and proximal (12). Both promoters exhibit characteristics of housekeeping genes lacking a TATA box, and the distal promoter is known to be 20-fold less active than the proximal promoter. The proximal promoter region of the MOR gene, which spans ~200 bp in length (from -450 to -249 bp, the translation start site is designated as +1) has proximal promoter activity to regulate MOR transcription (29). Thus, the effect of the NRSE DNA element on the proximal promoter activity (comprising -450 to +12 bp) of the mouse MOR gene was examined in MOR-positive (NMB) and MOR-negative (NS20Y and HeLa) cell lines (Fig. 2). The MOR promoter/reporter plasmids, pNRSE containing the native NRSE sequence (5′-GCTGCTGTCCATGGTCCTGGA-3′), pNRSEm (5′-CTGACAGAATGACCTCAATGCTGCTTG-3′), and pNRSEnn (deleted the NRSE), were each constructed with the 5′-flanking region (from -450 to -9) of the mouse MOR gene linked to a luciferase reporter gene in the pGL3-basic vector (Promega). The activity of the reporter in tested cell lines was negatively regulated when the NRSE DNA element was present in the MOR promoter construct, pNRSE (Fig. 2A). The promoter activity of pNRSE construct in NMB cell lines was ~5-fold lower than that of the pNRSEm and pNRSEnn constructs. This promoter activity of the pNRSE construct in NMB cells was relatively less than the activities of the pNRSEm and pNRSEn constructs. This promoter activity of the pNRSE construct in NMB cell lines was comparable to the activities of the pNRSE construct in NS20Y and HeLa cell lines. This might be a result of the low level of NRSE expression in NMB cell lines compared with its level in the other two cell lines.

![Fig. 1. Expression of NRSEF and MOR transcripts. A. The NRSE sequence of the mouse MOR is very similar to a consensus NRSE, and it is highly conserved among at least three species, mouse, rat, and human. The NRSE is located from -12 to +9 on the MOR promoter and contains a translation start site (ATG). The nucleotides shown in bold and capital letters are conserved in most functional NRSEs and in the MOR promoter from all species analyzed. B. NRSE mRNA is expressed in NMB, HeLa, and NS20Y cells. RT-PCR of RNA extracted from NMB, HeLa, and NS20Y cells was performed using intron-spanning primer sets, which are specific for NRSE, MOR, and β-actin to avoid chromosomal contaminations, as described under “Experimental Procedures.” Negative and positive controls contained either the pcDNA3 vector or the MOR expression construct (HA-MOR) as the PCR template, respectively. C. Analysis of NRSE/REST expression in NMB, HeLa, and NS20Y cells by Western blotting. Equal amounts of total protein (40 μg) were separated on a 10% SDS-PAGE gel and transferred to an ImmobilonTM-P (polyvinylidene difluoride membrane, Millipore) membrane. Monoclonal antibody against NRSE (a gift from D. J. Anderson) was used for this Western blotting. D. Quantitative data for lanes 1–3 of C were analyzed by using ImageQuant 5.2 software. Bars depict the sum of signal intensity in the same size area and S.D. between experiments. The average signal from NMB cells was set at 1.

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In addition, we used a heterologous promoter to examine the role of MOR NRSE (Fig. 2B). The NRSE DNA element of MOR and a mutated NRSE (the mutated site is the same as in pNRSEm) were each cloned into the multiple cloning site (Sacl/Smal) of the pGL3 basic luciferase reporter vector. The relative luciferase activity was shown after normalizing promoter activities with the pGL3 basic vector. Error bars indicate the range of S.E., and the activities of the luciferase reporter were expressed as n-fold relative to the activity of each corresponding luciferase reporter with vector alone transfected, which was assigned an activity value of 1.0. B, MOR NRSE silences the activity of the heterologous SV40 promoter. The wild type and mutated NRSE of the MOR promoter, pSVNRSE, and pSVNRSEm, respectively, were each cloned as monomers upstream from an SV40 promoter. These constructs were transfected into NMB, HeLa, and NS20Y cell lines. Values represent the average of three independent transfections, each of which was carried out in triplicate and normalized to a cotransfected β-galactosidase expression vector pCH110. Error bars indicate the range of S.E., and the promoter activity of the pGL3 promoter was set as 100%.

