

A Small Modulatory dsRNA Specifies the Fate of Adult Neural Stem Cells

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Summary

Discovering the molecular mechanisms that regulate neuron-specific gene expression remains a central challenge for CNS research. Here, we report that small, noncoding double-stranded (ds) RNAs play a critical role in mediating neuronal differentiation. The sequence defined by this dsRNA is *NRSE/RE1*, which is recognized by NRSF/REST, known primarily as a negative transcriptional regulator that restricts neuronal gene expression to neurons. The *NRSE* dsRNA can trigger gene expression of neuron-specific genes through interaction with NRSF/REST transcriptional machinery, resulting in the transition from neural stem cells with neuron-specific genes silenced by NRSF/REST into cells with neuronal identity that can express neuronal genes. The mechanism of action appears to be mediated through a dsRNA/protein interaction, rather than through siRNA or miRNA. The discovery of small modulatory dsRNAs (smRNAs) extends the important contribution of noncoding RNAs as key regulators of cell behavior at both transcriptional and posttranscriptional levels.

Introduction

The expression of cell type-specific differentiation genes is thought to depend on both positive and negative gene expression controls, which are implemented throughout the developmental history of cells. Numerous genetic studies provide evidence that cell type-specific gene expression activators and repressors are essential components of the process. In addition to the conventional transcription machinery, gene expression control by small noncoding RNAs, at the posttranscriptional level, appears to be essential (Eddy, 2001; Fire

et al., 1998; Pasquinelli, 2002). Base complementarity allows very small noncoding RNAs to be sequence-specific, and since they act both in *cis* and *trans*, their potential functional roles at specific regulatory stages can be expanded. Noncoding RNA genes, which produce transcripts, can function directly as structural, catalytic, or regulatory RNAs, rather than as expressed mRNAs that encode proteins. Recently, several groups have carried out systematic noncoding RNA gene identification screens (Dostie et al., 2003; Lau et al., 2001; Lee and Ambros, 2001). Studies indicate that the prevalence of noncoding RNA genes has indeed been underestimated. Plants, flies, worms, mice, and humans all harbor significant numbers of small RNAs that are likely to play regulatory roles. Although most of the identified noncoding RNAs have unknown functions, their sequences are typically conserved among different species, and many have intriguing expression patterns in different tissues or stages of development. Therefore, noncoding RNAs may have a general role in modulating gene expression in many aspects of development, such as tissue-specific patterning and cell fate specification.

The regulatory mechanisms of gene expression, which determine cell fates giving rise to each lineage, remain largely unknown. Cell fate decisions might involve the regulatory activities of noncoding RNAs. To examine whether noncoding RNAs contribute to cell fate specification of adult neural stem cells, we isolated small, noncoding RNAs from adult hippocampal neural stem cells during lineage-specific differentiation. To identify possible target nucleic acids, public database searches were used for genomic sequences. Among the large number of small noncoding RNAs that appeared, one unique sequence emerged from the “neurogenesis” noncoding RNA pools. This sequence defined the *NRSE/RE1* (neuron restrictive silencer element), which is recognized by the NRSF/REST transcriptional regulator (Chong et al., 1995; Schoenherr and Anderson, 1995).

In the CNS, neuronal restricted silencing factor/RE-1 silencing transcription factor (NRSF/REST) plays a critical role as a key transcriptional repressor for neuron-specific genes in nonneuronal cells (Chen et al., 1998; Huang et al., 1999; Palm et al., 1998; Schoenherr et al., 1996). NRSF/REST is a krüppel family zinc finger protein and binds specifically to a 21- to 23-base pair (bp) conserved DNA response element (*NRSE/RE1*). *NRSE/RE1* sequences are encoded within a broad range of genes involved in neuronal development and function, including ion channels, neurotransmitter receptors and their synthesizing enzymes, receptor-associated factors, neurotrophins, synaptic vesicle proteins, growth-associated and cytoskeletal and adhesion molecule factors involved in axonal guidance, transport machinery, transcription factors, and cofactors. The consensus *NRSE/RE1* sequence is conserved between *Xenopus*, mouse, rat, chicken, sheep, and human. NRSF/REST mediates transcriptional repression through the association of the N-terminal repressor domain with the mSin3/histone deacetylase-1/2 (HDAC1/2) complex and through the association of C-terminal repressor domain with the CoREST

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complex (by recruitment of MeCP2 or HDACs) (Huang et al., 1999; Lunyak et al., 2002; Naruse et al., 1999).

In this work, we report that the identified, noncoding RNA-containing *NRSE* sequence forms double-stranded RNA (dsRNA) in lengths of about 20 bp, and that the *NRSE* dsRNA activates expression of *NRSE/RE1*-containing genes during an early stage of neurogenesis. The *NRSE* dsRNA modulates the *NRSE/RE1* DNA-NRSF/REST protein machinery to switch neuronal gene expression from a repressed state in stem cells to an active state in early neurons. The *NRSE* dsRNA is necessary and sufficient to direct multipotent neural stem cells specifically down a neuronal lineage, suggesting it can function as an endogenous inducer of neuronal differentiation. The apparent gene activation effects of the *NRSE* dsRNA clearly distinguish it from the gene silencing effects of cellular miRNA/siRNAs and suggest a novel function for noncoding RNAs at a transcriptional level.

Results

Identification of a Neuron-Specific, Small Noncoding RNA from Adult Hippocampal Neural Stem Cells

To investigate the role of small, noncoding RNAs in the differentiation of neural stem cells, 20- to 40-nucleotide (nt) RNAs were cloned from total RNA extracted from adult hippocampal neural stem cells (HCN-A94; Gage et al., 1995). We obtained more than 50 unknown noncoding RNAs and decided to focus on one RNA sequence that contained a match to the 21 nt *NRSE/RE1* DNA sequence in the antisense orientation (*asNRSE*; Supplemental Figure S1 available at <http://www.cell.com/cgi/content/full/116/6/779/DC1>). The *NRSE/RE1* sequence is usually localized within promoter regions of neuron-specific genes and is recognized by the NRSF/REST protein to restrict neuron-specific gene expression; however, the function of a DNA element within an RNA sequence is unclear.

Northern analyses revealed an *asNRSE* RNA corresponding to about 20 nt in length within the neuronal population (cells treated with 1 μ M retinoic acid [RA] and 5 μ M forskolin [FSK] for 4 days). Surprisingly, our control probe for sense-strand revealed an \sim 20 nt sense *NRSE* RNA (*sNRSE*) within the same neuronal population (Figure 1A, probe for sense) suggesting that these RNAs might exist as double-stranded forms within the cell. Low amounts of both sense and antisense RNAs could also be detected within the progenitor population, but the expression levels in the neuronal population were much higher relative to the progenitor population. The expression of low levels of both RNAs within the progenitor cultures could be due to the presence of some cells already committed to specific lineages. Nevertheless, we could not detect any *NRSE* RNAs within an astrocyte population.

To determine which neuronal stages express the dsRNA, a time-course Northern blot analysis was performed after neuronal induction. Cells at 2 and 4 days after induction of neural differentiation (RA+FSK) contained the highest amounts of the *NRSE* dsRNA; as maturation proceeded, the levels of the *NRSE* dsRNA apparently decreased (Figure 1B). These data show that

the *NRSE* dsRNA appeared at an early stage of neurogenesis rather than at more mature stages.

Neuronal Lineage Induction by the *NRSE* dsRNA

To determine the function of the *NRSE* dsRNA, we expressed them in HCN-A94 cells. We made lentiviral vectors with U6 promoter-driven *sNRSE*, *asNRSE* and *NRSE* dsRNA expression cassettes. After infecting progenitor cells with virus, cells were maintained without FGF-2 for 4 days. In control infections (lentivirus with an empty U6 cassette), we did not observe any obvious effects on cell morphology (Figure 2A). Expression of single *sNRSE* or *asNRSE* RNA alone also had no obvious effects (Figure 2A). However, when we introduced both *sNRSE* and *asNRSE* RNA together, significant morphological changes were observed (Figure 2A). These cells extended processes indicative of differentiation and some cells made large but flat clusters with long processes.

We next performed immunocytochemistry with markers of various differentiated neural lineages. Introduction of *NRSE* dsRNA in progenitor cultures resulted in increased immunocytochemical staining of neuron-specific markers, including β III-tubulin (TUJ1), NF200, and calbindin (Figure 2B). Cells containing the *NRSE* dsRNA were completely negative for the astrocyte marker *GFAP* and oligodendrocyte marker *RIP* (Figure 2B).

Quantitative Analysis of *NRSE* dsRNA Activity as an Endogenous Inducer of Neuronal Differentiation

We next assessed the effects of noncoding *NRSE* dsRNA, both in progenitor cultures and during lineage-specific differentiation with reporter constructs. Stage-specific promoter-based reporter assays allowed us to quantify the activity of *NRSE* dsRNAs comparatively.

We used a *Sox2* promoter-driven luciferase construct as an undifferentiated neural progenitor-specific reporter construct. Luciferase values from cells 4 days after mock virus (control) infection were set as 100% (Figure 2C; the immunostaining is also shown in the right image). No obvious difference was observed for *sNRSE* RNA and *asNRSE* RNA; however, there was a significant decrease in luciferase activity in cells infected with *NRSE* dsRNA.