Dominant-negative NRSF (DN-NRSF) Reversed NRSF-mediated Suppression of MOR Promoter Activity in Several Cell Lines—To evaluate whether the suppression of MOR NRSE promoter activity is reversed by a DN-NRSF, we cotransfected a DN-NRSF expression plasmid with either reporter construct pNRSE or pNRSEm. DN-NRSF encodes only the DNA-binding domain of NRSF without the two repressor domains of the protein (10). As shown in Fig. 3, when the pNRSE construct was transfected into NMB, NS20Y, and HeLa cells in the presence or absence of DN-NRSF, all the MOR promoter activity was derepressed. This effect was absent when the mutated NRSE construct (pNRSEm) was cotransfected into these three cell lines. Because the DNA-binding domain of DN-NRSF was enough to compete with the endogenous NRSF for the NRSE binding sequence (15), these data indicated that the repression of pNRSE promoter activity in these cells was mediated by NRSF binding to the NRSE of the MOR promoter, resulting in antagonizing the transcriptional effects of the wild type NRSF protein. These results were further confirmed with the following experiments as shown in Fig. 4. We transfected NMB cells with either the DN-NRSF expression plasmid or the control vector for the analysis of endogenous MOR transcripts in NMB cells. Transfection of DN-NRSF resulted in markedly increased MOR transcript levels, compared with mock and vector control cells (Fig. 4). These results suggested that the repression of MOR transcription is mediated by NRSF binding to the NRSE of the MOR promoter in neuronal NMB cell lines.

Silencing MOR Promoter Activities by Overexpressed NRSF in NRSF-negative PC12 Cell Lines—We then tested whether exogenous expression of NRSF reproduces the attenuation of MOR promoter reporter activity in NRSF-negative cells. We
cotransfected either the MOR NRSE (pNRSE) or the mutated NRSE (pNRSEm) into PC12 cell lines (NRSF-negative cells) with either the pcDNA3.1 control vector or an NRSF expression vector (Fig. 5). We also included NRSF-positive NS20Y cells as a control. As expected, overexpression of NRSF in NS20Y cells did not affect the MOR expression from either promoter construct, pNRSE or pNRSEm, which is consistent with the saturated levels of endogenous expression of NRSF already present in this cell line (28, 29). In contrast, overexpression of NRSF in PC12 cells repressed the promoter activity of pNRSE construct by 5.4-fold, whereas the overexpression of NRSF had no effect on the promoter activity of the pNRSEm construct (Fig 5) suggesting that the direct binding of NRSF to the MOR NRSE is required for repression of MOR transcription.

**FIG. 3.** Expression of DN-NRSF (p73) derepressed the MOR promoter in vitro in NMB, HeLa, and NS20Y cells. Plasmid constructs, pNRSE and pNRSEm, were each transiently transfected with or without DN-NRSF into NMB, HeLa, and NS20Y cells. pNRSE activity was relieved with the coexpression of DN-NRSF. The promoter activity of each construct was expressed as relative luciferase activity, and transfection efficiencies were normalized to β-galactosidase activity by cotransfection of the internal control plasmid pCH110. Error bars indicate the range of S.E., and the activities of the luciferase reporter were expressed as n-fold relative to the activity of each corresponding luciferase reporter with vector-alone transfection, which was assigned an activity value of 1.0.

The MOR Transcriptional Repression Is TSA-sensitive through the NRSE of MOR Promoter—NRSF has been known to recruit histone deacetylases (HDAC) to act as repressors through chromatin remodeling (30). To assess whether the repression of MOR promoter activity is HDAC-dependent, we used TSA, a specific inhibitor of HDAC in NMB, NS20Y, and HeLa cells. These cell lines were transiently transfected either with pNRSE or pNRSEm constructs. Twenty-four hours after transfection, the cells were treated with 100 nM TSA according to methods published by Yoshida et al. (31). The luciferase activity was significantly increased in all TSA-treated cell lines, compared with non-treated cell lines. When either the MOR NRSE construct (pNRSE) or the mutated MOR NRSE construct (pNRSEm) was transfected into NMB cells, the pNRSE activity in TSA-treated cells was increased 3.9-fold compared with ethanol-treated cells as a control (TSA was dissolved in ethanol), whereas pNRSEm promoter activity in TSA-treated cells was not increased (Fig. 6A). Similar results of increased promoter activity by TSA treatment were obtained in two other cells (NS20Y and HeLa) with even greater increases, 5.29- and 7.56-fold, respectively (Fig. 6), indicating that TSA effects were higher in these cell lines (NS20Y and HeLa) than its effect in NMB cells, because the NRSF protein is expressed more in these two cell lines than in NMB cells. These data indicate that NRSF-mediated repression of the MOR promoter involves a TSA-sensitive mechanism mediated through the NRSE.