A β III-tubulin (TUJ1) promoter-driven luciferase construct was used for the neuron-specific reporter construct. RNA-expressing virus-infected progenitor cells were cultured in 1 μ M RA and 5 μ M FSK for 4 days. The luciferase activity increased more than 4 times when compared with the activity in the progenitor culture (data not shown). Many cells stained positive for TUJ1 (control with mock virus infection; Figure 2C); therefore, the luciferase value of the cell at this time point was taken as 100%. Expression of either *sNRSE* RNA or *asNRSE* RNA alone had no obvious effects on neuronal differentiation. In contrast, the *NRSE* dsRNA specifically increased the TUJ1 promoter-luciferase activity more than 2 times relative to control.

The *GFAP* and *MBP* promoters were prepared as lineage-specific luciferase assays for astrocyte and oligodendrocyte differentiation, respectively. To induce astrocyte differentiation, cells were treated with a combination of 50 ng/ml BMP-2, 50 ng/ml LIF and 1% FCS.

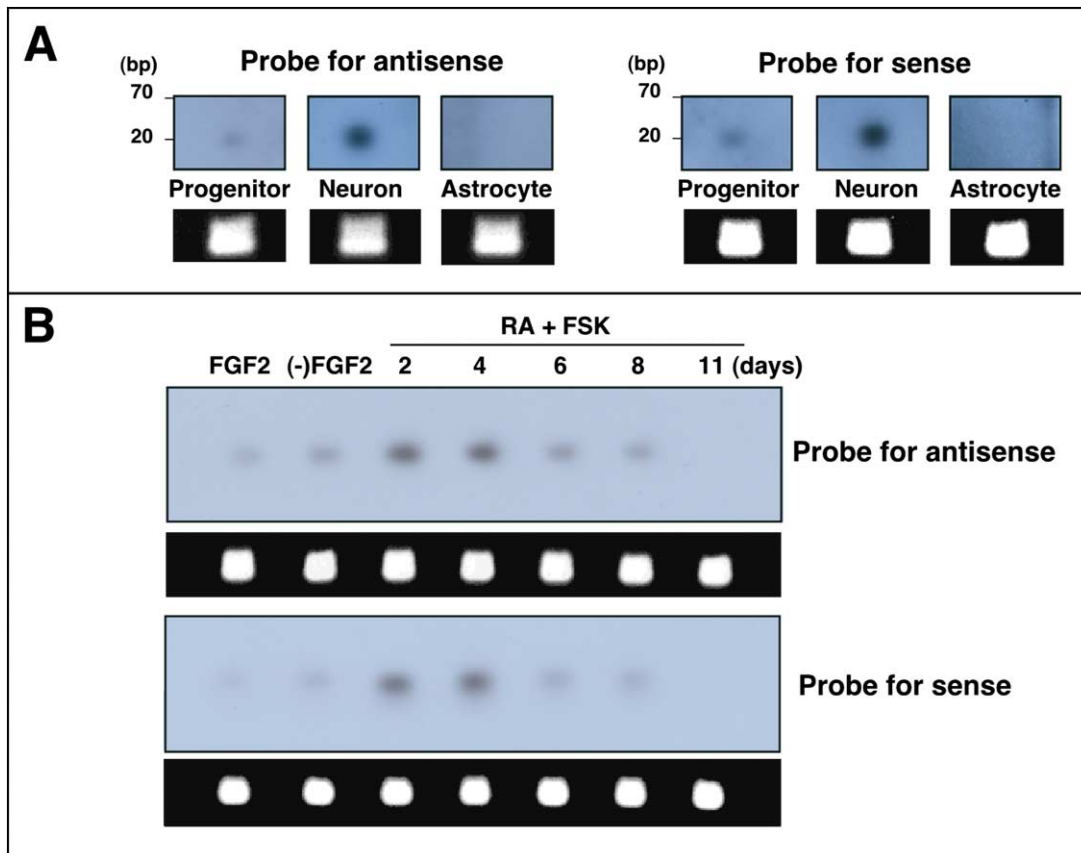


Figure 1. Identification of *NRSE* dsRNA from Adult Hippocampal Neuronal Cells

(A) Northern blot analysis of *NRSE* dsRNA. Both *asNRSE* and *sNRSE* RNA corresponding to approximately 20 nt in length exist in the neuronal population.

(B) Time-course Northern blot analysis after RA/FSK induction. Cells 2 and 4 days after induction of neural differentiation contain the highest amounts of the *NRSE* dsRNA, suggesting that *NRSE* dsRNA appears at an early stage of neurogenesis.

Four days later, the *GFAP*-luciferase activity increased more than 5-fold when compared with the activity in the progenitor culture (data not shown). To induce oligodendrocyte differentiation, FGF2 withdrawal of progenitor cultures results in some spontaneous differentiation. Two days later, the *MBP*-luciferase activity increased more than 3-fold when compared with the activity in progenitor cultures. We did not detect any obvious differences in the cases where *sNRSE* RNA and *asNRSE* RNA were expressed during astrocyte or oligodendrocyte differentiation. However, significant decreases in luciferase activity were detected when the *NRSE* dsRNA was introduced under each differentiation condition. Furthermore, *NRSE* dsRNA-expressing virus-infected cells that remained under each differentiation condition appeared to be neurons (TUJ1 positive, data not shown).

Increased Expression of Neuron-Specific Genes Containing *NRSE/RE1* by the *NRSE* dsRNA

To investigate the mechanism of action of the *NRSE* dsRNA, we first considered whether the *NRSE* dsRNA might function as a miRNA/siRNA. It has been discovered that miRNA/siRNAs exist as 21–25 nt dsRNAs and target cellular mRNAs in a complementary fashion, lead-

ing to a process of posttranscriptional gene silencing (Hutvagner et al., 2001; Pasquinelli, 2002).

Figure 2 demonstrates that neuronal lineage induction is one of the major effects of the *NRSE* dsRNA. If the *NRSE* dsRNA mediated the silencing of the *NRSF/REST* gene itself by a miRNA/siRNA-like function and if *NRSF/REST* functions as a repressor of neuronal gene expression, the repression of neuron-specific genes may be eliminated, resulting in neuronal lineage induction. However, there is no apparent *NRSE* sequence within the *NRSF/REST* mRNA, making it an unlikely target of the *NRSE* dsRNA at the posttranscriptional level. In fact, when total RNAs were extracted from HCN-A94 cells infected with *NRSE* dsRNA-expressing virus for 4 days, reverse transcription (RT) PCR analysis revealed that the introduction of the *NRSE* dsRNA did not appear to change the expression of the *NRSF/REST* itself (Figure 3A, top left).

NRSE sequences are preferentially localized within promoter regions of neuron-specific genes. We performed sequence database search, and found more than 60 *NRSE/RE1* sequences in the mouse genome. We next examined the direct effects of *NRSE* dsRNA on the expression of genes that have the *NRSE/RE1* element in their promoters (*SCG10*, *Synapsin I* *NaCh II*,

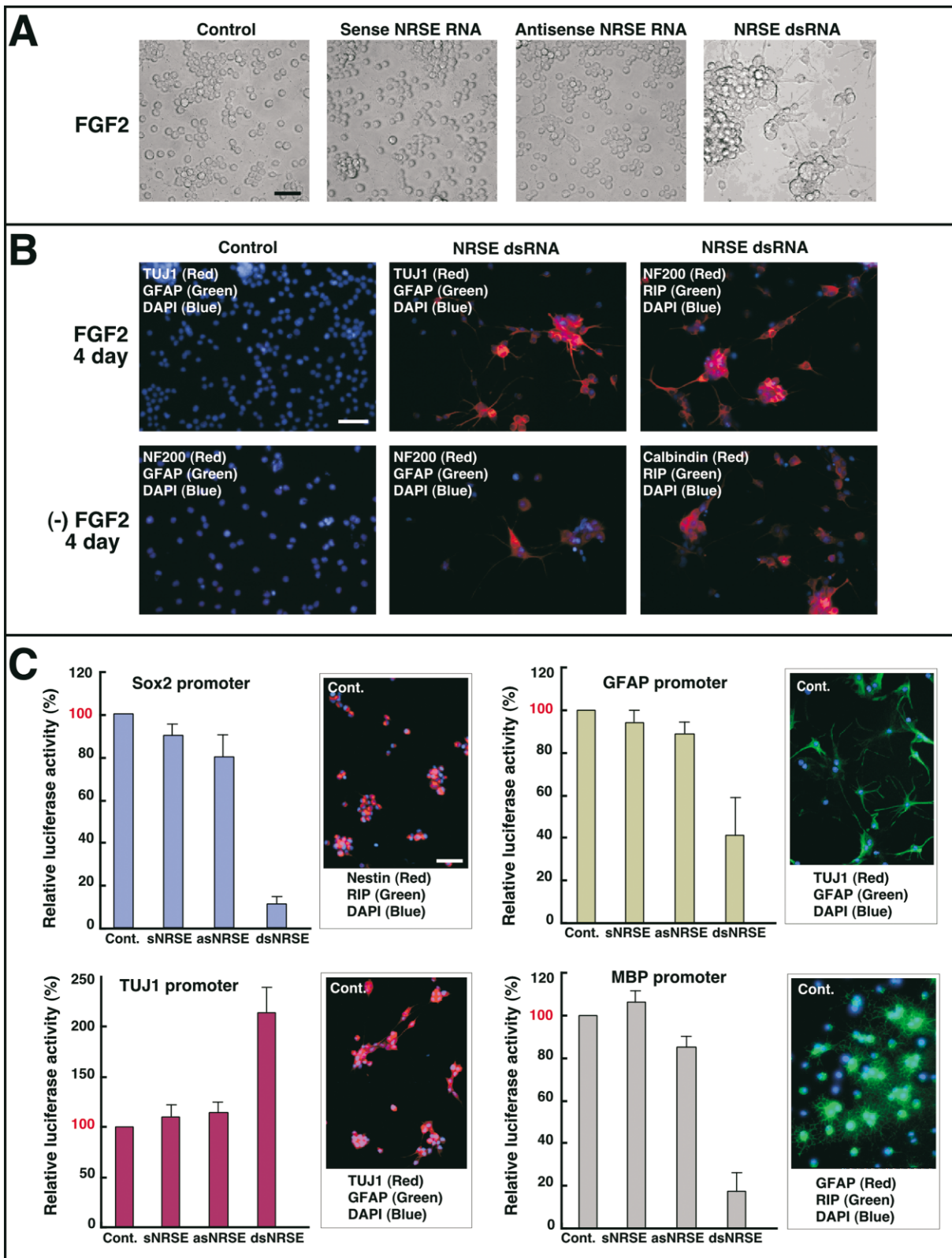


Figure 2. *NRSE* dsRNA Induces Neuronal Differentiation of Progenitor Cells

(A) Neural progenitor cells were infected with either a control virus or viruses expressing sense, antisense or *dsNRSE* RNAs.