We also performed experiments to investigate whether the transcriptional repression of the endogenous MOR gene can be relieved in NRSF-expressing cells (NMB cells) after TSA treatment (Fig. 6B). Total RNA from NMB cells either treated or not treated with TSA were analyzed by RT-PCR for MOR expression using a MOR-specific primer set. As shown in Fig. 6B, MOR mRNA levels were increased in cells treated with 100 nM TSA compared with untreated cells, although TSA did not affect the NRSF transcription (Fig. 6B). This observation was consistent with our data of TSA treatments in transient transfection assays (Fig. 6A), indicating that histone deacetylase activity is required for the repression of endogenous MOR gene transcription by NRSF.
Binding of NRSF to the NRSE cis-Acting Element of MOR Promoter—The NRSF gene was cloned on the basis of its ability to bind to NRSEs in the SCG10 and type II sodium channel genes (15, 27). We investigated whether this NRSF protein was able to interact with the NRSE of the MOR promoter. We performed a supershift assay with nuclear extracts from NS20Y cells expressing high levels of NRSF endogenously using the NRSE probe of the MOR promoter. As shown in Fig. 7, two major DNA-binding complexes from NS20Y nuclear extracts were observed (lane 2). The upper complex was abolished in the presence of monoclonal NRSF antibody, whereas incubation with preimmune serum (PI) and nonspecific antibody (IRF4) had no effect on the complex (Fig. 7, lanes 5, 6, and 7, respectively). The lower dense complex was not affected (Fig. 7, lane 6) by the antibody. Studies investigating the identity of the components of this complex are currently underway in our laboratory. Competitive binding experiments were also conducted in this electrophoretic mobility shift assay using a 500-
fold molar excess of a self-NRSE cold competitor and nonspecific SOX competitor (Fig. 7, lanes 3 and 4, respectively). The self-NRSE competitor competed for protein-DNA interaction, whereas the nonspecific SOX competitor did not affect the binding, indicating the specific interaction. In addition, this NRSF-associated DNA-binding complex was not detected in nuclear extracts obtained from the NRSF-negative PC12 cells (data not shown). In this experiment using the nuclear extracts of NS20Y cells, the anti-NRSF antibody caused a complete reduction in binding compared with preimmune serum and nonspecific antibody. These data demonstrated that the NRSF protein binds to the NRSE DNA element of the MOR promoter.

**Fig. 6.** The MOR promoter activity and endogenous MOR gene expression is increased in TSA-treated NMB cells. A, NMB cells were transfected with the MOR luciferase reporter gene constructs and treated with 100 nM TSA for 24 h. The relative luciferase activity of each construct was expressed as the -fold increase over the value for the non-TSA-treated control NMB cells. Data were normalized by protein concentration and expressed as the -fold activation of the luciferase activity of non-treated vector control, which is arbitrarily defined as 100%. Error bars indicate the range of S.E. In addition, the -fold increase in promoter activity by TSA compared with the corresponding untreated control is shown on the top of the error bars. B, total RNAs were isolated from NMB cells treated in the presence of ethanol (lane 2), 100 nM TSA (lane 3), or 200 nM TSA (lane 4). MOR gene expression was analyzed by RT-PCR using specific MOR primers (see the sequences under “Experimental Procedures”). Negative and positive controls were performed the same as in the legend to Fig. 1. C, quantitative data for lanes 2–4 of A were analyzed by using ImageQuant 5.2 software after β-actin normalization. Bars depict the sum of signal intensity in the same size area and S.D. between experiments. The average signal from non-TSA-treated cells was set at 1.

NRSF expression in NS20Y and HeLa cell lines, both of which express NRSF endogenously. The mouse NS20Y cells were transfected with either 1 μg of mouse NRSF siRNA or the scrambled siRNA (Fig. 8, A and B). RT-PCR was performed as described under “Experimental Procedures” after total RNA was isolated from the transfected cell lines. Each mRNA signal was quantified using ImageQuant 5.2 software and normalized to β-actin (Fig. 8, B and D). An induced level (~7-fold induction) of MOR mRNA was observed in the presence of NRSF siRNA duplexes compared with the scrambled control (Fig. 8, A and B).

In addition, we also included human HeLa cells as non-neuronal cells for the similar siRNA strategy (Fig. 8, C and D). We designed a human NRSF siRNA (hNRSFsi) and transfected different doses of human siRNA (1 and 3 μg) and the scrambled siRNA into the cells (Fig. 8, C and D). Transfection of hNRSFsi was also effective to repress the human NRSF expression by...
attenuating NRSF levels by 50–80% depending on siRNA concentration. In the presence of hNRSFsi, the human MOR mRNA level was increased 3–6-fold, depending on siRNA concentration, compared with scrambled siRNA control (Fig. 8, C and D). These results indicated that the NRSF protein regulates the endogenous MOR gene expression at the transcriptional level.

To further support the data that NRSF regulates endogenous MOR transcription, a ChIP assay was carried out to determine the interaction of the NRSF protein on the mouse MOR promoter (Fig. 9A). After the cross-linking of proteins and DNAs with formaldehyde, cell lysates from NS20Y cells were subjected to immunoprecipitation with the monoclonal mouse NRSF antibody. The precipitated DNA fragments were amplified with primers (see “Experimental Procedures” for details) spanning a 188-bp region covering the NRSE of the mouse MOR promoter. As shown in Fig. 9, ChIP PCR product was detected with the mouse NRSF antibody in NS20Y cells but not detected with preimmune serum (PI) as control. This suggests that the endogenous NRSF from the NS20Y cells specifically binds to the NRSE region of the MOR promoter. This is consistent with the in vitro results of our supershift assays shown in Fig. 7.

**DISCUSSION**

The proximal promoter of the mouse MOR gene is known to be regulated via various cis-acting elements and trans-acting factors, all of which are important for the proximal promoter activity (12–14). However, none of these studies define the tissue/cell-specific expression of the MOR gene.

A region overlapping the MOR start codon is substantially homologous to a DNA element named NRSE (also reported as the RE1 element) in the human MOR gene. It has a high homology with the mouse MOR NRSE, which is located from –9 to +12 bp of the MOR promoter, as well as with the rat MOR NRSE. The NRSE/RE1 element is a 21-bp DNA sequence that behaves as a regulatory element of several neuronal genes in non-neuronal cells, silencing their transcription by the binding of the NRSF/REST transcription factor (26, 32–34). NRSF is a protein containing C2H2 zinc fingers related to members of the Kruppel-like factor family. The protein contains a cluster of zinc fingers at the N-terminal end, which is required for binding to the NRSE DNA element. We have shown here that NRSF is able to bind to the NRSE DNA element of the MOR promoter and to modulate the gene promoter activity through this element. NRSF mRNA was expressed in all three cell lines used for our transfection studies, although the levels of NRSF.
mRNA in non-neuronal HeLa and NS20Y cells were substantially higher than in MOR-positive NMB cells (Fig. 1, B and C). The promoter activities of the MOR/luciferase reporter constructs in these cells decreased depending on the endogenous NRSF expression level. Previous studies (15, 27) have indicated that NRSF may act in an NRSF concentration-dependent manner and that a threshold level of NRSF may be required for efficient repression of transcription. Thus, the levels of NRSF found in some cells of neuronal origin may not be sufficient for transcriptional repression. In support of this hypothesis, we have found that overexpression of NRSF in PC12 cells (NRSF-negative cells) did suppress MOR promoter activity from a NRSE-linked promoter (Fig. 5) suggesting that endogenous levels of NRSF in these cells may be well below the threshold required for silencer activity. The expression of NRSF in cells of neuronal origin could also reflect alternative roles for NRSF in these cells and suggests that neurons may express additional factors that either cancel or modify the effects of NRSF. Recently, the NRSF/RE1 element has also been found in some non-neuronal genes (35). Although these elements present important changes with respect to the NRSE consensus sequence, they still have the capacity to interact with NRSF/REST, suggesting that the NRSE/RE1 may regulate non-neuronal as well as neuronal genes in neuronal and non-neuronal cells.

Expression of a dominant-negative form of NRSF (DN-NRSF) blocked the silencing activity of endogenous NRSF on its target genes (36, 37). In this study, cotransfection of DN-NRSF also derepressed NRSF-mediated suppression of the MOR promoter activity in all tested cell lines (Fig. 3) and induced the endogenous MOR mRNA level in NMB cells (Fig. 4). Although inhibition of NRSF function causes derepression of neuronal genes, it does not alter the fate of non-neuronal cells such as muscle (15). This suggests that NRSF acts to maintain repression of inappropriate tissue-specific terminal differentiation genes rather than to specify cell fate. For example, the fact that both the knock-out and DN-NRSF cause ectopic neuronal tubulin expression indicates that the latter blocks endogenous chicken NRSF function. The repression effect induced by NRSF is required for the interaction of NRSF with the corepressors, such as mSin3 and HDAC1, to form a complex that induces hypoacetylation of histones (16). Our data also supported this, because the promoter activity of MOR/luciferase construct was increased in all TSA-treated cells (Fig. 6A). These data showed that HDAC activity is required for the repression of MOR gene transcription by NRSF. To mediate the repression, NRSF recruits the HDACs and mSin3 by a mechanism similar to that described previously by others (16, 31).