(B) Immunocytochemical analysis of cells with *NRSE* dsRNA. Expression of *NRSE* dsRNA in progenitor cultures resulted in increased numbers of neuron-specific marker-positive cells.

(C) Quantitative analysis of *NRSE* dsRNA activity as an inducer of neuronal differentiation. The reporter assay was performed by using lineage-specific gene promoter-driven luciferase constructs. Scale bars are equal to 10 μ m.

M4 mAChR, and *mGluR2*). RT-PCR analysis revealed that the *NRSE* dsRNA increased expression levels of *NRSE/RE1*-containing genes (Figure 3A). In progenitor stages, expression levels of *SCG10*, *Synapsin I*, *NaCh II*, *M4 mAChR*, and *mGluR2* were very low. Upon introduction of *NRSE* dsRNA, significant transcriptional activation was observed (Figure 3A). These gene-activating events appeared to be *NRSE/RE1*-gene specific; no obvious increases in the expression of GAPDH and β -actin genes were detected.

To determine how widespread the *NRSE* dsRNA-dependent gene activation was, we monitored the activity with reporter assay using the *mGluR2* promoter. The *mGluR2* promoter containing an *NRSE/RE1* DNA element was fused to EGFP and this construct was transfected into various cell types infected with or without the *NRSE* dsRNA lentivirus. In HCN-A94 progenitor cultures, most of the cells were negative for GFP expression. In contrast, in progenitor cultures with the *NRSE* dsRNA, the number of GFP-positive cells increased (Figure 3B). Neurosphere cultures were prepared from whole brain of 10-day-old ICR strain mice, and primary neural stem cells were derived from ventricular zone, hippocampus, and whole brain of the 129/SvJ strain of adult mice. In all cases, the introduction of *NRSE* dsRNA mediated a substantial increase in *mGluR2* promoter activity (Figure 3B).

Critical Sequence Requirement of Both the *NRSE* dsRNA and *NRSE/RE1* DNA Element for Gene Activation

To investigate the sequence requirement and specificity, we prepared a set of simple reporter constructs. The *NRSE/RE1* element was fused upstream from the 260 bp CMV minimal promoter carrying a TATA box and linked to the luciferase gene (*NRSE-TATA*, Figure 3C). A mutated *NRSE/RE1* element was prepared similarly (*mtNRSE-TATA*). We also made expression cassettes for the *NRSE* dsRNA and a mutated *NRSE* dsRNA (*mtNRSE* dsRNA).

When we introduced TATA-luciferase constructs lacking the *NRSE/RE1* element, no differences in the luciferase activities were detected between cells infected with no RNA (control)-, *NRSE* dsRNA- and *mtNRSE* dsRNA-expressing virus constructs (Figure 3D, gray bars). In contrast, when we introduced *NRSE-TATA*-luciferase constructs, the *NRSE* dsRNA increased the expression levels of *NRSE-TATA*-luciferase gene more than 2.5 times (Figure 3D, orange bars). We next tested the effects of a mutated *NRSE* dsRNA on *NRSE-TATA*-luciferase activity. The mutations changed sequence specificity while preserving dsRNA structure. Interestingly, when the *NRSE* dsRNA was mutated (*mtNRSE* dsRNA), no additional increase relative to control was observed (Figure 3D). On the other hand, the introduction of a mutated *NRSE/RE1* DNA element (*mtNRSE-TATA*) in combination with an intact *NRSE* dsRNA was not enough to induce further gene activation (Figure 3D, green bars). These results show that *NRSE* dsRNA-dependent gene activation requires a critical sequence homology between the *NRSE/RE1* DNA element and the *NRSE* dsRNA.

RNA-Directed Chromatin Changes of *NRSE/RE1*-Containing Genes in Adult Hippocampal Neural Cells

NRSF/REST proteins interact with histone deacetylase (HDAC1) and methyl-CpG binding protein (MeCP2) to form a repressive chromatin state in nonneuronal cells (Huang et al., 1999; Lunyak et al., 2002). To investigate the nature of the transcriptional activation of *NRSE/RE1*-containing genes by the *NRSE* dsRNA, we performed chromatin immunoprecipitation (ChIP) assays. The promoter regions of *mGluR2* and *SCG10* genes were assessed as representative *NRSE/RE1*-containing genes, since these genes have been characterized in mechanistic studies of the NRSF/REST repressor complex (Myers et al., 1998; Naruse et al., 1999).

We prepared ChIP samples from HCN-A94 cells during progenitor (with FGF2) and differentiated stages (neurons, astrocytes, and oligodendrocytes). During stem/progenitor stages, as well as nonneuronal stages (oligodendrocytes and astrocytes), both *mGluR2* and *SCG10* genes were associated with NRSF/REST and HDAC1 (Figure 4A, second and third rows). Notably, NRSF/REST was always found to be associated with endogenous *mGluR2* and *SCG10* promoters, in the region of the *NRSE/RE1* (Figure 4A, second row). In the case of *mGluR2* promoter, in addition to HDAC1, methyl-DNA binding proteins of MeCP2 and MBD1 were found associated with the *NRSE/RE1* region (Figure 4A, fourth and fifth rows). As for the *SCG10* promoter, we observed decreased association of MeCP2 and MBD1, even though this gene is apparently repressed in the nonneuronal state, suggesting diversity of the repression machinery depending on specific gene/promoters containing *NRSE/RE1* elements.

In contrast, clear evidence of derepressed chromatin states was seen for both *mGluR2* and *SCG10* genes in neurons, where decreased association of HDAC1 with the *NRSE/RE1* element was found (Figure 4A, third row, third lane). The CREB binding protein (CBP)/p300 family of transcriptional coactivators possessing histone acetyltransferase activity has been shown to interact with various transcription factors to activate genes (Bannister and Kouzarides, 1996). We detected an increase in the association of CBP, acetylated histone H4, and acetylated histone H3 with both promoters when cells were in a state in which these genes are actively expressed (Figure 4A, sixth to eighth rows, third lanes). SWI/SNF chromatin-remodeling factors, BRG1 and BAF170, were also found to associate with the *NRSE/RE1* element, as part of a possible machinery to remodel the chromatin state for active expression of neuron-specific genes in neuronal cells (Figure 4A, eighth and ninth rows, third lanes).

Importantly, the ChIP assay revealed that, upon the introduction of *NRSE* dsRNA into progenitor cells, there was decreased association of the repressor proteins MeCP2, MBD1, and HDAC1 with the *NRSE/RE1*, resulting in the activation of neuronal genes. The fact that NRSF/REST still occupied the *NRSE/RE1* locus suggests that NRSF/REST may be involved in an alternative chromatin structure with acetylated histones to activate transcription. For this transition step to occur, chromatin-remodeling factors like BAF170 and BRG1, which had previously been shown to bind NRSF/REST (Battag-

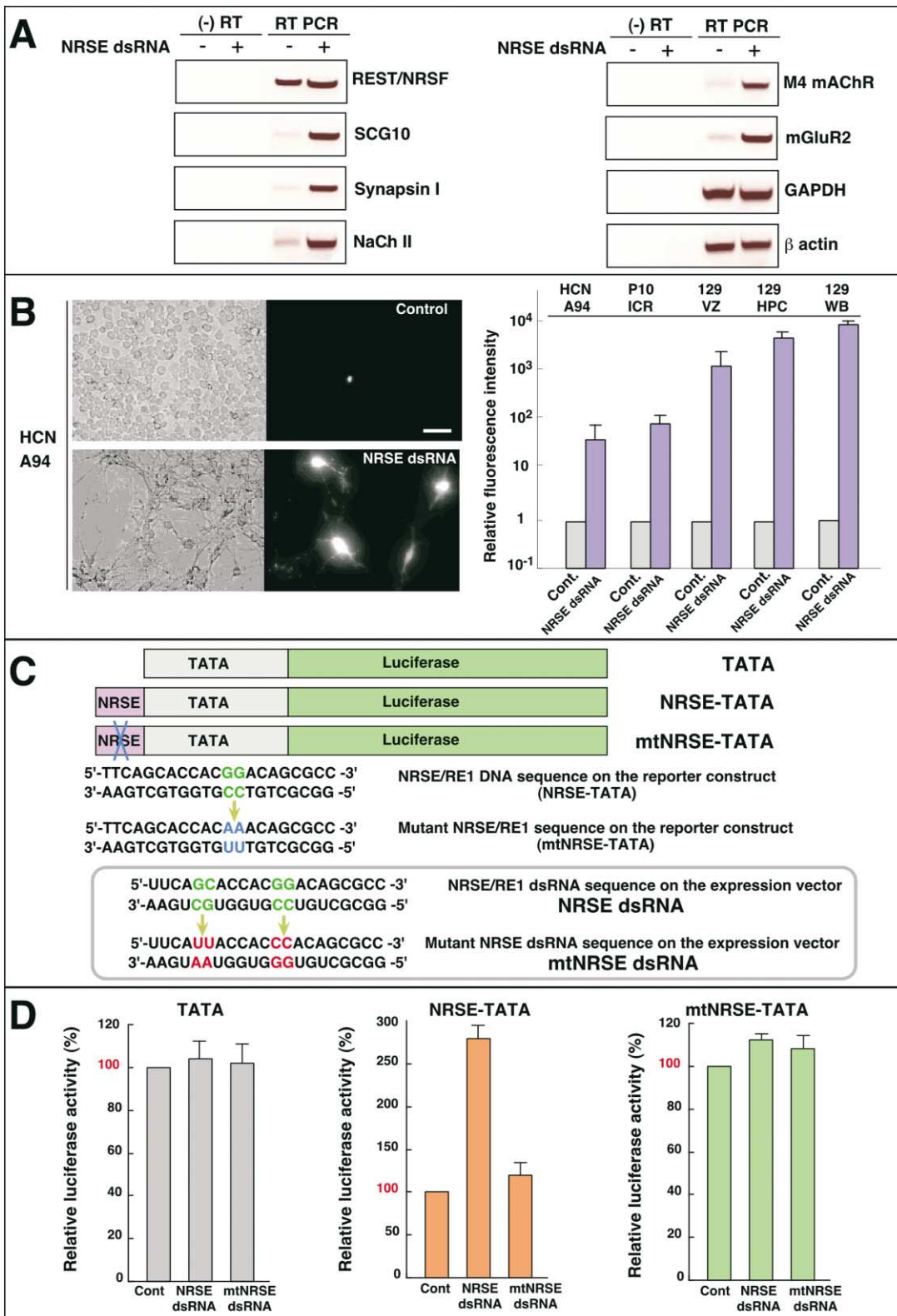


Figure 3. Effect of *NRSE* dsRNA on the Expression of Neuron-Specific Genes Containing the *NRSE/RE1* DNA Element

(A) RT-PCR analysis showed that *NRSE* dsRNA increased transcription levels of *NRSE/RE1*-containing genes without affecting the expression of *NRSF/REST*.

(B) Reporter assay for *NRSE* dsRNA activity using the mGluR2 promoter-driven EGFP construct. Effects of *NRSE* dsRNA on gene activation were assessed in neurosphere cultures and primary neural stem cells. Relative fluorescence intensity was plotted on a log scale (right). Scale bar is equal to 10 μ m.

(C) Schematic diagram of the constructs to examine the requirement of sequence specificity of *NRSE*. The *NRSE/RE1* element was fused

lioli et al., 2002), may be required for remodeling the chromatin through their ATPase activity.

We also examined the function of *NRSE* dsRNA in gene-specific activation. HCN A94 cells were treated with either an HDAC inhibitor trichostatin A (TSA) or with demethylation reagent 5'-aza-cytidine (5AzaC). Endogenous expression levels of mRNAs of mGluR2, NaCh II, *SCG10*, and *Synapsin I* were increased in TSA-treated cells, compared with untreated cells (Supplemental Figure S2 available on Cell website). The treatment with 5AzaC showed significant activation of the M4 AChR, mGluR2, and NaCh II mRNAs. Some of these differences in repression responses among neuronal genes reinforce the idea that there may be diversity in the REST/NRSF regulatory machinery.

NRSF/REST Is Converted from a Transcriptional Repressor to an Activator in the Presence of *NRSE* dsRNA

To determine whether the transactivation of genes by *NRSE* dsRNA was caused by derepression or by a functional switch of NRSF/REST from repressor to activator, we made GluR2 promoter-driven luciferase constructs [wild-type (GluR2-luciferase, Figure 4B) and mutated *NRSE* substituted with random nucleotides (mtGluR2-luciferase, Figure 4B)], and compared the level of luciferase activity with and without expression of *NRSE* dsRNA in adult neural stem cells (Figure 4B). NRSF/REST cannot bind to mutated *NRSE* sequences (Kraner et al., 1992). In the case of the mutated *NRSE* construct (mtGluR2-luciferase), the relative luciferase activity is seen at baseline levels, presumably due to a release of NRSF-mediated repression (derepression). We observed at least a 2-fold increase in the wild-type GluR2-luciferase construct upon introduction of the *NRSE* dsRNA, but not in the mtGluR2-Luciferase construct, indicative of an activation effect. This activation was never observed with the introduction of a mutant *NRSE* dsRNA or a control vector; in fact there was an active repression of GluR2-luciferase, consistent with NRSF/REST actions as a repressor. Taken together, these results suggest that: (1) NRSF/REST functions as a repressor in the absence of *NRSE* dsRNA, (2) NRSF/REST converts to an activator in the presence of *NRSE* dsRNA, and (3) the activator function of NRSF/REST is dependent on having both a wild-type *NRSE/RE1* DNA sequence and a wild-type *NRSE* dsRNA.

The Loss of Nuclear Localizing *NRSE* dsRNA Blocks Neuronal Differentiation in Adult Hippocampal Stem Cells

To determine if *NRSE* dsRNA is necessary for neuronal differentiation, we designed a ribozyme (Rz; Figure 5A) that can specifically cleave one of the strands of the dsRNA sequence, thus inactivating the expression of the *NRSE* dsRNA. For ribozymes, additional proteins are not needed for catalysis; they only require Mg^{2+} ions,

which are abundant in cells (Eckstein and Lilley, 1996; Warashina et al., 2000). Since it is important to select the appropriate promoter to express the ribozyme in the compartment of the cell where the target RNA is located (Koseki et al., 1998), we first analyzed the localization of *NRSE* dsRNA by Northern blotting. We found both antisense and sense *NRSE* RNAs dominantly expressed in the nuclear fraction (Figure 5A), reinforcing the finding that *NRSE* dsRNAs are not acting as miRNAs, which target cytoplasmic mRNAs to inhibit their translation. Treatment of progenitor cells with the ribozyme completely abolished expression of the *NRSE* dsRNA. An inactive ribozyme (I-Rz) with one nucleic acid substitution in the catalytic domain was prepared as a negative control and did not affect *NRSE* dsRNA expression.

We introduced both nuclear specific U6-driven functional Rz and I-Rz into HCN A94 cells by lentiviral infection. No obvious effects were detected at the progenitor stage (since *NRSE* dsRNAs are not expressed at this stage) compared with the cells in which the *NRSE* dsRNA had been introduced by lentivirus (Figure 5B). When the culture was switched into the neuronal differentiation condition, in the case of I-Rz, normal neuronal differentiation was observed (Figure 5B, bottom right). However, when the Rz targeting *NRSE* dsRNA was introduced, cells displayed strong antidifferentiation effects even with RA+FSK stimulation and resembled the morphology of cells in progenitor stages (Figure 5B, top right).

To determine the effect of Rz in each differentiation pathway, a cell type-specific promoter-based reporter assay was performed similar to that shown in Figure 2C. Luciferase values from cells 4 days after control mock virus-infection were taken as 100%. Under progenitor culture conditions, Sox2 promoter-driven luciferase values resulted in no difference in the cases of Rz and I-Rz treatment, probably due to the lack of endogenous dsRNA (Figure 5C).

Under the neuronal condition, the level of the *NRSE* dsRNA increased, as well as TUJ1 promoter-driven luciferase activity. Introduction of the Rz in this condition significantly reduced the TUJ1-luciferase activity, whereas the I-Rz had no effect (Figure 5C). Under astrocyte or oligodendrocyte differentiation conditions, no obvious differences were detected in the levels of luciferase driven from the *GFAP* or *MBP* promoter, respectively, upon either Rz or I-Rz introduction (Figure 5C). Mutant *NRSE* dsRNA (mt*NRSE* dsRNA) had no effect on various luciferase assays.

***NRSE* dsRNA in the Nuclei of Cells Differentiating into Neurons**

We next carried out in situ hybridization against *NRSE* dsRNA and immunostaining for NRSF/REST protein simultaneously. As illustrated in Figure 6A, DAPI (blue) and *NRSE* RNA (green) colocalized in the nucleus of HCN-A94 cells in neurons (RA+FSK for 4 days, upper

upstream of TATA box and linked to the luciferase gene. Mutated *NRSE/RE1* DNA element on the reporter construct (mt*NRSE*-TATA) and mutated *NRSE* dsRNA expression construct at a critical recognition site (mt*NRSE* dsRNA) were also prepared.

(D) Sequence requirement of both *NRSE* dsRNA and the *NRSE/RE1* DNA element for gene activation. Luciferase assay showed that *NRSE* dsRNA-dependent gene activation requires a specific sequence homology between the *NRSE/RE1* DNA element and *NRSE* dsRNA.