Our data also showed that the endogenous MOR gene is TSA-sensitive in NMB cells (Fig. 6B) but not in NS20Y and HeLa cell lines (data not shown), although the transiently transfected MOR NRSE promoter construct (pNRSE) is TSA-sensitive in NS20Y and HeLa cell lines (Fig. 6A). This may be a reflection of the differential nucleosomal structures between the transfected promoter construct and the endogenous MOR promoters from the NS20Y and HeLa cell lines. In addition, transfection with NRSF siRNA for silencing NRSF led to a remarkable increase in the endogenous MOR transcription in the NS20Y and HeLa cell lines (Fig. 8). These data suggested that the NRSF may act independently of HDAC recruitment in these two cell lines, compared with the NRSF-dependent repression in NMB cells, which is associated with HDAC proteins. Thus, in NMB cells, NRSF-induced histone hypoacetylation around the NRSE could change the local nucleosomal structure and therefore could have a direct effect on the TFIID-RNA polymerase II holocomplex decreasing access to the MOR promoter. The NRSE at the MOR promoter is located only at 268 bp downstream of the transcription start site, and so local changes to nucleosomal structure may affect the basal transcriptional complex. NRSF-dependent repression has been shown to be TSA-sensitive at a number of transiently transfected reporter genes, including the M4 (38), NaV1.2 (16, 23, 38), GluR2 (16), CRH (29), and ANP (30). By itself, TSA data must be treated with caution, because TSA can affect global acetylation patterns of core histones and non-histone targets (39–42), and consequently, its effects may not be solely attributable to targeted deacetylation by NRSF. Interestingly, one recent study reported that TSA relieved repression of the silent endogenous NaV1.2 in rat L6 cells, and ChIP assays showed the NRSE of NaV1.2 to be occupied by NRSF/REST, Co-REST, and HDAC2 (29). However, this same group later reported that the silent NaV1.2 in different Rat-1 fibroblasts recruited only NRSF/REST and Co-REST but no HDACs, and accordingly, expression of NaV1.2 was TSA-insensitive in Rat-1 fibroblasts (43). Thus, HDAC recruitment and an HDAC requirement do not always go hand in hand.

NRSF was originally defined as a repressor for neuronal specific gene in non-neuronal cells (15, 27). However, from recent studies, it has become clear that genetic context is extremely important in determining the function of the NRSF/NRSE system (26, 32–34, 44–47). Our results showed that the MOR gene is expressed differentially between neuronal NMB (MOR-positive) and NS20Y cells (MOR-negative) and this corresponds to the differing amount of NRSF protein expressed in these two cell lines. Results from the knock-out of NRSF in the mouse strongly suggest that tissue/cell type, as well as genetic context, is important for determining the effect of NRSF on gene expression (48). Our results underlined the fact that the
cellular context is critical for understanding and defining the function of the NRSF system. Not only is the distinction between neuronal and non-neuronal cell types important, but perhaps the distinction between different neuronal cell types is equally important. This result also suggested that although NRSF is not as abundant in brain as in peripheral tissue, small changes in the availability of NRSF to the nucleus may be sufficient to increase or decrease repression of its target genes. It is possible that NRSF protein modification is differently regulated between neuronal and non-neuronal cell lines and between two different neuronal cell lines.

So far, the post-translational and/or protein modification mechanisms of NRSF have not been known completely. In addition, a previous study has shown that the NRSF gene has two basal promoters but both promoters function equally in splicing, mRNA stability, and/or post-transcriptional level, e.g., splicing. Further mechanisms of NRSF have not been known completely. In between two different neuronal cell lines.

It is possible that NRSF protein modification is differently regulated between neuronal and non-neuronal cell lines and rather is modulated at the post-transcriptional level. This result also suggested that although NRSF is not as abundant in brain as in peripheral tissue, small changes in the availability of NRSF to the nucleus may be sufficient to increase or decrease repression of its target genes.

Acknowledgments—We thank Santosh Talreja and Dr. Ursula D’Souza for helpful suggestions and manuscript review. We also thank Dr. D. J. Anderson for providing the mouse NRSF monoclonal antibody and Dr. G. Mendel for kindly providing the dominant-negative and NRSF expression plasmids.

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Neuron-restrictive Silencer Factor (NRSF) Functions as a Repressor in Neuronal Cells to Regulate the \( \mu \) Opioid Receptor Gene

Chun Sung Kim, Cheol Kyu Hwang, Hack Sun Choi, Kyu Young Song, Ping-Yee Law, Li-Na Wei and Horace H. Loh

*J. Biol. Chem.* 2004, 279:46464-46473.  
doi: 10.1074/jbc.M403633200 originally published online August 18, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M403633200

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