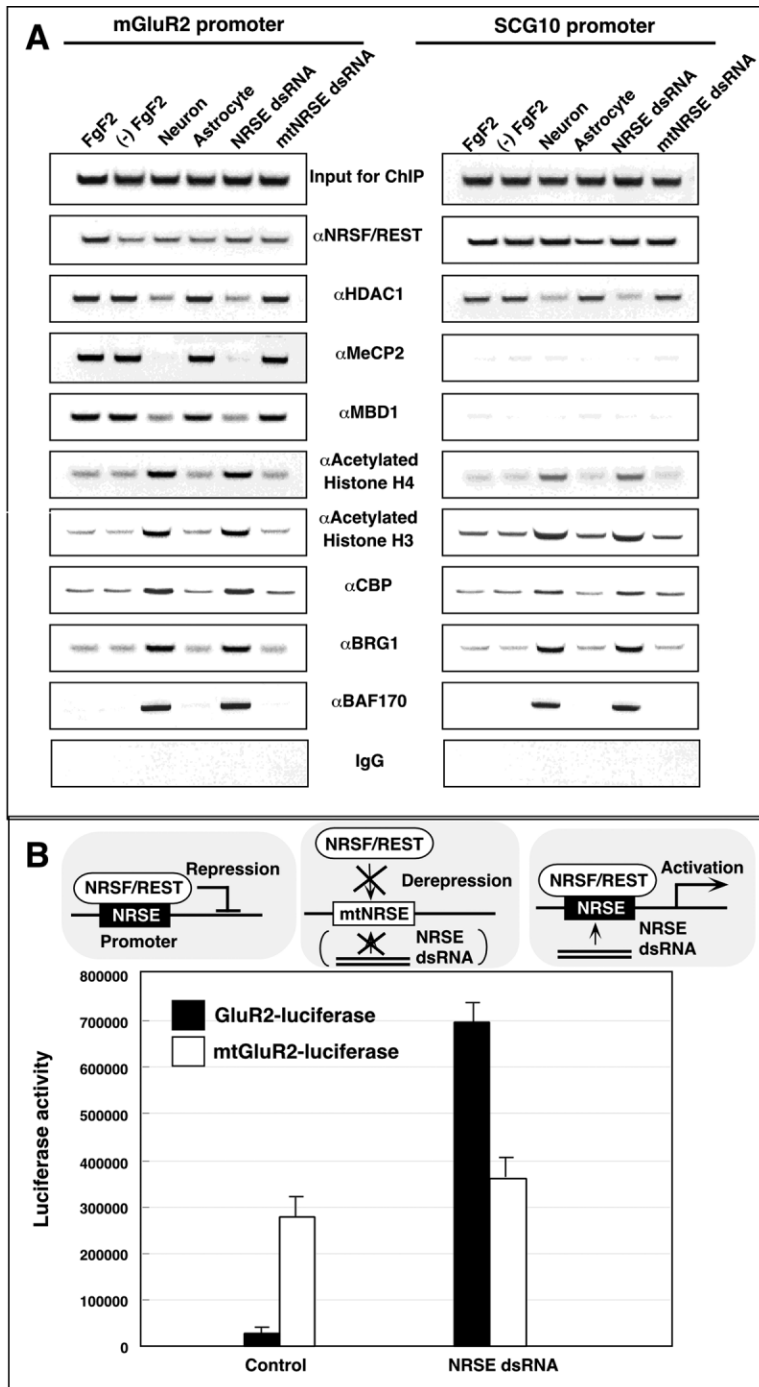


Figure 4. *NRSE* dsRNA-Directed Chromatin Changes of *NRSE/RE1*-Containing Genes

(A) ChIP assay for chromatin regulating factors. (B) Reporter assay for *NRSE* dsRNA function to convert *NRSF/REST* from a repressor to an activator using the *GluR2* promoter-driven luciferase construct. Intact and mutated *mGluR2*-luciferase constructs were prepared and the level of luciferase activity driven by each promoter in the presence or absence of *NRSE* dsRNA expression was compared.

images). However, colocalization did not occur when DNA was condensed during cell division (white arrow, Figure 6A, upper image). During DNA condensation, *NRSE* RNAs remained in the nuclear domain but appeared to be outside of the condensed chromosomal region (white arrows, Figure 6A, upper image). *NRSE* dsRNA localization in mitotic cells seems to reflect the localization of histone acetylase/proteins, which also appear beyond the condensed chromosomal region. The nature of their actions on transcriptional regulation is in accord with the finding that transcription is repressed during mitosis (Kruhlik et al., 2001). Molecules

smaller than 50~70 kDa can translocate back and forth through the nuclear pore through a process of natural diffusion (Stehno-Bittel et al., 1995). Since *NRSE* dsRNAs are ~20 bp in length (less than 20 kDa), they would likely diffuse throughout the cell. However, *NRSE* dsRNAs were located specifically in the nucleus, suggesting as yet unknown molecule(s) restricting the localization of the dsRNA to the nucleus.

NRSF/REST proteins are mainly localized in the nucleus, regardless of cell division (magenta, Figure 6A). Even though *NRSF/REST* is expressed in all of the cells, the cells expressing higher amounts of *NRSE* RNAs were

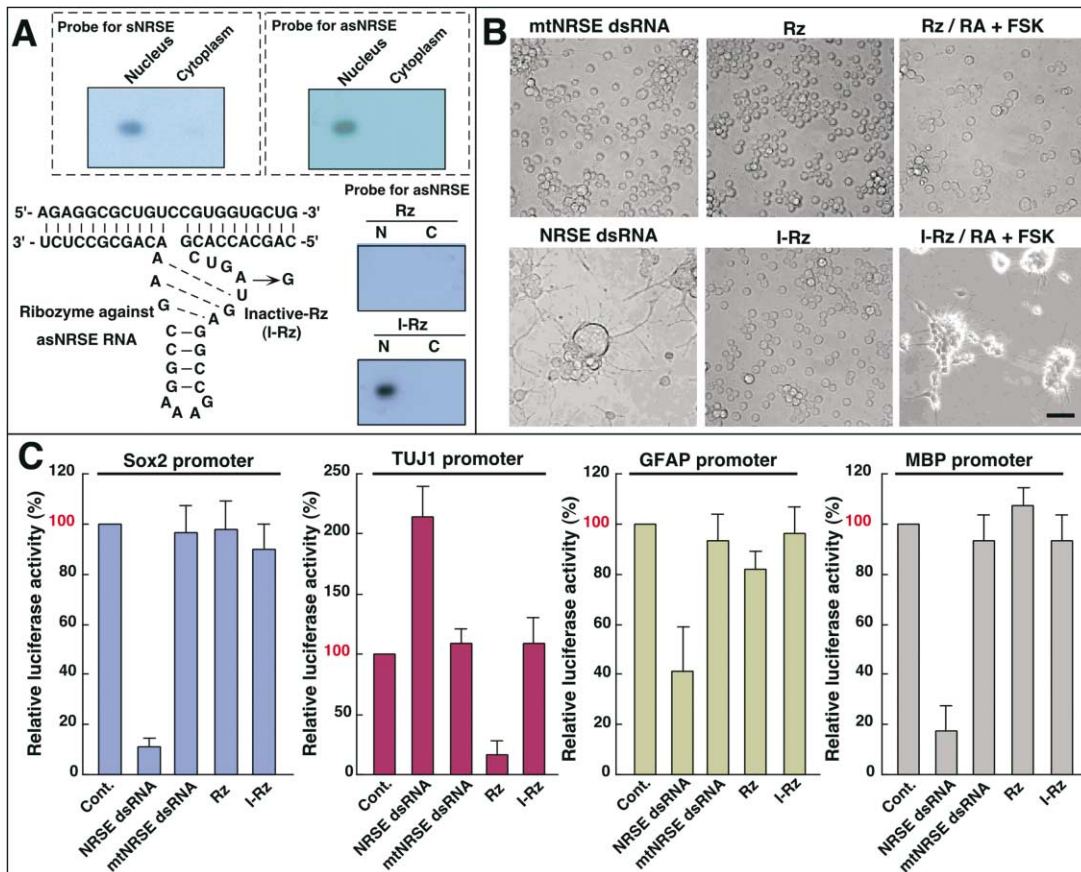


Figure 5. Loss of *NRSE* dsRNA Blocks Neuronal Differentiation

(A) Nuclear localization of the *NRSE* dsRNA was confirmed by Northern blot analysis. The ribozyme (Rz) sequence was designed to cleave the *asNRSE* RNA, and the effect was assessed by Northern blot using fractionated RNAs extracted from 4-day RA+FSK-treated HCN cells. Nuclear fraction, (N); cytoplasmic fraction, (C).

(B) Cells with Rz cleaving antisense *NRSE* RNA showed strong antidifferentiation effects. Scale bar is equal to 10 μ m.

(C) The effect of Rz on the function of *NRSE* dsRNA in each lineage. Cell type-specific promoter-based reporter assay was performed in HCN A94 cells. The results shown are the averages of results from three sets of experiments.

also TUJ1-(yellow) positive (red arrows). Conversely, cells expressing *NRSE* RNAs at the lowest levels were TUJ1-negative (blue arrows).

Interaction between NRSF/REST and the *NRSE* dsRNA

To examine the potential interaction between NRSF/REST protein and *NRSE* dsRNA, we incubated the cell extract with biotin-labeled *NRSE* dsRNAs. We also prepared biotin-labeled *NRSE* dsDNAs as a positive control. To assess the specificity in the interaction, negative controls with the partial sequence of the multicloning site (MCS) in pBlueScript II SK+ were prepared. Bound proteins were “pulled down” and analyzed by Western blot (Figure 6B). The immunoblot revealed that both the *NRSE* dsDNAs and dsRNAs bound NRSF/REST, demonstrating an interaction between NRSF/REST and *NRSE* dsRNA.

To compare the affinity between *NRSE* dsDNA and *NRSE* dsRNA to NRSF/REST protein, we carried out electrophoretic mobility shift assay (EMSA). NRSF/REST with cMyc-tag was expressed in 293T cells and immunoprecipitated with anti-cMyc antibody. After purification, the protein was incubated with either *NRSE* dsDNAs

or dsRNAs. We tested a range of NRSF/REST protein concentrations; the highest one tested produced a shift in dsDNA migration, whereas a 16-fold lower concentration of NRSF/REST protein was enough to produce a shift in dsRNA (Figure 6C). Surprisingly, these data revealed that the affinity of NRSF/REST to the *NRSE* dsRNAs was much higher than the affinity to *NRSE* dsDNAs. Binding of NRSF/REST to the sequence of the MCS control dsRNAs or dsDNAs was not observed. Furthermore, no apparent band-shift was observed in samples of bovine serum albumin (BSA) incubated with *NRSE* dsRNAs or dsDNAs. This highly specific binding between *NRSE* dsRNA and NRSF/REST protein may contribute to a functional switch of the NRSF/REST machinery from transcriptional repressor to activator.

Expression of NRSF/REST Protein and *NRSE* dsRNA in the Adult Hippocampus

We next examined the expression of NRSF/REST mRNA and *NRSE* dsRNA in the adult mouse hippocampus by in situ hybridization. Although NRSF/REST mRNA was expressed in nonneuronal glial cells (data not shown), the mRNA was highly expressed in hippocampal neurons (Figure 7), suggesting that NRSF/REST is playing

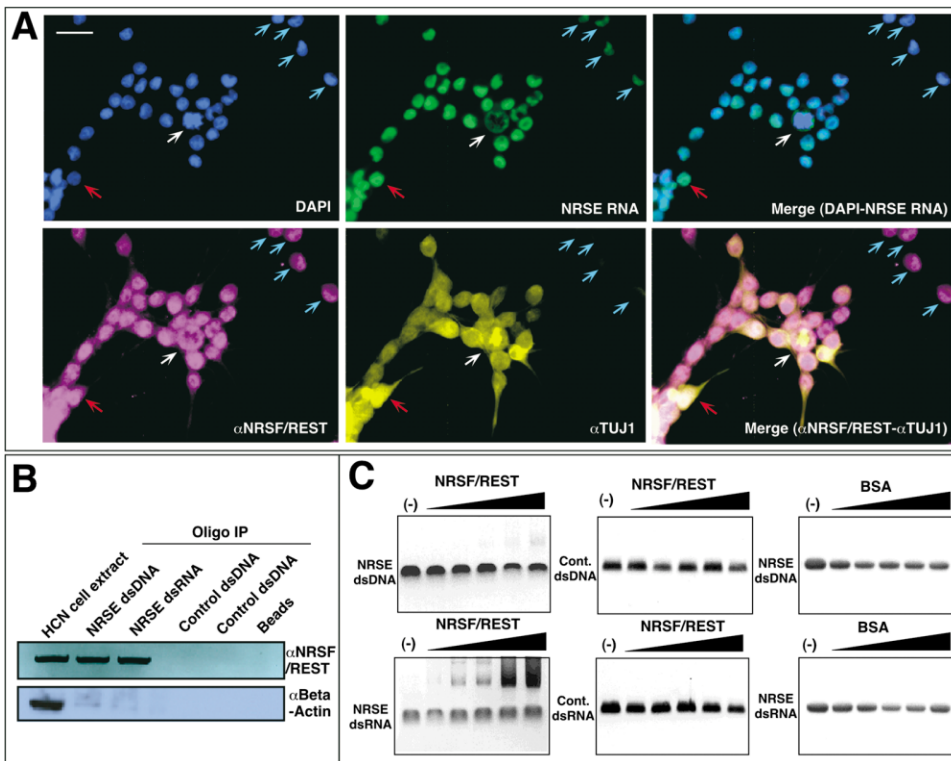


Figure 6. Localization of *NRSE* dsRNAs in the Nucleus of Neuronal Cells and Their Interaction with NRSF/REST Protein

(A) Nuclear localization of *NRSE* dsRNAs and NRSF/REST. Scale bar is equal to 10 μ m. (B) Binding of *NRSE* dsRNAs to endogenous NRSF/REST proteins. Proteins that had bound to the biotin-labeled oligonucleotides were “pulled down” with streptavidin beads and were analyzed by Western blot. (C) EMSA of NRSF/REST protein against *NRSE* dsRNA and dsDNA. While the concentration of each nucleotide was fixed as 20 μ M, protein amount was increased 2-fold by each lane depending on arrow direction.

not only a role as a transcriptional repressor in nonneuronal cells but also a role in neurons *in vivo*. To verify the specificity of the *in situ* experiments, we did additional experiments with two negative control probes: a probe with the same nucleotide content containing a scrambled sequence and a probe with the same sequence in the reverse direction. There was no detectable signal with either negative control probe (data not shown). An enlarged view of NRSF/REST and *NRSE* dsRNA expression is shown in Figure 7B. Similar expression patterns of NRSF/REST have been previously documented (Kalunki et al., 1998; Palm et al., 1998; Timmusk et al., 1999). Interestingly, the expression of *NRSE* dsRNA was highly restricted in the subgranular region of dentate gyrus, in a region where adult neurogenesis is continuously occurring (van Praag et al., 2002; Kempermann, 2003) (Figure 7), supporting the *in vitro* data that *NRSE* dsRNAs function at an early stage in neuronal differentiation (Figures 1 and 2).

These results suggest the existence of interactions between proteins (NRSF/REST complex) and dsRNAs (the *NRSE* dsRNA) in addition to dsDNAs (*NRSE/RE1* element). After the participation of dsRNAs in cells at early stages of neurogenesis, the NRSF/REST complex alters binding partners from repressors to activators to initiate transcription of neuron-specific genes. Derepression events might include global changes in cells, but at least the key players—proteins, dsDNAs, and

dsRNAs—could recognize each other within the nucleus in order to direct neurogenesis.

Discussion

NRSE dsRNA Stimulates Neuronal Differentiation through an Interaction with NRSF/REST Complex

In this study, a dsRNA with a restrictive silencer element was identified as a functional transcriptional activator. Genes important for neuronal properties contain the *NRSE* sequence, which is recognized by the protein NRSF/REST (Palm et al., 1998; Schoenherr et al., 1996). The maintenance of neuronal gene repression in nonneuronal cells depends on the ability of NRSF/REST to bind the *NRSE* sequence (Chen et al., 1998; Huang et al., 1999; Lunyak et al., 2002). To repress gene expression, NRSF/REST recruits negative transcriptional regulators such as HDACs and methyl-DNA binding proteins (Lunyak et al., 2002; Naruse et al., 1999). The question of how multipotent adult neural stem cells switch from actively repressing neuron-specific genes in the “stem cell state” to actively expressing neuron-specific genes in the “differentiated state” appears to be explained, at least in part, by the *NRSE* dsRNA. The cell that will become a neuron activates transcription of genes marked by the *NRSE*. These cells supply noncoding RNA that forms dsRNA with an *NRSE* sequence. The

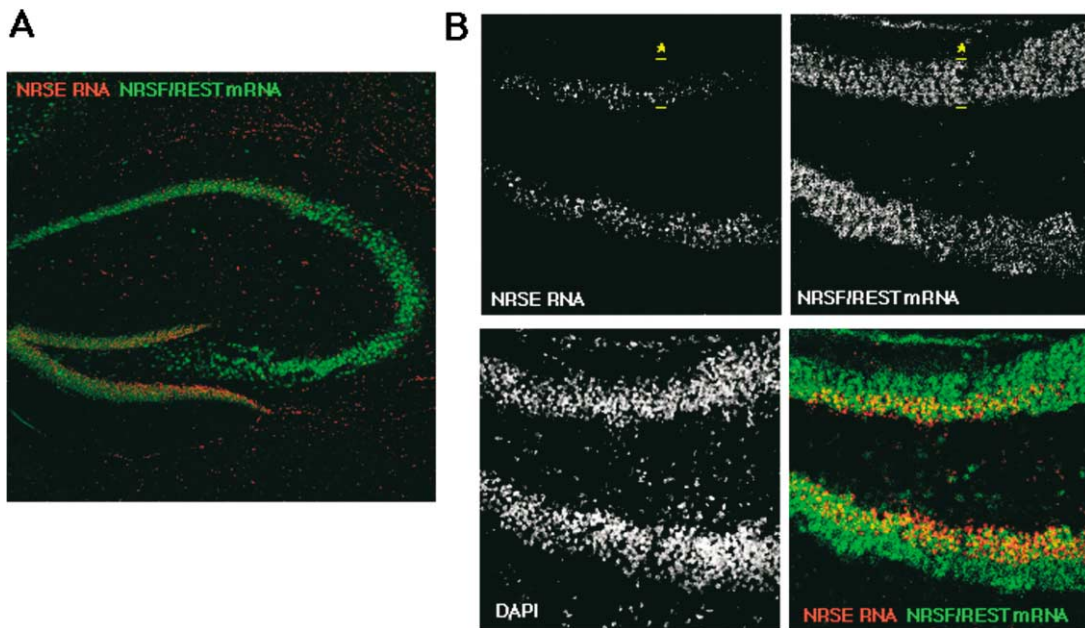


Figure 7. In Situ Hybridization Analysis for NRSF/REST mRNA and *NRSE* dsRNA in Adult Mouse Hippocampus (A and B) *NRSE* dsRNA expression was highly restricted within the subgranular layer of the dentate gyrus, whereas NRSF/REST mRNA was expressed in a widespread neuronal area in adult hippocampus. (B) Higher magnification view.

dsRNA interacts with NRSF/REST machinery, resulting in the NRSF/REST switching cofactors from repressors to activators. This intrinsic ability of the NRSF/REST machinery implies that NRSF/REST can function as a flexible mediator of *NRSE* regulatory elements.

Multipotent neural stem cells require a highly selective gene regulation system to achieve uniquely different fates. During the uncommitted stem cell state, the genes required for neural differentiation are repressed (Figure 8), whereas commitment to the neuronal lineage requires repression of stem cell-, astrocyte-, and oligodendrocyte-specific genes and activation of neuron-specific genes (Figure 8). Adult hippocampal neural stem cells would be considered one of the cell types that need selective gene regulation for endogenous fate determination. Noncoding dsRNAs encoding *NRSE* sequences play a unique role in *NRSE* element dependent gene regulation, without alteration of the expression of a key protein player, NRSF/REST, at the transcriptional level. The expression of *NRSE* dsRNA modulates the function of NRSF/REST between activator and repressor of neurogenesis.

We have shown that *NRSE* dsRNAs can act as inducers of neuronal differentiation. Interestingly, introduction of *NRSE* dsRNA alone is sufficient to activate *NRSE/RE1*-containing neuron-specific genes and induce neuronal differentiation. Furthermore, introduction of a ribozyme targeted against the *NRSE* dsRNA has antineuronal differentiation effects, suggesting that *NRSE* dsRNAs are also necessary to induce neuronal differentiation. It should be noted that the NRSF/REST mRNA is highly expressed in adult hippocampal neurons as well as in nonneuronal cells, and the expression of *NRSE* dsRNAs was highly restricted in the subgranular layer

of dentate gyrus, one of the neurogenic regions in the adult mammalian brain (van Praag et al., 2002; Kempermann, 2003). These findings imply that *NRSE* dsRNAs also participate in neuronal differentiation in vivo.

Possible Mechanism of *NRSE* dsRNA-Mediated Neuronal Differentiation

NRSE dsRNA binds NRSF/REST as well as the *NRSE* dsDNA (Figure 6B). A simple model of the mechanism of dsRNA-dependent activation is that the dsRNA captures NRSF/REST as a decoy and releases the genome from the repression. However, the ChIP analysis (Figure 4A) indicates that NRSF/REST protein remains stably associated with the *NRSE/RE1* machinery in both the “stem cell state” and the “differentiated states.” These results also show that the nature of the *NRSE*-containing chromatin changes from a repressed state (association with HDACs, MBD1, and MeCP2) to an activated state (association with acetylated histones) and does not involve a change in the association of the NRSF/REST protein itself. How can we explain a change in NRSF/REST function, even though NRSF/REST proteins appear to remain physically associated at *NRSE/RE1* sites within different cell stages? We postulate several models (Figure 8). Model A, based on pull-down experiments (Figures 6B and 6C) and mutation analyses (Figure 3D), proposes that there is a physical interaction between the *NRSE* dsRNA and NRSF/REST protein, suggesting a critical sequence dependency between dsRNAs and proteins. Basically, *NRSE/RE1*-containing neuronal genes are actively repressed by the NRSF/REST machinery (through the association of HDACs and methyl-DNA binding proteins). At the onset of neuronal differentiation, the dsRNA interacts directly with *NRSE* dsDNA-NRSF/REST ma-

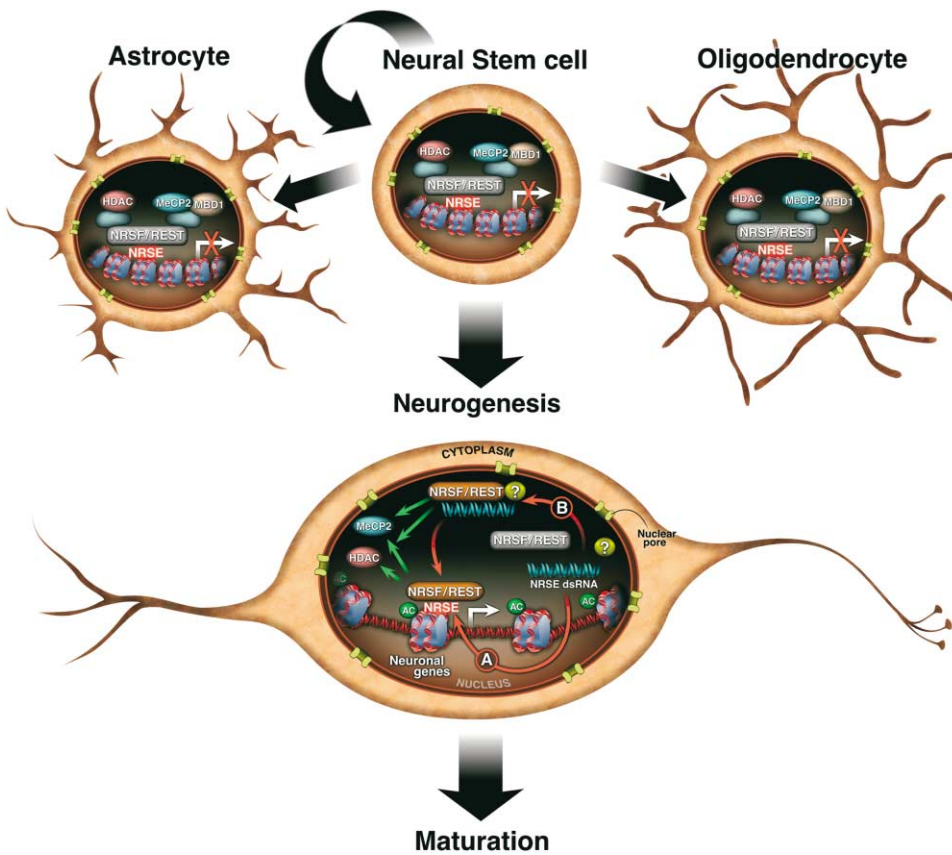


Figure 8. Schematic Representation of Activation Events by *NRSE* dsRNA

NRSE dsRNA can trigger gene expression of neuron-specific genes through interaction with NRSF/REST transcriptional machinery. This interaction results in the NRSF/REST complex no longer binding to HDACs, MeCP2, and MBD1.

chinery within the genome and triggers an organizational change in transcriptional activation (Figure 8, Model A). Another possibility is that the NRSF/REST protein acts as a homodimer. In this case, the *NRSE* dsRNA could bind one monomer of NRSF/REST while the other monomer remains physically associated with dsDNA/chromatin. In Model B, through physical interactions with the NRSF/REST complex, *NRSE* dsRNAs alter NRSF/REST function, possibly by inducing a conformation change in NRSF/REST and/or associated proteins (Figure 8, Model B). In both cases, after an interaction with *NRSE* dsRNAs, the NRSF/REST complex can no longer associate with repressor proteins, such as HDACs, MeCP2, and MBD1.

NRSF/REST is a krüppel family zinc finger protein that contains a DNA binding domain with an eight zinc-finger cluster and one zinc finger at carboxyl terminal (Palm et al., 1998; Shimojo et al., 2001; Tapia-Ramirez et al., 1997). A zinc finger domain includes 2 conserved cysteine and 2 conserved histidine residues in a C-2-C-12-H-3-H (C₂H₂) type motif. Zinc finger domains have been found in numerous nucleic acid binding proteins and interact with nucleotides in the major groove of the nucleic acid. They have the ability to bind to both RNA and DNA. C₂H₂ motif zinc finger proteins represent one of the most common nucleic acid binding motifs found in nature (Hoovers et al., 1992). Proteins containing these motifs are generally DNA binding transcription factors

that recognize specific sequences in the context of the B-form helix. However, some zinc finger proteins are also able to bind single-stranded RNA, double-stranded RNA, and RNA-DNA hybrids. Recently, a zinc finger protein was identified that possessed higher binding affinity to A-form dsRNA and RNA-DNA hybrids than to B-form dsDNA helix (Finerty and Bass, 1999). Interestingly, the NRSF/REST protein contains similar features to these RNA binding motifs. Although as mentioned above, Figures 6B and 6C showed possible interactions between NRSF/REST and the *NRSE* dsRNA, it remains to be determined whether NRSF/REST alone is capable of binding to the dsRNA or if this association involves a larger protein complex capable of binding *NRSE* dsRNA. Some proteins may specifically and dominantly recognize the short dsRNA form itself in the nucleus, and this complex interacts with NRSF/REST within the genome at *NRSE/RE1* loci, as a common machinery for the dsRNA-dependent transcriptional regulation (Figure 8, Model B).

Noncoding Small dsRNA Regulates Gene Expression at a Transcriptional Level

In animals, the dsRNA-specific endonuclease, Dicer, produces miRNAs and siRNAs for gene silencing (Bernstein et al., 2001; Hutvagner et al., 2001). miRNA/siRNAs target mRNAs through their sequence homology, leading to gene silencing via the Dicer complex within the

cytoplasm. Posttranscriptional gene silencing by non-coding RNA critically contributes to regulation of developmental timing, spatial patterning of cell fates, and cellular physiology (Eddy, 2001; Fire et al., 1998; Pasquelli, 2002).

Pre-miRNAs approximately 70 nt in length are made within the nucleus, and a protein complex(es) recognizes them, exports them to the cytoplasm, and passes them to the next players for various gene silencing events (Lee et al., 2002). The identified 21–25 bp *NRSE* dsRNA is smaller; if there are no molecules to keep them within the nucleus, they should naturally diffuse through the nuclear pore and out into the cytoplasm. Since *NRSE* dsRNAs are clearly localized only in the nucleus (Figures 5A and 6A), there must be molecule(s) involved in sequestering their localization. One major candidate is the NRSF/REST protein, since it can recognize the *NRSE* dsRNA through sequence specificity by the zinc-finger motifs. Other candidates are the above-mentioned specific proteins that recognize the short dsRNA form itself in the nucleus and perhaps act as global regulators, like Dicer for a miRNAs/siRNA regulatory mechanism (Figure 8, Model B).

The dsRNAs so far identified may regulate mRNA expression at a posttranscriptional step in the cytoplasm. The currently reported *NRSE* dsRNAs appear to function exclusively at the transcriptional level, suggesting a novel aspect of noncoding dsRNA function. Even though their functions are different, their nucleotide lengths are almost the same (21–25 bp), enabling them to diffuse in cells without limitation. Therefore, dsRNAs might be sequestered in specific cellular compartments through interaction with their cognate protein partner(s) to mediate effects in a spatial-temporal and sequence-dependent manner of target mRNA, DNA, and proteins. Many questions remain, including the exact mechanism that produces small modulatory dsRNAs within the nucleus, and whether a noncoding *NRSE* gene(s) exists. We believe that the *NRSE* dsRNA defines a class of functional noncoding RNAs that have primary roles in regulating gene expression at the transcriptional level, and we propose that this class be named small modulatory RNAs (smRNAs).

Experimental Procedures

Cell Culture

HCN A94 cells were cultured as described (Gage et al., 1995). For neuronal differentiation, cells were cultured in N2 medium (Invitrogen) containing RA (1 μ M, Sigma) and forskolin (5 μ M, Sigma). For astrocyte differentiation, cells were cultured with 50 ng/ml BMP-2 (R&D systems), 50 ng/ml LIF (Chemicon), and 1% FCS (HyClone) for 4–10 days. For oligodendrocyte differentiation, cells were cultured in N2 medium after FGF-2 withdrawal for 2–4 days. Cell imaging was performed using microscope (Nikon TE300) with a SPOT camera.

Construction of Plasmids

The *sNRSE* RNA- and *asNRSE*-expressing lentiviral vectors were constructed by using CSC PW, a lentiviral vector. The CMV promoter was digested out. U6 promoter drives each *NRSE* RNA sequence with the terminator at the 3' end first amplified by PCR. Each U6 cassette was subcloned into CSC PW. Ribozyme-expressing vectors were constructed similarly. The production of lentivirus has been described elsewhere (Pfeifer et al., 2001), and infections were

almost 100% (viral titers were $>1.5 \times 10^4$ Tu/ng defined by the P24 assay).

Murine *Sox2* promoter on *Sox2* pBS SK (gift from Dr. Rizzino) was inserted into pNeoLuci at the site of MCS (Clontech). Murine *TUJ1*-, *GFAP*-, *MBP*- and rat *GluR2*-promoters were cloned by PCR from genomic DNA and each promoter was inserted into pNeoLuci. The *mtGluR2*-luciferase construct with mutated *NRSE/RE1* substituted with random nucleotides (from TTCAGCACACGGACAGCGCC to GCATCCGCACCGCTAGCGCAG), was also prepared. The TATA, *NRSE*-TATA and *mtNRSE*-TATA luciferase reporter plasmids were constructed in pGL2-basic plasmid (Promega).

Northern Blotting Analysis

Total RNA was extracted with TRIzol reagent (Gibco-BRL). To prepare cytoplasmic fraction, cells were incubated in digitonin lysis buffer (50 mM HEPES/KOH, [pH 7.5], 50 mM potassium acetate, 8 mM $MgCl_2$, 2 mM EGTA, and 50 μ g/ml digitonin) on ice for 10 min. The lysate was centrifuged at $1,000 \times g$ and the supernatant was collected as the cytoplasmic fraction. The pellets resuspended in NP-40 buffer (20 mM Tris-HCl, [pH 7.5], 50 mM KCl, 10 mM NaCl, 1 mM EDTA, and 1% NP-40) were used as the nuclear fraction. Purified RNA was loaded on a 3.5% NuSieve-Seakem agarose gel (FMC Inc.) and transferred to a Hybond-N nylon membrane (Amersham Biosciences). The membrane was probed with synthetic oligonucleotides that were complementary to the sequences of each *sNRSE* or *asNRSE* that had been labeled with ^{32}P by T4 polynucleotide kinase (NEB). Prehybridization and hybridization were carried out using EazyHyb solution (Clontech) following manufacturer's instructions.

In Situ Hybridization and Immunofluorescence Studies

Cells were fixed in fix/permeabilization buffer (50 mM HEPES/KOH, [pH 7.5], 50 mM potassium acetate, 8 mM $MgCl_2$, 2 mM EGTA, 2% paraformaldehyde, 0.1% NP-40, and 0.02% SDS) for 15 min. The FITC-/rhodamine-labeled oligodeoxynucleotide probes matching complementary to *asNRSE* and NRSF/REST mRNA were denatured for 10 min at 70°C and chilled. Hybridization buffer, containing 20% dextran sulfate and 2% BSA in $4 \times$ SSC, with probes were placed on the cells for 16 hr. Cells were rinsed in $2 \times$ SSC/50% formamide and in $2 \times$ SSC for 20 min each.

Immunofluorescence studies were performed basically as described (Gage et al., 1995): rabbit anti- β tubulin-III (TUJ1; 1/7500, Covance), guinea pig anti-*GFAP* (1:500; Advanced Immunochemical, Inc.), rabbit anti-NF200 (Advanced Immunochemical, Inc.), mouse anti-RIP (1/250, Immuno), rabbit anti-calbindin (Advanced Immunochemical, Inc.) and DAPI (Sigma). All secondary antibodies were from Jackson ImmunoResearch. Images were analyzed using Bio-Rad Radiance confocal imaging system (Hercules, CA).

Luciferase Assay

Luciferase activity was measured with Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's protocol. The luminescent signal was quantitated with a luminometer (Lumant LB 9501). As an internal control, a plasmid containing Renilla luciferase gene was cotransfected.

Chromatin Immunoprecipitation (ChIP), RT-PCR

ChIP assay was done essentially as described (Takizawa et al., 2001) by using ChIP assay kit (Upstate). We used the monoclonal 12C11 antibody (gift from Dr. Anderson) and NRSF-P18 antibody (Santa Cruz), as the antibody for NRSF/REST. RT-PCR was performed by using total RNA extracted from HCN A94 cells. 1 μ g RNA was used for first-strand cDNA synthesis with SuperScript II (GibcoBRL). PCR primer sequences are available upon request.

Pull-Down Assay with Biotin-Labeled Oligonucleotide

Biotin-labeled RNA was synthesized with an AmpliScribe T7 transcription kit (Epicentre Technologies). Streptavidin-agarose beads (Gibco BRL) were washed with binding buffer (20 mM Tris-HCl, [pH 7.5], 60 mM KCl, 2.5 mM EDTA, and 0.1% Triton X-100) and suspended. While the beads were kept on ice, cell extract was mixed with 70 μ g of biotinylated RNA. After incubation on ice for 10 min, the total volume was adjusted to 1 ml with binding buffer.

Then the sample was transferred to the tube with agarose beads, and the tube was rotated slowly overnight at 4°C. The beads were washed 5 times with wash buffer (20 mM Tris-HCl, [pH 7.5], 350 mM KCl, and 0.01% NP-40) and resuspended in binding buffer. Proteins were eluted by boiling the beads and were separated by SDS-PAGE.

EMSA

The expression plasmid of cMyc-tagged NRSF/REST (gift from Dr. Anderson) was transfected in 293T cells, and the lysate was incubated with cMyc-antibody in RB buffer (20 mM Tris-HCl, [pH 7.5], 50 mM MgCl₂, 10 mM NaCl, and 1 mM EDTA) overnight at 4°C. After 1 hr of incubation with Fast Flow protein agarose beads (Upstate) at room temperature, associated proteins were precipitated. The beads were washed 3 times with wash buffer and resuspended in RB buffer. Proteins were eluted by pH 2.0 elution buffer (Upstate) and were neutralized immediately with Tris-Cl pH 8.5 buffer. The protein solution was purified and concentrated with Millipore Centricon (Amicon). Resultant protein solutions were sequentially diluted and each solution was incubated with preannealed and prestained 20 μM oligonucleotides for 30 min at room temperature. Oligonucleotide prestaining was done with SYBR green I (Molecular Probes) for DNA and SYBR green II (Molecular Probes) for RNA. Samples were loaded on 2% Nusieve agarose gel and the image was developed by Eagle Eye II (Stratagene).

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